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Title	Meropenem, levofloxacin and linezolid in human plasma of critical care patients: a fast semi-automated micro-extraction by packed sorbent UHPLC-PDA method for their simultaneous determination
Article type	Full length article

Abstract

An ultra high-performance liquid chromatographic (UHPLC) method with PDA detection was developed and validated for the simultaneous quantification of meropenem, linezolid, and levofloxacin in human plasma and applied in human plasma of critical care patients. A semi-automated microextraction by packed sorbent (MEPS) for sample preparation was used. All parameters in the extraction step (pH, sample volume, sample dilution and number of aspiration - ejection cycles) and in the desorption step (percentage of acetonitrile in the solvent of elution and number of aspirations of elution solvent through the device) were statistically significant when the recovery was used as response. The method showed good linearity with correlation coefficients, r2>0.9991 for the three drugs, as well as high precision (RSD%< 10.83% in each case). Accuracy ranged from -7.8% to +6.7%. The limit of quantification of the three drugs was established at 0.01 □g/mL for linezolid and levofloxacin and 0.02 □g/mL for meropenem. Linezolid, meropenem, levofloxacin and the internal standard were extracted from human plasma with a mean recovery ranged from 92.4% to 97.4%. During validation, the concentration of meropenem, linezolid and levofloxacin was found to be stable after 3 freeze-thaw cycles and for at least 24 h after extraction. This method will be subsequently used to quantify the drugs in patients to establish if the dosage regimen given is sufficient to eradicate the infection at the target site.

Keywords	meropenem; linezolid ; levofloxacin; MEPS; UHPLC; critical care patients
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Dear Editor,

We are sending our manuscript entitled “Meropenem, levofloxacin and linezolid in human plasma of critical care patients: a fast semi-automated micro-extraction by packed sorbent UHPLC-PDA method for their simultaneous determination” to your journal after all the authors have seen and approved the final form of it. This paper reports the development of an analytical method with a novel sampling and clean-up procedure of biological samples using the micro extraction with packed sorbent (MEPS). At the best of our knowledge there is no MEPS-UHPLC-PDA method in literature for the simultaneous determination of meropenem levofloxacin and linezolid in human plasma.

We declare that we have not published it anywhere else before nor is it being considered simultaneously in another journal and we have no conflict of interest to declare. Also, it is an honour to publication of my paper in your excellent journal.

The following address can be used for any kind of negotiations.

gcarlucci@unich.it

We do look forward to hearing from you soon.

Sincerely yours

Prof. Giuseppe Carlucci

Full Professor of Analytical Chemistry

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Response to Editor

Dear Editor

The authors thank the referees and the editor, for your excellent revision of our paper. In complying with your remarks we have answered point-by-point all their queries. Please find enclosed our responses.

Combined comments from the reviewers:

-In the guide for authors the following restriction is reported:"In the case of bioanalytical (pharmacokinetic, bioequivalence) studies, too general, oversimplified sentences such as "The methods in the literature are not sensitive enough, require tedious sample preparation steps; the retention times are too long and the method requires expensive instrumentation and special skill." are not acceptable."This means that the sentence reported at lines 84-86 should be cancelled, or rephrased.

Thanks for the comment. The authors agree with the referees, according with their comment the sentences reported at line 84-86 have been cancelled.

-Furthermore, searching in the literature was not accurate, i.e. the following paper reporting the simultaneous bioanalysis of antibiotics (including those considered in the present papers) by UHPLC-MS/MS was not referenced:

Simultaneous analysis of antibiotics in biological samples by ultra high performance liquid chromatography-tandem mass spectrometry, Cazorla-Reyes R., Romero-Gonzalez R., Frenich A.G., Rodriguez Maresca M.A., Martinez Vidal J.L. (2014) Journal of Pharmaceutical and Biomedical Analysis, 89, pp. 203-212.

The authors thanks the referees for their suggestion. Two new references for the analysis of antibiotics (including those considered in the present papers) by UHPLC-MS/MS have been added.

-Line 108: regimes ??

The authors apologize for the typo. Regimens has been added instead of regimes

-Lines 110-111: the sentence was not grammatically correct and should be amended.

The authors thank for the comment. The sentence has been corrected.

Experimental

-Line 145: the core-shell technology of the column should be emphasized.

Thanks for the insightful comment. Core shell technology has been emphasized in the result and discussion section.

-Line 150: One full stop was enough.

The authors apologize for the typo. According with the referees the sentence has been corrected

-Lines 177 and 195: g should be reported instead of rpm.

According with the referee *g* units has been used instead of *rpm*

-A section dealing with the real sample analysis and reporting the administered dose of the drugs, the blood volume collected, etc., was missing.

Thanks for the comment. A new section dealing with real sample analysis has been added.

Results and discussion
-Line 247: Fused-Core® is a trademark of Advanced Materials Technology, Inc., otherwise, core-shell could be used instead.

Thanks for the comment. Core-shell has been used instead of fused-core
-Section 3.3 was a bit confusing. It should report only the validation issues. A new section dedicated to the results and discussion of real samples analysis should be added.

The authors agree with the referees. Section 3.3 has been edited according with the comments. Furthermore, a new section dedicated to the results and discussion of real sample analysis has been added.

Figures
-A chromatogram at the LOQ should be presented in Fig. 5.

A chromatogram at the LOQ has been added to Fig.5 according with the comment.

We do look forward to hearing from you soon.

Sincerely yours

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Meropenem, levofloxacin and linezolid in human plasma of critical care patients: a fast semi-automated micro-extraction by packed sorbent UHPLC-PDA method for their simultaneous determination

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Abstract

An ultra high-performance liquid chromatographic (UHPLC) method with PDA detection was developed and validated for the simultaneous quantification of meropenem, linezolid, and levofloxacin in human plasma and applied in human plasma of critical care patients. A semi-automated microextraction by packed sorbent (MEPS) for sample preparation was used. All parameters in the extraction step (pH, sample volume, sample dilution and number of aspiration - ejection cycles) and in the desorption step (percentage of acetonitrile in the solvent of elution and number of aspirations of elution solvent through the device) were statistically significant when the recovery was used as response. The method showed good linearity with correlation coefficients, r^2>0.9991 for the three drugs, as well as high precision (RSD%< 10.83% in each case). Accuracy ranged from -7.8% to +6.7%. The limit of quantification of the three drugs was established at 0.01 μg/mL for linezolid and levofloxacin and 0.02 μg/mL for meropenem. Linezolid, meropenem, levofloxacin and the internal standard were extracted from human plasma with a mean recovery ranged from 92.4% to 97.4%. During validation, the concentration of meropenem, linezolid and levofloxacin was found to be stable after 3 freeze-thaw cycles and for at least 24 h after extraction. This method will be subsequently used to quantify the drugs in patients to establish if the dosage regimen given is sufficient to eradicate the infection at the target site.

***Keywords**:* meropenem, linezolid , levofloxacin, MEPS, UHPLC, human plasma, critical care patients.

1. Introduction

The growing drug resistance of *Gram-positive* (e.g. *Staphylococcus Aureus*) and *Gram-negative* (e.g. *P. Aeruginosa*) has become an issue of concern for clinicians. In presence of growing resistance, how to optimize the better efficacy or shorter course and how to minimize the emergence of drug resistance and reduce adverse reactions are issues worthy of further discussion [1-4]. Combinatory regimes of linezolid with fluoroquinolones (levofloxacin) or beta-lactam antibiotics (meropenem) are frequently used for initial treatment of severe multidrug-resistant nosocomial infections, because they provide considerably large broad and partly complementary antibacterial spectra.

Meropenem (MERO), or (4*R*,5*S*,6*S*)- 3-[[(3*S*,5*S*)-5-[(Dimethylamino)carbonyl]-3-pyrrolidinyl]thio]-6-[(1*R*)-1-hydroxyethyl]-4-methyl-7-oxo-1-azabicyclo[3.2.0.]hept-2-ene-2-carboxylic acid (Fig.1A), is a semi synthetic antibiotic member of a class of β-lactam antibiotics, the carbapenems, having a broad-spectrum and a concentration-dependant bactericidal activity against Gram-positive and Gram-negative respiratory pathogens. Unlike imipenem, meropenem proves a good stability against human renal dehydropeptidase I and does not require the coadministration of a dehydropeptidase enzyme inhibitor [5].

Lizenolid (LINZ) or N-[[(5S)-3-[3-Fluoro-4-(4-morpholinyl)phenyl] -2-oxo-5-oxazolidinyl] methyl] acetamide (Fig.1B) is an oxazolidinone derivative with a predominantly bacteriostatic effect against *Gram-positive* bacteria, including methicillin-resistant Staphylococcus aureus (MRSA), vancomycin-resistant enterococci and cephalosporin-resistant Streptococcus pneumonia [6]. Levofloxacin (LEVO) or (-)-(S)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid (Fig.1C) is a fluoroquinolone antibiotic which is a concentration-dependant antibacterial agent with its therapeutic results closely related to the ratio of the area under the concentration time curve to the minimum inhibitor concentration (AUC/MIC) for the organism. The quantification of the plasma concentrations for MERO, LINZ and LEVO could be an important pharmacological tool in order to optimize the drug dosage regimens and to maximize the ratio of efficacy to resources. Various analytical methods used for the determination of MERO [7-10] LEVO [11-13] and LINZ [14-17] concentration in biological matrices have been reported. High-performance liquid chromatography (HPLC) coupled with photodiode array detector (PDA) [7-9,11] and fluorescence [13] detection are the most

widely used techniques. Recently, HPLC coupled with mass spectrometry (LC-MS/MS) for determination MERO,LEVO or LINZ in human plasma have also been published [18,19].

A selective and sensitive ultra-high performance liquid chromatography (UHPLC-PDA) methods are preferable to more expensive LC-MS techniques. Furthermore, most of the proposed methods are still quite time-consuming and laborious, requiring large amount of sample and often also the use of toxic organic solvents in order to achieve satisfactory analytical limits. Clearly, this macro-approach to sample preparation is not sensitive enough when low volumes of sample are available. It is well known that recent trends in the field of sample preparation have been focused on miniaturization, automation, high-throughput performance, on-line coupling with analytical instruments and cost-effectiveness through extremely low or no solvent consumption in order to reduce laboratory workload and increase analytical performance. In such settings, micro extraction by packed sorbent (MEPS) is an attractive option and powerful sample-preparation approach suitable for accomplishing analytical and bioanalytical challenges. MEPS is a miniaturized form of the solid-phase extraction (SPE) technique. In MEPS, compared with SPE, the sorbent material (about 2 - 4 mg) is integrated directly into the syringe and not in a separate column and can be connected on-line to gas chromatography (GC), liquid chromatography (LC), LC coupled to mass spectrometry (LC-MS) or GC-MS [20-23].

Many sorbent materials, such as silica and silica-based (C_2, C_8, C_{18}), strong cation exchangers (SCX), polymeric HDVB and SDVB or molecular imprinted polymers (MIPs) can be used as packing bed or as a coating. The MEPS technique has been used to extract a wide range of analytes from biological sample, such as plasma, dialyzed, urine and blood [24-28]. The current research study describes the development and validation of a sensitive, fast, efficient, reliable and high throughput semiautomatic MEPS-based methodology in combination with UHPLC-PDA for the simultaneous determination of MERO, LINZ and LEVO in human plasma to establish if the dosage regimens given are sufficient to eradicate the infection at the target site. This method was validated according to the FDA [29] and ICH [30] guidelines. At present, no papers have been published for the simultaneous analysis of MERO. LEVO, LINZ in human plasma sample using MEPS-UHPLC-PDA

2. Materials and methods

2.1 Chemicals and reagents

Meropenem trihydrate (CAS 119478-56-7), linezolid, (CAS 165800-03-3) and levofloxacin (CAS 100986-85-4) were purchased from Sigma-Aldrich (Sigma-Aldrich, Milan, Italy), while ulifloxacin (CAS 112984-60-8), used as the internal standard (IS) to compensate for variability in extraction efficiency, was supplied from Suzhou Bichal Biological Technology (Jiangsu, China). Hydrochloric acid triethylamine and ammonium acetate were obtained from Carlo Erba Reagents (Carlo Erba, Milan, Italy). Methanol, acetonitrile HPLC grade, were purchased from VWR International (VWR International, Milan, Italy). All chemicals were of analytical-reagent grade or better. Pooled drug-free human plasma was obtained from Sigma-Aldrich (Sigma-Aldrich, Milan, Italy).

2.2 Instrumentation

The analysis of MERO, LINZ, LEVO and the IS, was performed on a Waters Ultra Performance Liquid Chromatography (ACQUITY H-Class) with column heater, degassing system combined with a Acquity quaternary solvent manager equipped with Acquity UPLC sample manager and a Waters 2996 PDA detector. Empower v.3 software (Waters) was used for setting-up the analysis and for data management. A XS104 Mettler Toledo analytical balance was used to weigh the analytes for the preparation of stock solutions and calibration standards. Water HPLC-grade water obtained by passage through an Elix 3 and Milli-Q Academic water purification system (Millipore, Bedford, MA, USA). Extraction was performed using the MEPS 250 μL syringe and the MEPS BIN (barrel insert and needle) containing 4 mg of silica C_{18} sorbent from SGE Analytical Science (LabService Analytica, Italy). Pooled drug-free human plasma was obtained from Sigma-Aldrich (Sigma-Aldrich, Milan, Italy). A CMA 100 microinjection pump (CMA Microdialysis AB, Kista, Sweden) was used for semiautomatic control of the flow-rate during aspiration and elution. All solvent evaporations were carried out in a Visiprep Vacuum Manifold equipped with a Visidry Drying Attachment System (Supelco, Bellefonte, USA). Samples were sonicated in an ultrasonic bath Labsonic (FALC, Milan, Italy).

142 *2.3 Analytical and chromatographic conditions*

143

144 Chromatographic separation was achieved using a core shell Ascentis Express C_{18} (50 x 2.1 mm I.D.
145 2.7 µm particle size) column protected by a disposable Guard Cartridge Ascentis Express C_{18} (5.0 x
146 2.1 mm) (Sigma-Aldrich, Milan, Italy) maintained at 25±1°C, using a thermostatically controlled
147 column heater. Mobile phase consisted in 10 mM ammonium acetate buffer adjusted to pH 5.5
148 with hydrochloric acid (phase A) and a mixture of acetonitrile and methanol in a ratio of 80/20
149 (v/v) (phase B), both phases were added with 0.1% (v/v) of triethylamine. To perform the best
150 separation of the analytes a linear gradient elution program was used. Starting from 97% and 3%
151 of phase A and B, respectively, within 2.5 min the composition of the eluting mixture was turned
152 to 65% and 35% of phase A and B, respectively, then an isocratic plateau was programmed for 0.8
153 min and returned back to the original ratio between 3.5 and 3.6 min, followed by 1.5 min of re-
154 equilibration of the column to the initial condition.The flow rate was set at 0.6 mL/min. The
155 solvents were filtered before use through a 0.45 µm PTFE membrane (Waters, Milan, Italy), while
156 ammonium acetate solution was filtered through a 0.45 µm GH-Polypro membrane (VWR
157 International, Milan, Italy). For quantification purposes, the UV detection was conducted using the
158 maximum absorbance wavelength of each analyte as follows: 300 nm for meropenem, 254 nm for
159 Linezolid, 290 nm for levofloxacin and 272 for the IS. Under these conditions, the total run time
160 was 5 min. The system suitability test (SST) was performed under optimised chromatographic
161 conditions. It was evaluated as the reproducibility of retention time and area expressed as RSD%.
162 The results are shown in Table 1.

163

164 *2.4 Preparation of calibration standard and quality controls*

165

166 Stock solutions of MERO, LINZ, LEVO and the IS at the concentration of 2.0 mg/mL were
167 individually prepared by dissolving 20 mg of each reference powder into 10 mL volumetric flask
168 with a mixture of methanol and hydrochloric acid 0.1M (95/5 v/v) and stored at -20°C; under these
169 conditions they were stable for over 4 months. The working solutions were prepared by dilution of
170 the stock solutions (calibration and quality control) in water. A working solution of I.S. (2.5 µg/mL)
171 was prepared in water. Standards for the calibration curves and quality control samples were then

prepared using serial dilutions of stock solution in drug-free human plasma. A seven-point calibration curve was built by spiking the blank plasma with appropriate aliquots of the working solutions to obtain final concentration in the range 0.02 to 30.0 μg/mL for MERO, 0.01 to 10.0 μg/mL for LEVO and 0.01 to 30.0 for LINZ, respectively. 50 μL of plasma sample were diluted to 150 μL with water, sonicated for 10 min at room temperature, then centrifuged (4320 *g*, 10 min, 4°C). The MEPS needle was fitted with a barrel insert and needle assembly (BIN) containing a C_{18} sorbent. The CMA/100 microinjection pump is a digitally-controlled dispensing unit used to draw and discharge samples and solutions through the BIN. Before being used for the first time, the sorbent was conditioned firstly with 250 μL of methanol and then with a mixture water-methanol (95/5, v/v). This step activates the sorbent and ensures reproducible retention of the analytes. Aliquots of 50 μL of spiked samples and standards were diluted to 150 μL with water and then passed through the C_{18} sorbent 10 times at a flow rate of 7.5 μL/sec. The sorbent was then washed with 100 μL of a mixture of 100 mM ammonium acetate buffer (pH 5.5) and methanol (95/5, v/v) to remove interferences, at a speed of 7.5 μL/sec. Then the analytes were eluted with 150μL of methanol at a flow rate of 10.0 μL/sec. Pooled quality control (QC) samples of MERO, LINZ and LEVO were prepared independently in the same way described above at concentration levels representing the lower limit of quantification (LLOQ), the low (LQC), middle (MQC) and high (HQC) ranges of the calibration curves to determine the intra- and inter-assay precision and accuracy of the method. In all measurements (standards and samples) 20 μL of ulifloxacin (giving a 2.5 μg/mL concentration) were added as internal standard (IS) prior to extraction.

2.5 Sample collection

Blood samples were collected at "*S.S. Annunziata*" Hospital (Chieti, Italy) 4h after an intravenous administration of MERO (Merrem 1g three times a day), LEVO (Levoxacin 400mg twice daily) and LINZ (Zyvox 600mg twice daily). Sample were collected at the same time as routine TDM samples in glass tube containing EDTA as the anticoagulant and did not require extra visits to the clinic or additional fingerpricks. Plasma samples were obtained after centrifugation at 1800 *g* for 10 min at 4°C, then, the supernatant (plasma) was stored at -20°C. Samples were thawed just before the extraction procedure and subjected to the method described above.

3. Results and discussion

3.1 Optimisation of MEPS extraction procedure

MEPS presents many more optimization opportunities to be investigated. Several parameters namely, sorbent type, sample dilution, number of extraction cycles, loading volume, sampling flow rate, elution solvent and its volume, elution flow rate and solvent for conditioning and washing step .The sorbents used are obviously a critical parameter and the correct choice of the sorbent will define the analytical performance of the whole methodology. So far, fistly several sorbent materials, such as silica and silica-based (C_2, C_8, C_{18}), strong cation exchangers (SCX), polymeric HDVB and SDVB were investigated in order to evaluate which had the best recoveries, C_{18} achieved best recoveries as shown in Fig 2.

Samples can be loaded directly or diluted if they are too concentrated or viscous in order to avoid the BIN saturation and the consequently the limitation on MEPS extraction efficiency. Two plasma dilutions with Milli-Q water (1:3, 1:2, v/v) and undiluted plasma were tested. Plasma diluted with Milli-Q water 1:2 (v/v) was the optimum in terms of reproducibility and sensitivity, undiluted plasma was unsuitable due to the short sorbent life and the higher irreproducibility of the MEPS extraction while when plasma diluted 1:3 (v/v) was investigated, lower sensitivity was observed.

The sample volume used was investigated in order to obtain the best equilibrium between good analytical performance and good extraction yield in the 50-250 µL range. In MEPS the analytes preconcentration on the sorbent phase is affected by the number of extraction cycles performed. According to Rani et al. (2012), the analytes extraction increased as the number of extraction cycles enhanced up to ten [31]. Fig 3 shows the results obtained. 150 µL was found to be a good choice as it was an appropriate volume for all evaluated dilutions. So far, plasma diluted 1:2 and a sampling volume of 150 µL could be set as optimal conditions. Sampling flow rate was also evaluated within 1-20 µL/s in order to investigate and modulate the interaction of target analytes to the sorbent. The optimum was achieved using 10 µL/s, higher values promoted great signal irreproducibility for the majority of tested analytes (Fig.4). The elution capacity of different solvents with different polarity was evaluated in term of peak area of each analyte, better results were obtained when methanol was used; consequently, latter one was selected as the elution solvent (150 µL). Different washing solutions were evaluated, including neat ultrapure water and

ultrapure water containing methanol at different proportions (5, 10, 25 and 50% v:v). In all cases, 100 μL of the washing solution was passed through the cartridge. Best results were obtained when 100 μL of a mixture of 100 mM ammonium acetate buffer (pH 4.0) and methanol (95/5 v/v) was used due to the highest analytical signal of the analytes and the lowest interference of co-eluting analytes. In order to avoid or reduce problems related to carryover, 6 wash-discard cycles (3 with 100 μL methanol and 3 with 100 μL water were implemented after elution). This cleaning procedure removed most of the residual analytes, as the carryover checked after the washing procedure was reduced to less than 0.5% peak area of the initial amount of analytes in all cases. The sample clean-up by MEPS is very quick, and additional operations between sample preparation and injection were not required. The small amount of stationary phase can be easily and effectively cleaned, avoiding carry-over effect so a single BIN can be used up to 80 times without loss in extraction performance.

3.2 Optimization of the chromatographic conditions.

Chromatographic separations were tried by using different compositions of mobile phases at different pH, different ratios and different analytical columns in order to achieve good resolution, selectivity and efficiency in a short run time. Higher separation efficiency was obtained using Ascentis express C_{18}. This column, packed with core shell particles, avoids the band broadening caused by multiple inter-particle paths, reduces the longitudinal diffusion and allows to work at higher flow rates with lower working pressures compared to totally porous columns. Different organic modifiers *e.g*. methanol and acetonitrile were tried in isocratic mode firstly then in gradient elution. Due to the different chromatographic properties of LINZ, MERO and LEV, isocratic separation was found to be unsuitable. The optimal mobile phase consisted of a mixture of methanol-acetonitrile and 10 mM ammonium acetate buffer (pH 4.0) in a gradient elution. Several gradient elution were explored, best separation was obtained using the gradient elution reported in Section 2.3. Furthermore to increase the peak shape, different triethylamine percentage in the range of 0.05 and 0.5 % (v/v) was added to the mobile phase. The optimum was achieved by using 0.1% (v/v) of triethylamine. Under this condition,MERO, LEVO and LINZ , were well separated and their peak shape were symmetric. Each drug was detected at its massimum wavelength in order to obtain higher sensitivity. By applying the chromatographic condition herein reported, the total run time in this assay was 3 min and the retention times for MERO, LEVO and LINZ were 1.55(±0.03),

2.36(±0.02) and 2.82(±0.02) min respectively while for the IS was 2.58(±0.02) min with consistently excellent reproducibility of less than 1.3%. All the evaluate parameters were within the required range, as established in FDA guidelines [29,30].

3.3 Method validation

In order to demonstrate the suitability of the developed analytical method, validation was carried out according to the International Guidelines [29,30].Selectivity of the proposed method was assessed by analyzing six different batches of control blank samples. The absence of any signal at the same retention time of the selected antibiotics suggested that there were no matrix interferents that may give false positive.The intra- and inter-day precision (RSD values) and precision (BIAS %), obtained from the analysis of three batches of LLOQ, QCL,QCM and QCH, in duplicate, on the same day and for five consecutive days, did not exceed 10.83 % for precision and ranged between -7.8 and +6.7 for accuracy, as shown in Table 2.

The LOQs of the method were 0.02 μg/mL for MERO and 0.01 μg/mL for LINZ and LEVO, respectively, with good precision and accuracy. The LODs were 7.0 ng/mL for MERO and 3.0 ng/mL for LEVO and LINZ. Results are shown in Table 3.

Calibration graphs were obtained by a linear regression analysis with weighting factors consisting in $1/x^2$ values in order to describe the relationships between concentration of the analytes and their peak area ratio to I.S. as analytical response. Quantitative analyses for MERO, LEVO and LINZ were performed at different wavelengths, as reported in Table 1**,** in order to obtain higher sensitivity and better signal-to-noise ratio.

Carry-over was investigated by injecting into the UHPLC two extracted blank plasma spiked with MERO, LEVO and LINZ at the ULOQ concentration, followed by three blank samples. No significant carry over effect (< 0.5%) was evident.

Three dilutions (1:4, 1:3 and 1:2) with the blank matrix were evaluated, with at least five determinations for each dilution factor in order to demonstrate the dilution integrity. For all evaluated dilutions accuracy was within ± 8%, while precision, expressed as relative standard deviations, was less than 6%. The extraction recovery of MERO, LEVO and LINZ from plasma was determined by assaying aliquots of drug-free plasma samples spiked at the QCL, QCM and QCH concentrations, results are reported in Table 4. Stability of MERO, LEVO and LINZ was investigated during sample collection, after long-term storage and short-term storage, and through several

freeze and thaw cycles according to the guidance for industry on the validation of bioanalytical methods [29,30]. The stock solution was stable at room temperature for 48 h and at 4 ◦C for two weeks. MERO, LEVO and LINZ were also stable up to 24 h at room temperature in plasma; it remained intact at −20 ◦C for up to five weeks. No degradation was observed after three cycles of freezing and thawing. The stability of MERO, LEVO and LINZ in the extracts was confirmed after a 24 h storage at 4 ◦C.

3.4 Application of the proposed method

The proposed method was applied for the determination of MERO,LEVO and LINZ in human plasma samples from patients 4h after being treated with an intravenous administration of MERO (Merrem 1g three times a day), LEVO (Levoxacin 400mg twice daily) and LINZ (Zyvox 600mg twice daily). To ensure quality results, an internal quality control was performed in each batch of samples, which implies a blank sample, a matrix matched calibration and a spiked blank samples at 7.5 µg/mL, 2.5µg/mL and 15µg/mL for MERO, LEVO and LINZ, respectively (Fig.5). The results obtained are shown in table 5.

4.Conclusion

The determination of drugs in biological matrix is the keystone in drug discovery and drug development as well as for pharmacodynamic and pharmacokinetic studies. Patients attending hospital emergency services with severe multi-drug resistant infections need rapid dosage adjustment in order to eradicate the infection at the target site. So far, a simple, fast, precise and accurate MEPS-UHPLC-PDA method for the determination of MERO,LEVO and LINZ in human plasma of critical care patients has been developed and applied. Nowadays no paper has been published for the determination of these drugs. MEPS in combination with UHPLC-PDA has shown to be adequate for the determination of MERO,LEVO and LINZ in plasma sample achieving good LOQs and high recovery.

About 100 extraction cycles were carried out using the same MEPS sorbent with no loss in performance. This novel microextraction technique has demonstrated to be simple, cost

effectiveness, precise and accurate requiring only small volume of sample and a short run time (5 min).

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Figure caption

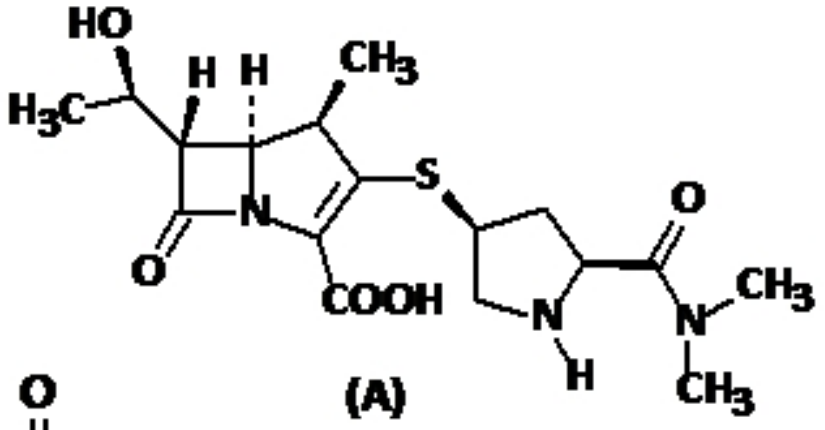
Figure 1: Chemical structure of Meropenem,(A) Levofloxacin (B) linezolid (C) and the internal standard ulifloxacin (D, IS)

Figure 2: Comparison of extraction recovery percentage obtained from analysis of spiked plasma samples as a function of the used sorbent (n=5)

Figure 3: Mean absolute recovery of MERO, LEVO, LINZ as a function of the extraction cycles and sample volume.

Figure 4: Reproducibility of MERO, LEVO, LINZ and the internal standard (I.S.) as a function of elution flow rate.

Figure 5: Chromatograms from human plasma extracts. (a) a blank plasma; (b) a blank plasma spiked with 2.5 µg/mL of internal standard; (c) a blank plasma spiked at the LOQ value, (d) a blank plasma spiked with MERO (7.5 µg/mL) LEVO (5 µg/mL) I.S. (2.5 µg/mL) and LINZ (15 µg/mL)and (e) a human plasma sample collected 4h after an intravenous administration of MERO (Merrem 1g three times daily) LEVO (Levoxacin 400mg twice daily) and LINZ (Zyvox 600mg twice daily) containing MERO (5,7 µg/mL) LEVO (1.2 µg/mL) and LINZ (7 µg/mL). Horizontal axis, retention time (min).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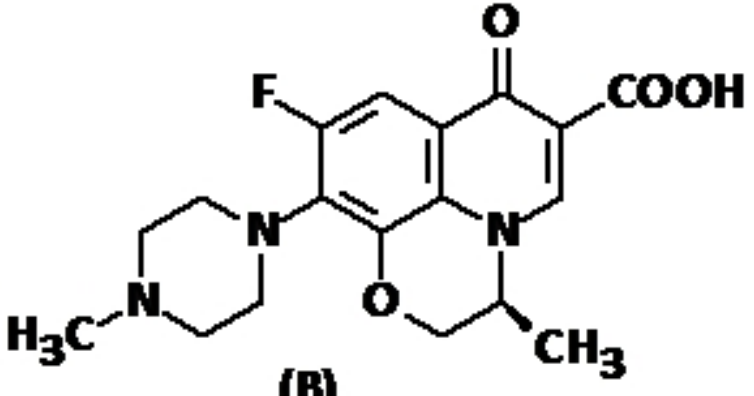
CH3
H3C
S
N
O
O
COOH
N
CH3
H
N
CH3

(A)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N
N
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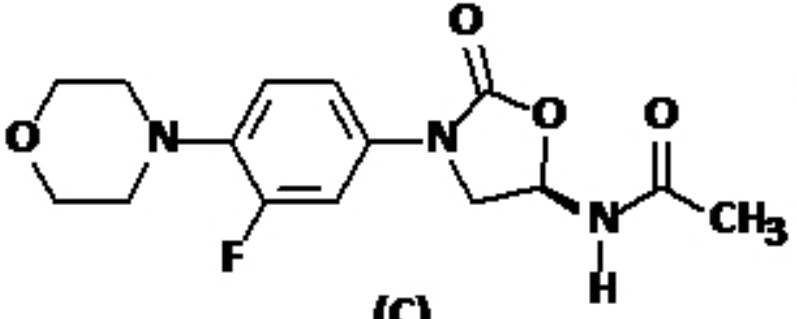
H3C
O
CH3

(B)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N
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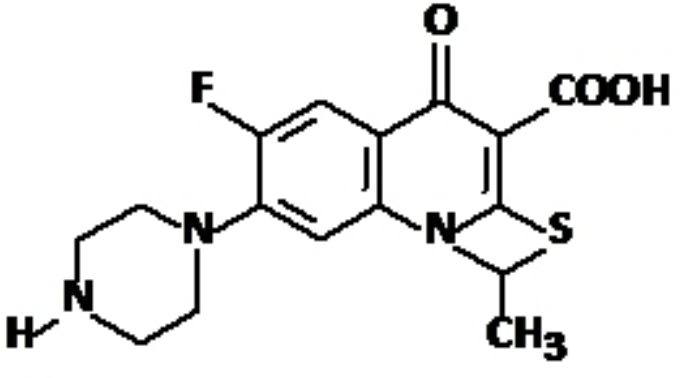
O
O
N
F
H
CH3

(C)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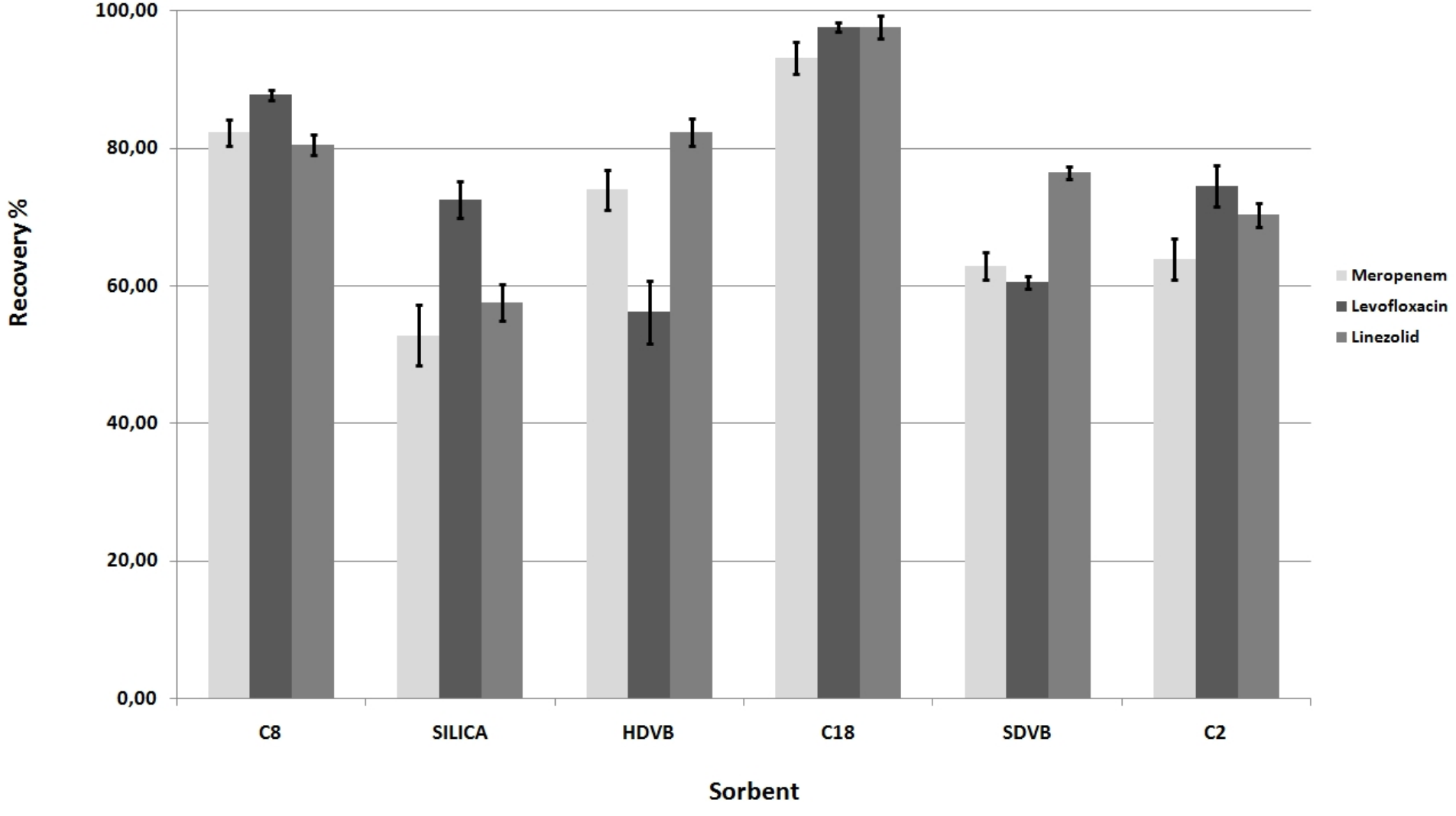
F
COOH
N
N
S
N
H
CH3

(D, I.S.)


100,00
80,00
60,00
40,00
20,00
0,00
Recovery %
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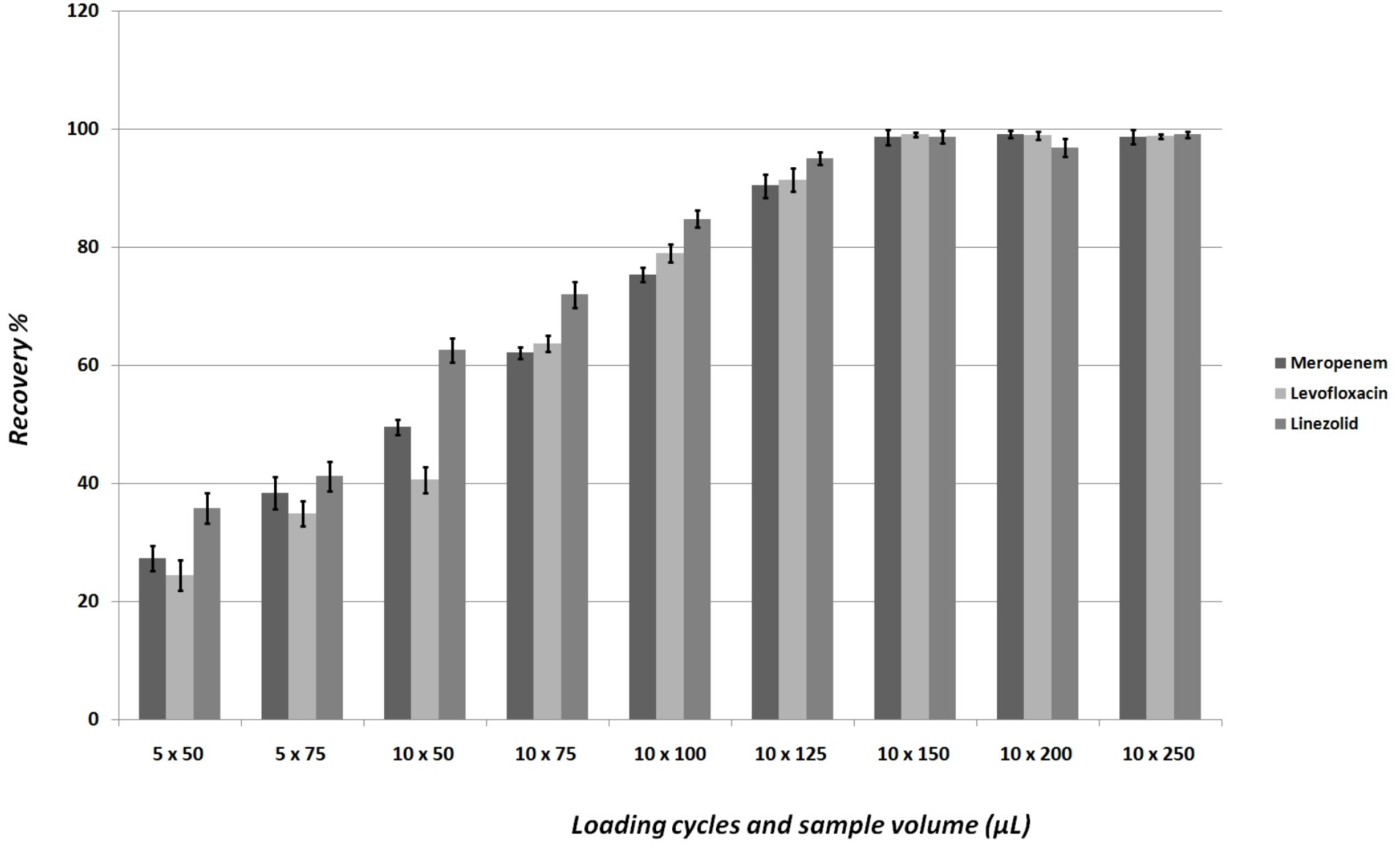
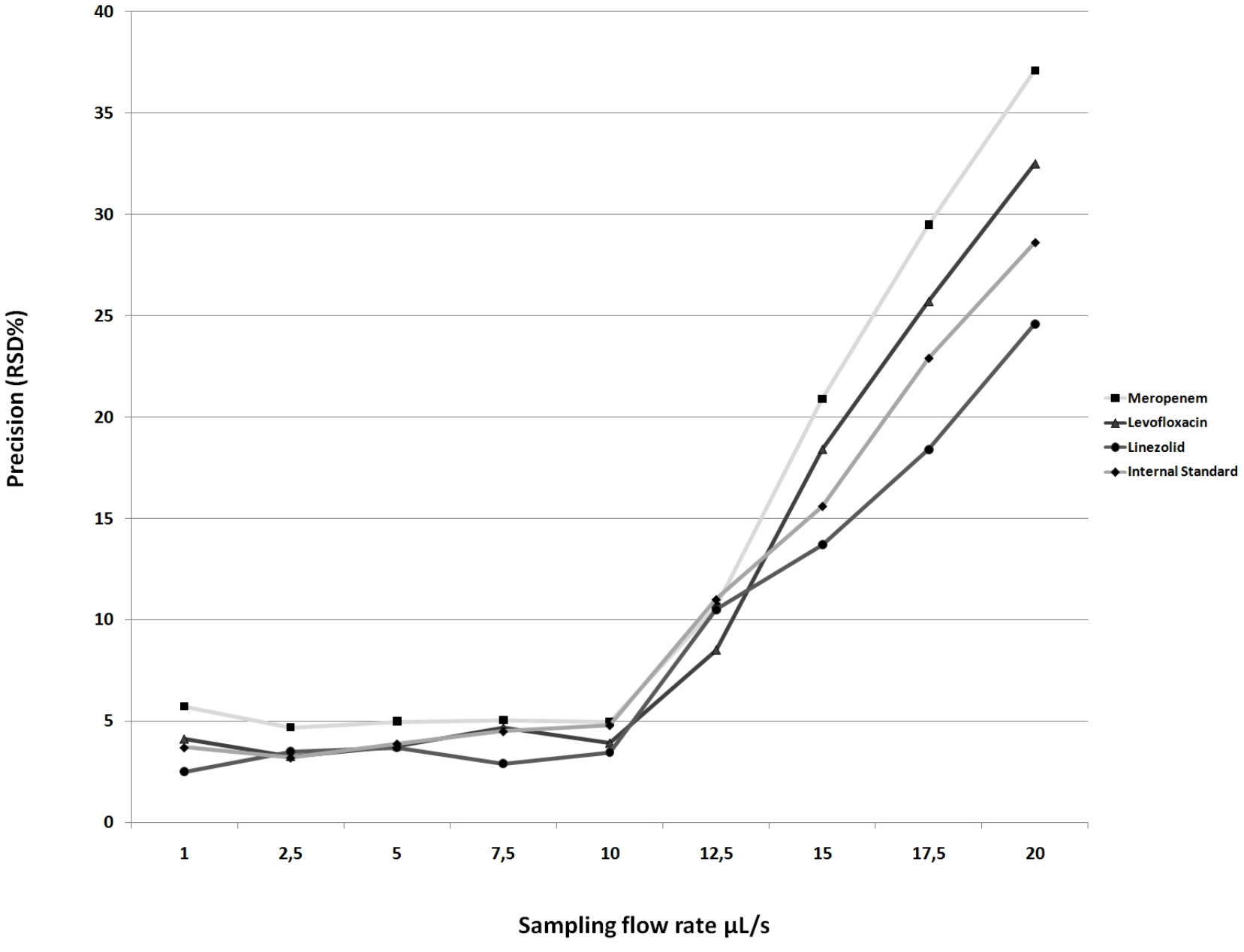
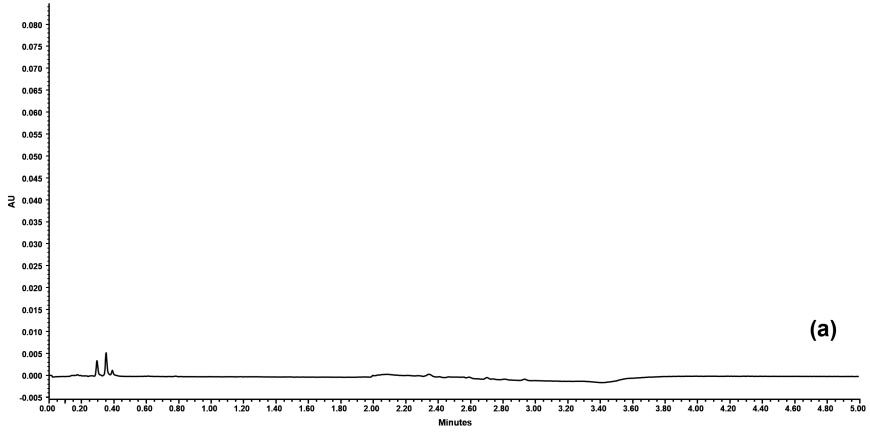
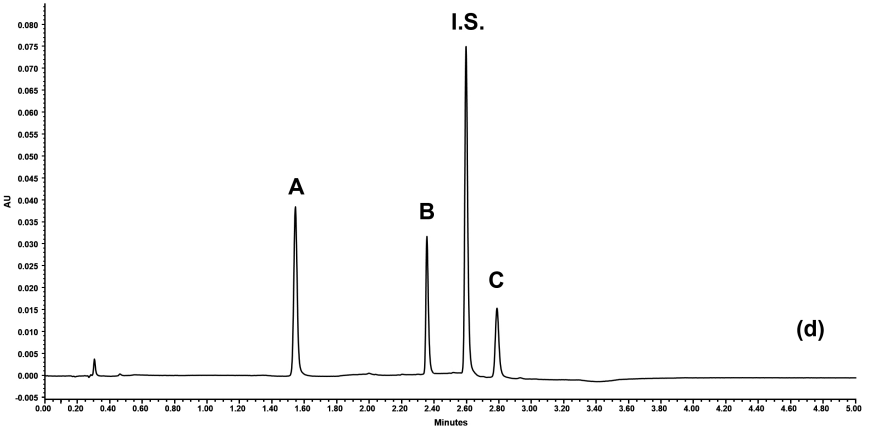
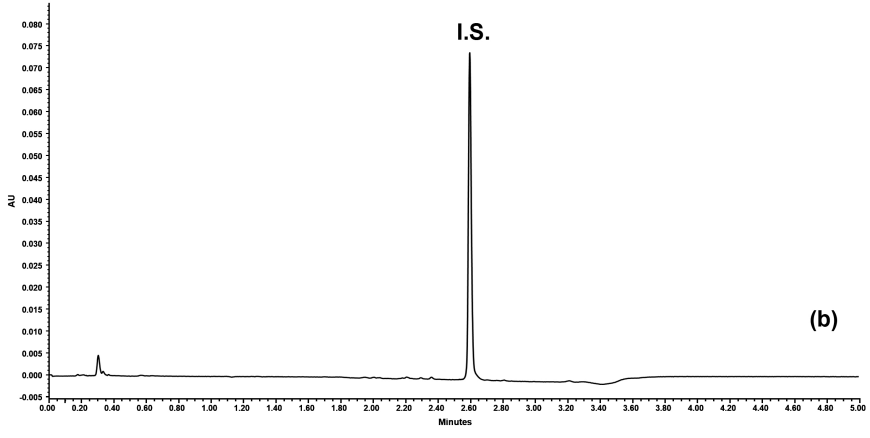
SILICA
HDVB
C18
SDVB
C2
Sorbent
Meropenem
Levofloxacin
Linezolid

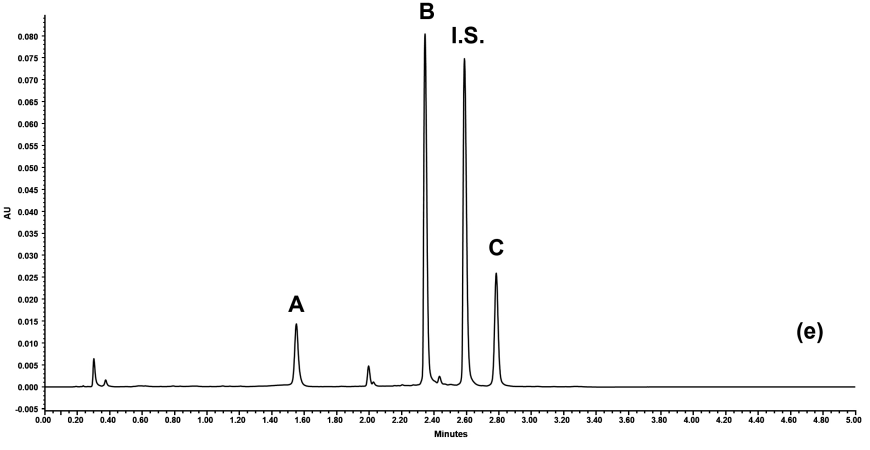

120
100
80
60
40
20
0
Recovery %
5 x 50
5 x 75
10 x 50
10 x 75
10 x 100
10 x 125
10 x 150
10 x 200
10 x 250
Loading cycles and sample volume (µL)
Meropenem
Levofloxacin
Linezolid


Precision (RSD%)
40
35
30
25
20
15
10
5
0
1
2,5
5
7,5
10
12,5
15
17,5
20
Sampling flow rate µL/s
Meropenem
Levofloxacin
Linezolid
Internal Standard


AU
Minutes
(a)


I.S.
A
B
C
AU
Minutes
(d)


I.S.
AU
Minutes
(b)


B
I.S.
C
A
AU
Minutes
(e)

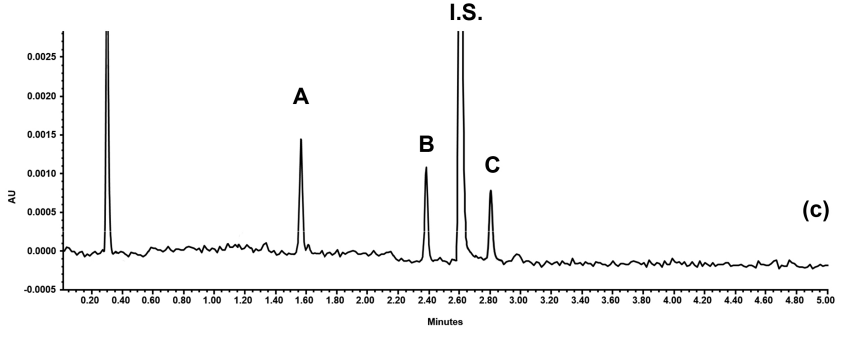

I.S.
A
B
C
AU
Minutes
(c)

Table 1: Summary of the system suitability test for the proposed method

Analyte	RT[a]	α[b]	Rs[c]	As[d]	N[e]
Meropenem	1.55	-	12.46	1.11	6051
Levofloxacin	2.36	1.68	3.01	1.08	8911
Ulifloxacin (I.S.)	2.58	1.11	2.84	1.16	13148
Linezolid	2.82	1.14	-	1.09	15708

[a] Retention time (minutes) [b] Selectivity [c] Resolution [d] Asymmetry [e] Theoretical plates

Table 2: Intra-day and inter-day accuracy and precision for linezolid and Levofloxacin and meropenem at the LLOQ and QCs value.

	Intra-day (n=6)			Inter-day (n=6)		
	Mean Measured concentration (µg/mL) ± S.D.	**Accuracy BIAS (%)**	**Precision (RSD%)**	**Mean Measured concentration (µg/mL) ± S.D.**	**Accuracy BIAS (%)**	**Precision (RSD%)**
Linezolid theoretical concentration (µg/mL)						
0.01[A]	0.0098 ± 0.0003	-7.4	3.06	0.0099 ± 0.0004	+6.7	4.04
0.02	0.019 ± 0.001	-3.7	5.26	0.018± 0.002	-7.8	10.83
1.00	1.03 ± 0.06	+3.4	5.82	1.05 ± 0.06	+5.3	5.71
25.00	24.87 ± 0.57	-0.5	2.29	25.4 ± 1.15	+1.6	4.61
Levofloxacin theoretical concentration (µg/mL)						
0.01[A]	0.0095 ± 0.002	-4.7	7.42	0.021 ± 0.03	+4.0	7.17
0.02	0.019 ± 0.002	-3.7	10.50	0.021 ± 0.002	+5.9	9.52
0.75	0.74 ± 0.02	-2.2	2.70	0.78 ± 0.07	+4.3	8.97
7.50	7.53 ± 0.19	+0.5	2.52	7.51 ± 0.43	+0.1	5.72
Meropenem theoretical concentration (µg/mL)						
0.02[A]	0.0191 ± 0.002	-4.3	10.40	0.021 ± 0.03	+4.0	7.17
0.05	0.048 ± 0.003	-3.5	6.25	0.053 ± 0.005	+6.2	9.43
1.00	0.94 ± 0.07	-5.8	7.44	0.78 ± 0.07	+4.3	8.97
25.0	24.1 ± 0.6	+3.6	2.48	26.5 ± 0.9	+6.0	3.39

[A] **LLOQ concentration**

Table 3: Analytical performance of the MEPS-UHPLC-PDA method

	Meropenem	**Linezolid**	**Levofloxacin**
λ_{max}[a] (nm)	300	254	292
Conc. Range (µg/mL)	0.02-30	0.01-30	0.01-10
Intercept (± SD)	2.18 (±0.09) · 10^{-2}	7.89 (±0.03) · 10^{-3}	4.14 (±0.07) · 10^{-3}
Slope (± SD)	0.0182 (±0.008)	0.093 (±0.004)	0.0182 (±0.008)
r^2	0.9996	0.9998	0.9991
LOQ (µg/mL)	0.02	0.01	0.01
LOD (µg/mL)	0.004	0.007	0.004

[a] wavelenght of the PDA detection

Table 4: Mean recovery of Meropenem, Levofloxacin ,linezolid and the internal standard from human plasma at the QCs concentrations.

Nominal concentration	Mean Measured concentration (µg/mL) ± S.D.	Recovery (%)	RSD%
	MEROPENEM		
0.05	0.0493 ± 0.003	98.6	6.00
1.00	0.982 ± 0.08	98.2	8.24
25.00	24.51 ± 0.34	98.0	1.36
	LEVOFLOXACIN		
0.02	0.0191 ± 0.0012	92.5	6.31
0.75	0.71 ± 0.05	94.7	7.04
7.50	7.23 ± 0.44	96.4	5.86
	LINEZOLID		
0.02	0.0193 ± 0.0010	96.5	5.23
1.00	0.96 ±0.09	96.7	9.12
25.00	24.10 ± 0.36	96.4	1.44
	INTERNAL STANDARD		
2.50	2.39 ± 0.05	95.6	2.13

Table 5: Plasma concentration of meropenem, levofloxacin and linezolid obtained from real sample subjected to the MEPS-UHPLC-PDA method

Sample	Age	Meropenem (µg/mL)	Levofloxacin (µg/mL)	Linezolid (µg/mL)
#1	72	18.7	1.50	7.8
#2	53	24.1	2.20	22.4
#3	64	9.8	0.98	14.3
#4	67	11.7	3.47	11.6
#5	51	6.1	1.11	9.6

Highlights

- A novel sampling and clean-up procedure using microextraction by packed sorbent.
- UHPLC-PDA method development and validation
- First MEPS-UHPLC-PDA method for determination of meropenem, levofloxacin, linezolid in human plasma
- Therapeutic drug monitoring in critical care patients