Manuscript Details

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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 metronidazole, meropenem, ciprofloxacin, linezolid and piperacillin in human plasma and applied to patients suffering from hospital acquired pneumonia (HAP). The method uses an air assisted dispersive liquid-liquid microextraction for sample preparation. All parameters in the extraction step, including selection of extractant, amount of extractant, ionic strength, pH, and extraction cycles, were investigated and optimized. Chromatography was carried out using a Poroshell 120 SB C18 (50 x 2.1 mm I.D. 2.6 µm particle size) column and a gradient mobile phase consisting of ammonium acetate buffer (10 mM, pH 4.0) (eluent A); and a mixture of acetonitrile-methanol in a ratio (80/20)(eluent B). Ulifloxacin was used as internal standard. The method demonstrated good linearity with correlation coefficients, r2>0.9995 for the drugs, as well as high precision (RSD%≤ 9.87%), accuracy ranged from -8.14% to +8.98. The enrichment factor (EF) obtained ranged within 87 and 121. During the validation, the concentrations of the analytes were found to be stable after 3 freeze-thaw cycles and for at least 24 h after extraction. Subsequently, this method was used to quantify the drugs in patients with HAP in order to establish if the dosage regimen given was sufficient to eradicate the infection at the target site.

Keywords	Antibiotics ; AA-DLLME-SFO; UHPLC-PDA; method validation; human plasma analysis ; 1-dodecanol
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Dear Editor,

We are sending our manuscript entitled “Air assisted dispersive liquid-liquid microextraction with solidification of the floating organic droplets (AA-DLLME-SFO) and UHPLC-PDA method: Application to antibiotics analysis in human plasma of hospital acquired pneumonia patients” to your journal after all the authors have seen and approved the final form of it. At the best of our knowledge there is no AA-DLLME-SFO-UHPLC-PDA method in literature for the simultaneous determination of metronidazole, meropenem, ciprofloxacin, linezolid and piperacillin 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.

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We do look forward to hearing from you soon.

Sincerely yours

Prof. Giuseppe Carlucci

Full Professor of Analytical Chemistry

Department of Pharmacy

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Response to editor

Dear Editor

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

"AA-DLLME-SFO method is popular, documented in many journals. Authors repeatedly claimed that it is the first application of AA-DLLME-SFO in the preparation of human plasma. While the application of AA-DLLME-SFO in plasma samples using 1-dodecanol was previously investigated in a missing paper [Low-toxic air-agitated liquid-liquid microextraction using a solidifiable organic solvent followed by gas chromatography for analysis of amitriptyline and imipramine in human plasma and wastewater samples. Asghari A., Saffarzadeh Z., Bazregar M., Rajabi M., Boutorabi L., Microchem. J. 130 (2017) 122-128]. Hence, I think this manuscript was suffering from lack of novelty in the analytical aspects. However, investigated antibiotics have high therapeutic index and AA-DLLME-SFO can be used for the determination of these drug in the human plasma"

Thanks for the comment. The authors agree with the referee in the importance of the AA-DLLME-SFO as a tool for the determination of the investigated antibiotics which have high therapeutic index. Furthermore, as described in the introduction only few papers report the development of the DLLME-SFO as a sample preparation technique for the analysis of biological sample. According with the referee the reference missing as been added in the manuscript.

Further comments:

- The first highlight should be reconsidered

The first highlight has been modified according with the referee.

- Provided figures of merit were incomplete. ER, PF RSD and … should be added in the abstract, and related tables.

Thanks for the suggestion, according to the referee ER,PF and RSD have been added in the abstract and related tables.

- "Table 1: Analytical data of the MEPS-UHPLC-PDA method". What is MEPS?

Table 1 has been corrected.

- Table 2 was not mentioned in the text.

Table 2 has been mentioned in the text in Section 3.7.

- Did the method provide the linearity up to 50 (µg mL-1)?

Dilution integrity was evaluated and validated for concentration above the ULOQ. The MIC for the investigated antibiotics are within the linearity range. Furthermore, the therapeutic ranges of the selected antibiotics are also within the investigated range for all the antibiotics.

- Chromatograms related to near to LOQ should be added.

A chromatogram near the LLOQ as been added in figure 4 according with the referee.

- Description about real samples analysis is incomplete.

The authors agree with the referee. A better description of the real sample analysis as been added in section 2.3 and 3.9

- pKa, Log P, therapeutic index and other information on drugs should be added.

According with the referee a supplementary table 1 containing pka, Log P, trade name and therapeutic range of the investigated antibiotics has been added.

Highlights

- Development of sampling and clean-up procedure using AA-DLLME-SFO.
- UHPLC-PDA method development and validation
- Determination of several antibiotics in human plasma
- Therapeutic drug monitoring in patients with hospital acquired pneumonia.

Air assisted dispersive liquid-liquid microextraction with solidification of the floating organic droplets (AA-DLLME-SFO) and UHPLC-PDA method: Application to antibiotics analysis in human plasma of hospital acquired pneumonia patients

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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 metronidazole, meropenem, ciprofloxacin, linezolid and piperacillin in human plasma and applied to patients suffering from hospital acquired pneumonia (HAP). The method uses an air assisted dispersive liquid-liquid microextraction for sample preparation. All parameters in the extraction step, including selection of extractant, amount of extractant, ionic strength, pH, and extraction cycles, were investigated and optimized. Chromatography was carried out using a Poroshell 120 SB C_{18} (50 x 2.1 mm I.D. 2.6 µm particle size) column and a gradient mobile phase consisting of ammonium acetate buffer (10 mM, pH 4.0) (eluent A); and a mixture of acetonitrile-methanol in a ratio (80/20)(eluent B). Ulifloxacin was used as internal standard. The method demonstrated good linearity with correlation coefficients, r^2>0.9995 for the drugs, as well as high precision (RSD%≤ 9.87%), accuracy ranged from -8.14% to +8.98. The enrichment factor (EF) obtained ranged within 87 and 121. During the validation, the concentrations of the analytes were found to be stable after 3 freeze-thaw cycles and for at least 24 h after extraction. Subsequently, this method was used to quantify the drugs in patients with HAP in order to establish if the dosage regimen given was sufficient to eradicate the infection at the target site.

Keywords: Antibiotics ; AA-DLLME-SFO; UHPLC-PDA; method validation; human plasma analysis ; 1-dodecanol

1. Introduction

The growing drug resistance of *Gram-positive* (e.g. *Staphylococcus Aureus*) and *Gram-negative* (e.g. *P. Aeruginosa*) bacteria has become, during the last decade, an issue of concern for clinicians. However, new and alternative empirical treatments not currently outlined in the published guidelines could be used in the public interest for critical care patients. So far, the therapeutic drug monitoring (TDM) of antimicrobial agents could be a useful tool against drug-drug interactions and might increase the therapeutical efficacy in patients suffering from nosocomial infection [1-3].

Meropenem (MER) is a widely used carbapenem with antibacterial activity against a wide range of *Gram-positive* and *Gram-negative* bacteria[4]. Metronidazole (MET), the reference agent of the nitroimidazole antibiotic family [5], is used in the therapy of several contagious and infectious diseases, such as amoebiasis, trichomoniasis, lambliasis and anaerobic infections [5]. Ciprofloxacin (CIP), a fluoroquinolone antibiotic, demonstrates a concentration-dependent bactericidal activity, which depends on the ratio of the maximum drug concentration to the minimum inhibitory concentration (MIC) [6]. Linezolid (LIN) is an oxazolidinone derivative with a predominantly bacteriostatic effect against severe infections caused by methicillin or vancomycin resistant *Gram-positive* bacteria [7]. Piperacillin (PIP), a β-lactam antibiotic, proves a time-dependent bactericidal activity. Maximizing the time above the minimum inhibitory concentration (MIC) for a pathogen is the best pharmacodynamic predictor of efficacy [8]. The combination of these drugs is widely used for the treatment of severe multidrug resistant infections. Several HPLC assays with ultraviolet (UV) detection for the determination of these drugs, alone or in combination, have been reported [9-17]. These assays have some disadvantages in terms of extensive chromatographic run times [9,11-13, 15, 16] a large consumption of non environmentally friendly solvents [11] or a large amount of sample [11,15,16]. Recently, HPLC methods coupled with mass spectrometry (LC-MS/MS) for determination of the selected drugs in human plasma have also been published [18-23]. Nowadays, more and more scientists focus 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 in many settings. In such settings, ultra high performance liquid chromatography with photodiode array detection (UHPLC-PDA) is the most commonly used due to its low cost and easy accessibility compared to LC-MS.

Assadi *et al*. in 2006 developed a novel microextraction techinque called dispersive liquid-liquid microextraction [24-26]. In this technique, a cloudy solution of fine organic droplets is formed when a mixture of extraction solvent (immiscible with water) and a dispenser solvent (miscible with both water and the extractant solvent) is rapidly injected into an aqueous solution sample containing the analytes. In DLLME, large surface contact between fine droplets and the analytes, speeds up the processes regarding mass-transfer of analytes from aqueous phase to organic phase which greatly enhances extraction efficiency. Nevertheless the extraction solvent used in DLLME is generally highly toxic (chlorobenzene, chloroform or carbon disulfide), not environmentally friendly and incompatible with reverse phase HPLC. Recently, in order to solve this problem, a new microextraction technique has been developed by using extracting solvents which have lighter density than water integrated with the solidification of the organic floating droplet (DLLME-SFO) [27]. In DLLME-SFO the extraction solvent after DLLME, is collected in the top of the test tube and then is cooled by inserting it into an ice bath for 5 min.

DLLME-SFO is widely applied for environmental water samples but rarely it is used for the analysis of drugs in complex matrixes such as biological fluids [28-30]. Recently, three new DLLME techniques have been introduced to eliminate dispersive solvents: air assisted (AA-DLLME), vortex assisted (VA-DLLME) and ultrasound assisted (UA-DLLME). Recent literature reports the comparison between AA-DLLME, VA-DLLME and UA-DLLME [31]. Although AA-DLLME must be manually performed, it demonstrates lower centrifugation time, less analysis time without relevant differences in enrichment factor and recovery. In the present work a fast, sensitive, precise and accurate method for the determination of MER, MET, CIP, LIN and PIP in human plasma by UHPLC-PDA using a combination of protein precipitation (PP) and air assisted dispersive liquid-liquid microextraction with solidification of the floating organic droplet (AA-DLLME-SFO) has been developed. Various extraction parameters including selection of extractant, amount of extractant, ionic strength, pH and extraction cycles, were tested and optimized. As far as we know, no papers have been published for the simultaneous analysis of these drugs in human plasma sample by using AA-DLLME-SFO coupled with UHPLC-PDA.

2. Experimental

2.1. Chemicals and reagents

The standards of MER, MET, CIP, LIN and PIP, 1-undecanol, 1-dodecanol and hexadecane were purchased from Sigma Aldrich (Milan, Italy) while ulifloxacin, the internal standard (I.S.), was supplied from Suzhou Bichal Biological Technology (Jiangsu, China). Methanol, acetonitrile HPLC grade, triethylamine, sodium chloride, sodium hydroxide, ammonium acetate were purchased from Carlo Erba Reagents (Milan, Italy). HPLC-grade water was obtained by passing through an Elix 3 and Milli-Q. Pooled drug-free human plasma was obtained from Sigma-Aldrich (Milan, Italy). Stock solutions of MER,MET,CIP,LIN,PIP and the I.S. were individually prepared by dissolving 20mg of each reference powder into 10 mL volumetric flask with a mixture of methanol and hydrochloric acid 10mM (95/5 v/v) and stored at -20°C. Working solutions were obtained by serial dilutions of the stock solution with ultra-pure water.

2.2 Instrumentation and chromatographic conditions

The analysis of the investigated analytes and the internal standard were performed on a Waters Ultra Performance Liquid Chromatography (ACQUITY H-Class) with column heater, degassing system combined with an Acquity quaternary solvent manager equipped with Acquity UPLC sample manager and a Waters 2996 photodiode array 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 a stock solution and calibration standard.

Chromatographic separation was achieved by using a Poroshell 120 SB C_{18} (50 x 2.1 mm I.D. 2.6 µm particle size) column protected by a disposable Security Guard Poroshell 120 SB (2.1 x 5.0 mm) (Agilent, Santa Clara , CA, USA) maintained at 25±1°C. Mobile phase consisted in 10 mM ammonium acetate buffer adjusted to pH 4.0 with hydrochloric acid (phase A) and a mixture of acetonitrile and methanol in a ratio of 80/20 (v/v) (phase B). Both phases were added with 0.1% (v/v) of triethylamine. To perform the best separation of the analytes a linear gradient elution program was used. Starting from 95% and 5% of phase A and B,respectively, within 2.5 minutes became 65% and 35% of phase A and B, respectively, then an isocratic plateau was programmed for 0.8 minutes and came back to original condition between 3.5 and 3.6 minutes, followed by 1.4 minutes of re-equilibration of the column to the initial condition. The flow rate was set at 0.5 mL min^{-1}. For the quantification purposes, the UV detection was conducted by using the maximum absorbance wavelength of each analyte as follows: 315nm for MET, 300nm for MER, 277nm for CIP, 251nm for LIN, and 235 for PIP, respectively, while 272nm for the I.S.. Under these conditions,

the total run time was 5 minutes. The autosampler temperature was set at 20°C to avoid the solidification of the collected organic droplet. The system suitability test (SST) ensures that a complete testing system (including instrument, reagents and columns) is suitable for the intended application.

2.3. Sample preparation

Blank human plasma sample (drug-free) was purchased from Sigma-Aldrich while the real samples were collected from patients at "*S.S. Annunziata*" Hospital (Chieti, Italy). The blood samples were usually drawn in the morning from patients and stored into glass tubes containing EDTA as anticoagulant until been centrifuged at 1300*g* for 10 min at 4°C. Then, the supernatant (plasma) was stored at -20°C. In order to eliminate proteins and other substances 2 mL of plasma was placed into 10 mL glass vial and 2 mL of a mixture containing 15% (w/v) of zinc sulfate and 20% of trichloroacetic acid solutions (60/40, v/v). Shortly after, the glass tube was vortexed for 20s and centrifuged at 5600*g* for 5 minutes. The supernatant was collected and diluted to 5 mL with water in order to decrease the matrix effects. The resulting solution was subjected to the AA-DLLME-SFO process.

2.4. Air assisted DLLME-SFO extraction

For the AA-DLLME-SFO, in a 10 mL glass test tube, 200 mg of sodium chloride and 100µL of sodium hydroxide 1.0M, were added to a 5.0 mL of a pretreated spiked plasma sample. 30µL of the extraction solvent (1-dodecanol) was added and the mixture was rapidly pulled into a 10mL glass syringe from the tube and then pushed out into it (repeated eight times) via syringe needle. A cloudy solution was formed due to the dispersion of fine droplets of 1-dodecanol and the analytes were rapidly extracted.

After centrifugation at 4200*g* for 4 min, the test tube was cooled in an ice bath and the organic droplets of 1-dodecanol floating on the surface solidified and formed a small ball due to a solidification point of 12°C. The solidified 1-dodecanol phase was transferred to an Eppendorf 1.5 mL tube and mixed with 50 µL of methanol due to the high viscosity of 1-dodecanol, 5 µL of the mixture were injected into the UHPLC for analysis. A schematic procedure of the AA-DLLME-SFO is illustrated in Fig.1.

184 *2.5. Evaluation of the enrichment factor, extraction recovery and relative recovery*

185

186 In order to obtain the optimized extraction conditions, the enrichment factor (EF) was used to
187 evaluate the extraction efficiency under different conditions. The EF was defined as the ratio
188 between the analyte concentration in the organic floating phase ($C_{floating}$) after extraction and the
189 initial concentration of the analyte in the sample (C_o), as follows:

190

191 $$EF = \frac{C_{floating}}{C_o}$$

192

193 The $C_{floating}$ was obtained from a calibration graph of a direct injection of the analyte standard
194 solution in the extraction solvent. The extraction recovery was defined as follows:

195

196 $$ER = EF \times \frac{V_{floating}}{V_{Aq}} \times 100$$

197

198 where $V_{floating}$ and V_{Aq} are the volumes of the floated phase and sample solution, respectively.
199 The relative recovery was obtained by the following equation:

200

201 $$RR = \frac{C_{founded} - C_{real}}{C_{added}}$$

202

203 $C_{founded}$ represents the concentration of the analyte after adding a known amount of standard to
204 the real sample C_{real} is the concentration of the analyte in the real sample and C_{added} are the
205 concentrations of the analyte after the addition of known amount of standard in the real sample
206 [25].

207

208 **3. Results and discussion**

209

210 3.1. Optimization of protein precipitation

211

To eliminate proteins and other substances from plasma samples and facilitate an efficient AA-DLLME-SFO procedure, different protein precipitation procedures were tested including methanol, acetonitrile, trichloroacetic acid (20% w/v) and zinc sulfate (15% w/v) alone or in combination.
Incomplete protein precipitation was observed using methanol, trichloroacetic acid and zinc sulfate, alone, while acetonitrile used at two-fold of the sample volume yielded satisfactory precipitation efficiency; yet this procedure was found to be unsuitable due to the limitation of the extractant in the forming of fine droplets for the AA-DLLME-SFO.
Combination of trichloroacetic acid (20% w/v) and zinc sulfate (15% w/v) (60/40, v/v) in a 1:1 v/v ratio with the sample volume achieved similar precipitation efficiency compared to the acetonitrile precipitation. Furthermore no limitation of the extractant in the forming of fine droplets was observed. In this respect, to a 2 mL of human plasma, 2 mL of a mixture of trichloroacetic acid and zinc sulfate above reported was added as the protein precipitation solvent.

3.2. Selection of the extraction solvent and its volume

The selection of the appropriate extraction solvent for the AA-DLLME-SFO must meet several requirements: low volatility, low toxicity, low solubility in water and a solidification point near the room temperature in order to easily collect the solvent by solidification. For all the evaluated AA-DLLME-SFO parameters, plasma spiked with the investigated analytes (5µg/mL) and I.S. (2.5 µg/mL) was used.
Three different extractants were investigated (1-dodecanol, 1- undecanol , hexadecane). Among these solvents as shown in Fig.2, best results were obtained using 1-dodecanol. The extraction efficiencies of 1-undecanol and hexadecane were lower compared to 1-dodecanol. Furthermore lower repeatability was achieved using hexadecane as extracting solvent. Hence, 1-dodecanol was selected as extractant due to its suitable solidification point and excellent extraction efficiency.
Various volumes of 1-dodecanol were tested during the optimization (30, 50, 70, 100 µL): the results indicated the decrease of the peak area with the increase of the 1-dodecanol volume. Although the use of lower volumes of extraction solvent may lead to high enrichment factor, it is not easy to handle extracts with volumes less than 30 µL. Consequently, 30 µL was selected as the optimal extraction solvent volume.

3.3. Optimization of the number of extraction cycles

For the proposed method, the number of extraction was defined as the number of repeatedly sucking extraction solvent and sample solution mixture into a 10mL glass syringe and then its injecting into the test tube. It is predictable that by increasing the number of extraction cycles, recoveries should be increased too. The range of 1-10 extractions cycles was investigated. The peak areas of the analytes increased up till seven extraction cycles then a plateau was observed. Moreover by increasing the number of extraction the repeatability improved, yet in case of extraction cycles higher than eight the repeatability decreased. Hence, eight extraction cycles were selected as optimum for the AA-DLLME-SFO, it was noted that this step was performed in less than 1 min.

3.4. Effect of ionic strength

The salting-out effect has been used universally in LLE. Generally, addition of a salt decreases the solubility of analytes in the aqueous sample and enhances their partitioning into the organic phase (in LLE). Different concentrations of sodium chloride ranging from 0 to 9% (w/v) were added into the sample solution. Each experiment was performed in quintuplicate. The results demonstrated an improvement on the formation of floated drop and extraction efficiency for all the analytes up to 4.0% (w/v) while for concentration higher than 4% they decreased gradually. Therefore 4% NaCl (w/v) was used in further experiments.

3.5. Effect of pH

Starting from a pH=2 solution obtained after trichloroacetic acid addition for protein precipitation, the effects of the sample pH on the performance of AA-DLLME-SFO were tested at various pH within the range of 2-9 by adding sodium hydroxide and keeping constant the other experimental conditions. According to these experiments the peak area of the investigated analytes had a slightly increase within 2 to 7 while from 7 to 9 the peak area for both the analytes sharply declined. According to the results, the pH 7 was chosen as the optimum pH value for further studies.

276 *3.6. Optimization of the UHPLC-PDA conditions*

277

278 During the method development four reversed-phase columns Waters BEH C_{18} (75 x 2.1mm I.D.,
279 2.6 μm), Fortis Speedcore C_{18} (100 x 4.6 mm I.D., 2.6 μm), Kinetex EVO C_{18} (100 x 4.6 mm I.D., 2.6
280 μm) and Poroshell SB C_{18} (50 x 2.1mm I.D0,. 2.6 μm) were tested with different mobile phase
281 compositions. Good separation of all analytes and internal standard was obtained using a
282 Poroshell SB C_{18} column. This semi-porous silica column (packed with core-shell particles) provided
283 a shorter diffusion path for analytes, minimised peak broadening and had a high permeability. In
284 comparison to the results obtained by using other columns, the application of Poroshell SB C_{18}
285 provided a higher separation efficiency. Hence, a quicker analysis of all the analytes with good
286 resolution and peak shape was possible. In order to achieve the efficient separation of the
287 analysed compounds and the IS, different mobile phases (methanol, acetonitrile, water with
288 additive ammonium acetate) firstly in isocratic mode, then with a gradient program, were tested.
289 Isocratic separation was found to be unsuitable for the selected compounds due to their different
290 chromatographic properties resulting in a long run-time analysis. It was determined that the
291 optimal mobile phase consisted of a mixture of methanol- acetonitrile and 10 mM ammonium
292 acetate buffer (pH 4.0) in a gradient elution program as reported in Section 2.2. Gradient elution is
293 typically used to improve the peak separation and eliminate significant matrix effects due to co-
294 elution of the analyte and the endogenous plasma components. Furthermore in order to increase
295 the peak shape, different triethylamine percentages in the range of 0.05 and 1.0 % (v/v) were
296 added to the mobile phase. The optimum was achieved by using 0.4% (v/v) of triethylamine.
297 Under this condition, the peak shape of the analytes was symmetric and the analytes were well
298 separated. To detect the analytes with high sensitivity, each drug was monitored at its maximum
299 wavelength. By applying the chromatographic condition herein reported, the total run time in this
300 assay was 5.0 min and the retention times for, metronidazole, meropenem, ciprofloxacin, linezolid
301 and piperacillin were 1.13(±0.02), 1.76 (±0.04), 2.32 (±0.04), 2.79 (±0.03) and 3.48 (±0.01)
302 respectively, while was 2.56 (±0.02) min with consistently excellent reproducibility of less than
303 1.8% for the internal standard. The resolution of the developed UHPLC-PDA method was
304 satisfactory, as all analytes were well base-line separated. Chromatograms are shown in Fig 3.

305

306 *3.7. Method validation*

All calibration curves in human plasma for the investigated analytes, obtained by plotting concentration (μg mL^{-1}) versus drug-internal standard peak area ratio, were linear over the range tested for each analyte (Table 1). In order to describe the relationship between the concentration of the analytes and the detector response, a linear regression analysis with weighting factors consisting in $1/x^2$ values was used. The limits of detection (LODs) and quantification (LOQs) of the method are reported in Table 1, while the chromatogram near the LLOQ are reported in figure 4. The data for intra- and inter-day precision and accuracy, obtained from the analysis of three batches of LLOQ and the quality control (QC) samples at three different levels of analytes (LLOQ, LQC, MQC and HQC) in duplicate on the same day and for five consecutive days, are shown in Table 2. The intra- and inter-day precision (RSD values) did not exceed 9.87 %, the intra- and inter-day accuracy (BIAS %) varied between -8.14 and +9.34%. According to the guidelines [32-33], these results suggested that the method assessed in this study had satisfactory accuracy, precision and repeatability. Carry over was investigated on column by injecting into the UHPLC two extracted blank plasma spiked with the analytes at the ULOQ concentration, followed by, at least, three extracted blank biological samples. Non significant carry over effect (< 0.5%) was evident. Dilution integrity was demonstrated by spiking the matrix with concentration above the ULOQ and diluting the sample with blank matrix. Three dilutions (1:4, 1:3 and 1:2) with the blank matrix were evaluated, with at least five determinations for each dilution factor. For all the evaluated dilutions accuracy was within ± 8%, while precision, expressed as relative standard deviation, was less than 6%. SST was performed under optimised chromatographic conditions, results are shown in Table 3.

The stability of the investigated analytes was determined under different storage conditions in the studied matrix using the QC samples, which were analysed immediately after being prepared and after applying the storage conditions to evaluate (temperature, freeze-thaw cycles, short-term and long-term storage). QC samples were analysed against a calibration curve and the concentrations obtained were compared with the nominal concentrations. The stock solution was stable at room temperature for 48 h and at 4 ◦C for two weeks. The analytes 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 the investigated analytes in extracts was confirmed after 24 h storage at 4 ◦C.

3.8. Comparison of the proposed method with existing methods

The performance of the proposed extraction method based on AA-DLLME-SFO was compared with the previously reported extraction methods for the analysis of the selected antibiotics. As can be seen in Table 4, the proposed method proved better LOQ, RSD% , EF and extraction time compared with the other methods. The enrichment factor in AA-DLLME-SFO was very high and the extraction time was very short, compared to literature reports. The main advantages of AA-DLLME-SFO were the use of solvents with densities lower than water and with a lower toxicity compared to classical DLLME solvents. Furthermore extraction solvent in DLLME is not compatible with reverse-phase HPLC and evaporation is required after the extraction.

3.9. Application in real samples

Real blood samples were collected from patients at "*S.S. Annunziata*" Hospital (Chieti, Italy) 4h after an intravenous administration of metronidazole (Deflamon 500 mg three times daily), meropenem (Merrem 1 g three times daily), linezolid (Zyvox 600mg twice daily), ciprofloxacin (Ciproxin 400mg twice daily) and piperacillin (Tazocin 4g three times daily) at the same time as routine TDM samples for plasma and did not require extra visits to the clinic. Samples were drawn in the morning and stored in glass tubes containing EDTA as anticoagulant until been centrifuged at 1300*g* for 10 min at 4°C. Then, the supernatant (plasma) were subjected to the proposed AA-DLLME-SFO method. All human plasma samples were analyzed within 48h from the collection (Fig 3d). The proposed AA-DLLME-SFO followed by UHPLC with PDA detection was successfully applied to the simultaneous determination of meropenem, metronidazole, ciprofloxacin, linezolid and piperacillin in human plasma. Log P, pKa and therapeutic index of the investigated drugs are reported in supplementary table 1.

4. Conclusions

In this study the protein precipitation was combined with the AA-DLLME-SFO for the determination of antibiotics in human plasma using UHPLC-PDA. This combination not only resulted in a high enrichment factor but also offered numerous advantages such as simplicity, ease of operation, low detection limits , short analysis time using extraction solvent with lower toxicity instead of highly toxic solvents. Adequate accuracy, repeatability and linearity demonstrated that

371 the proposed method is feasible for quantitative analysis in real plasma samples. As far as we
372 know, no papers have been published for the simultaneous analysis of these drugs in human
373 plasma sample using AA-DLLME-SFO coupled with UHPLC-PDA.
374
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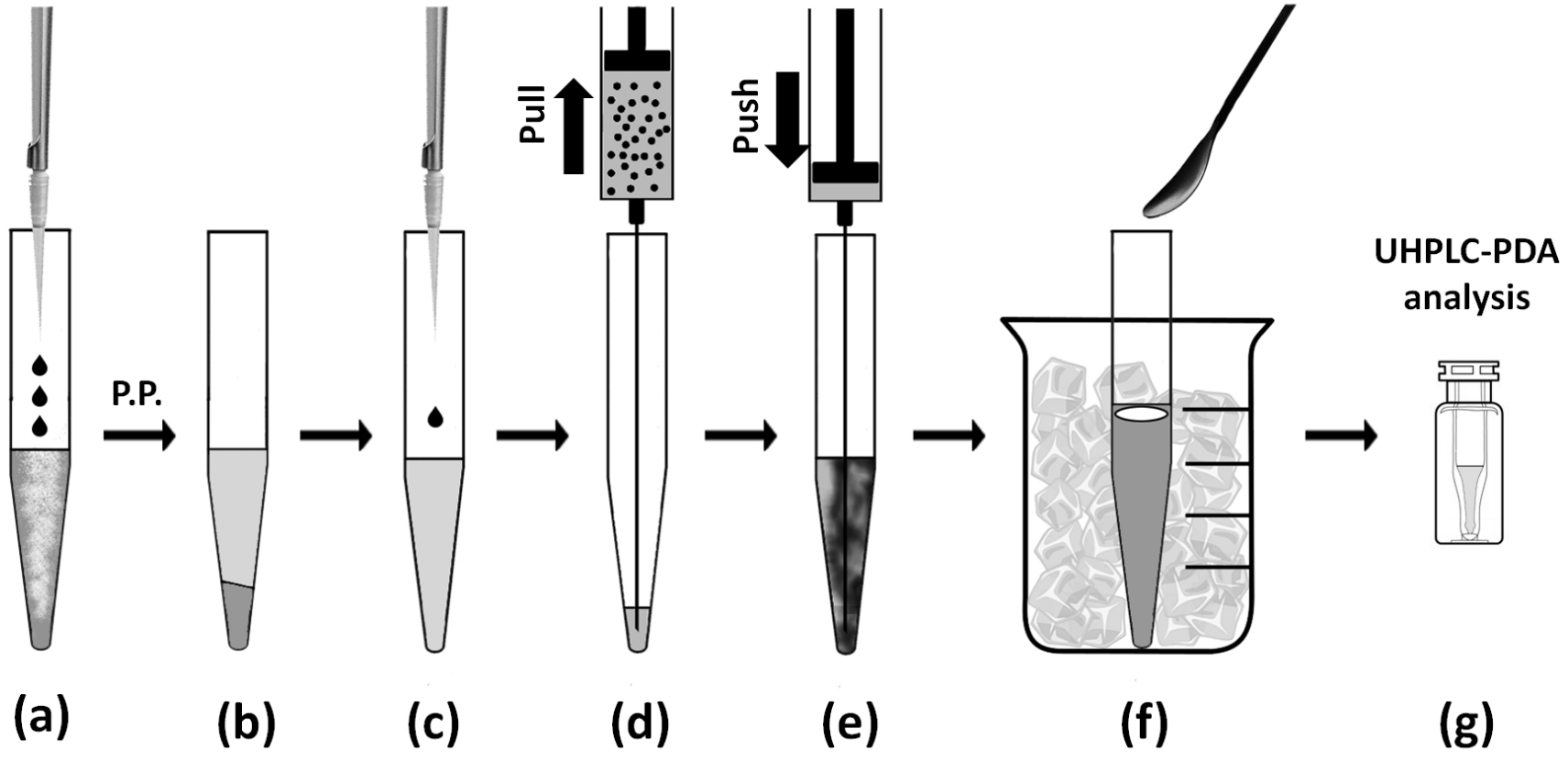
Figure captions

Figure 1: The experimental procedure of the proposed AA-DLLME-SFO in human plasma. (a) to 2 mL of human plasma was added 2 mL of a mixture containing 15% (w/v) of zinc sulfate and 20% of trichloroacetic acid solutions (60/40, v/v), then the glass tube was vortexed and centrifuged at 5000rpm for 5 minutes. (b) The supernatant was collected in another glass test tube. (c) The extraction solvent was added into the supernatant. (d) The Sample was pulled into a 10 mL glass syringe from the tube. (e) The mixture was pushed out via syringe needle into the tube forming a cloudy solution. (f) The extraction solvent droplets floating on the surface after solidification were solidified in an ice bath, collected and transferred. The extract was analyzed using UHPLC-PDA.

Figure 2: Effect of extraction solvent. Extraction conditions: sample volume 4 mL; extraction solvent volume, 30 µL; extraction cycles, 8, NaCl concentration, 4% (w/v)

Figure 3: Chromatograms from human plasma subjected to the AA-DLLME-SFO method at 275 nm. (a) a blank plasma spiked with 2.5 µg mL^{-1} of internal standard; (b) a blank plasma; (c) a blank plasma spiked with MET (2 µg mL^{-1}), MER (1 µg mL^{-1}), CIP (3 µg mL^{-1}), LIN (10 µg mL^{-1}), PIP (25 µg mL^{-1}) and I.S. (2.5 µg mL^{-1}) and (d) a human plasma sample collected 4h after an intravenous administration of metronidazole (Deflamon 500 mg three times daily), meropenem (Merrem 1 g three times daily), linezolid (Zyvox 600mg twice daily) and ciprofloxacin (Ciproxin 400mg twice daily) and piperacillin (Tazocin 4g three times daily).

Figure 4: Chromatograms from human plasma subjected to the AA-DLLME-SFO method at 275 nm at concentration near to LLOQ.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analysis
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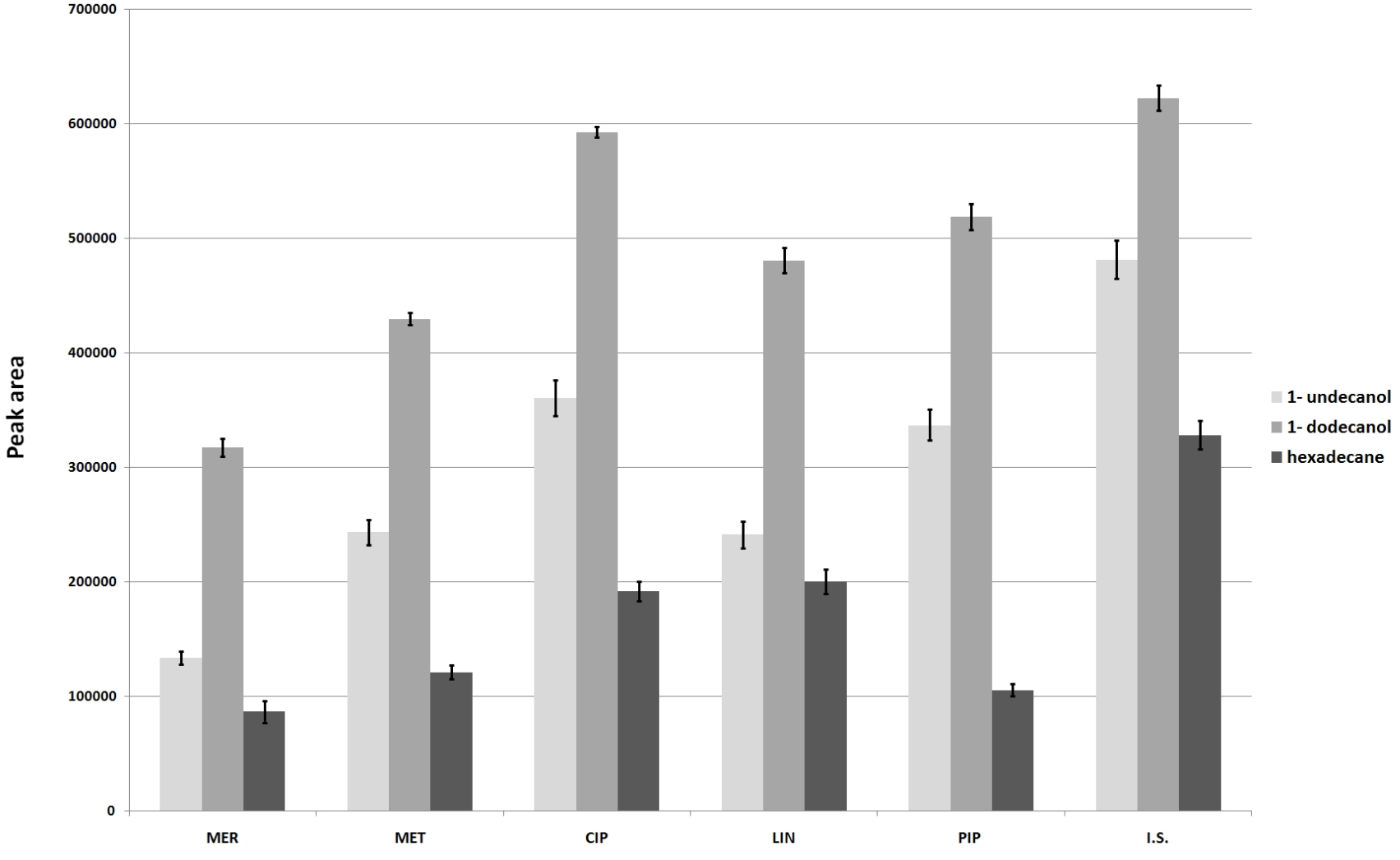
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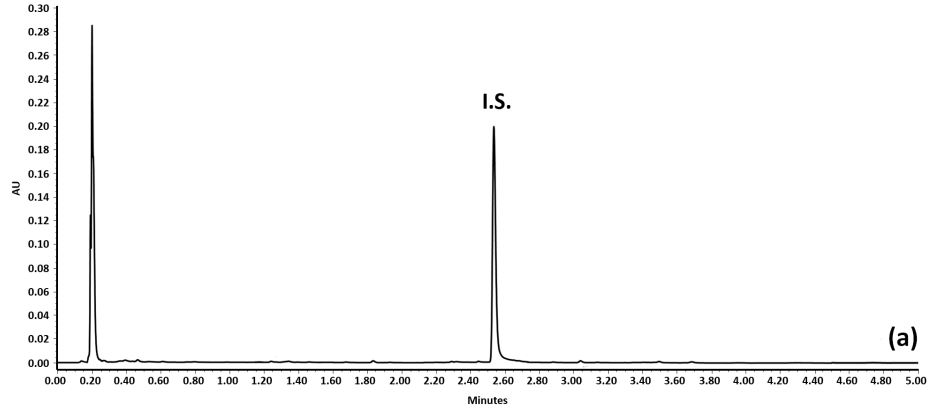
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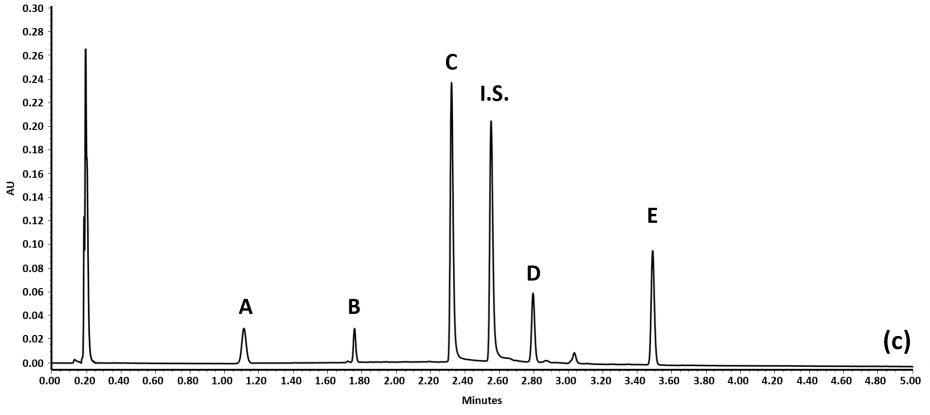
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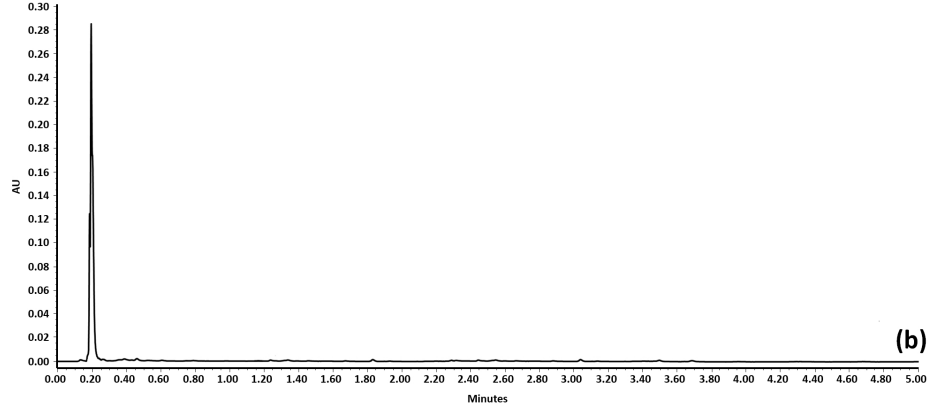
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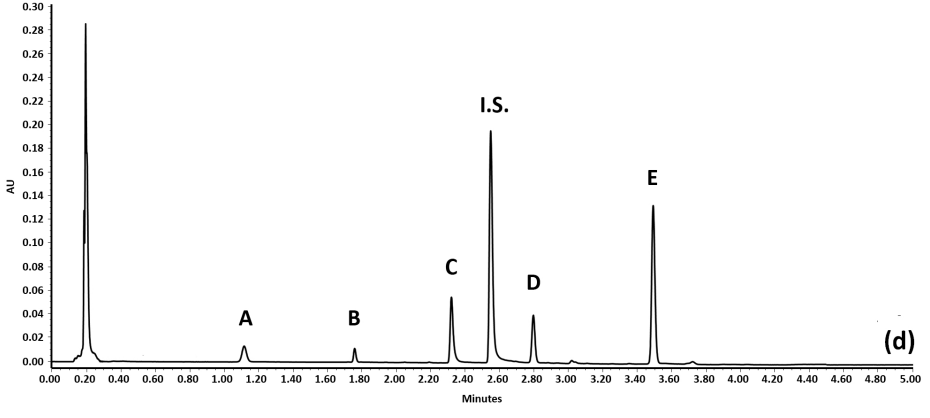
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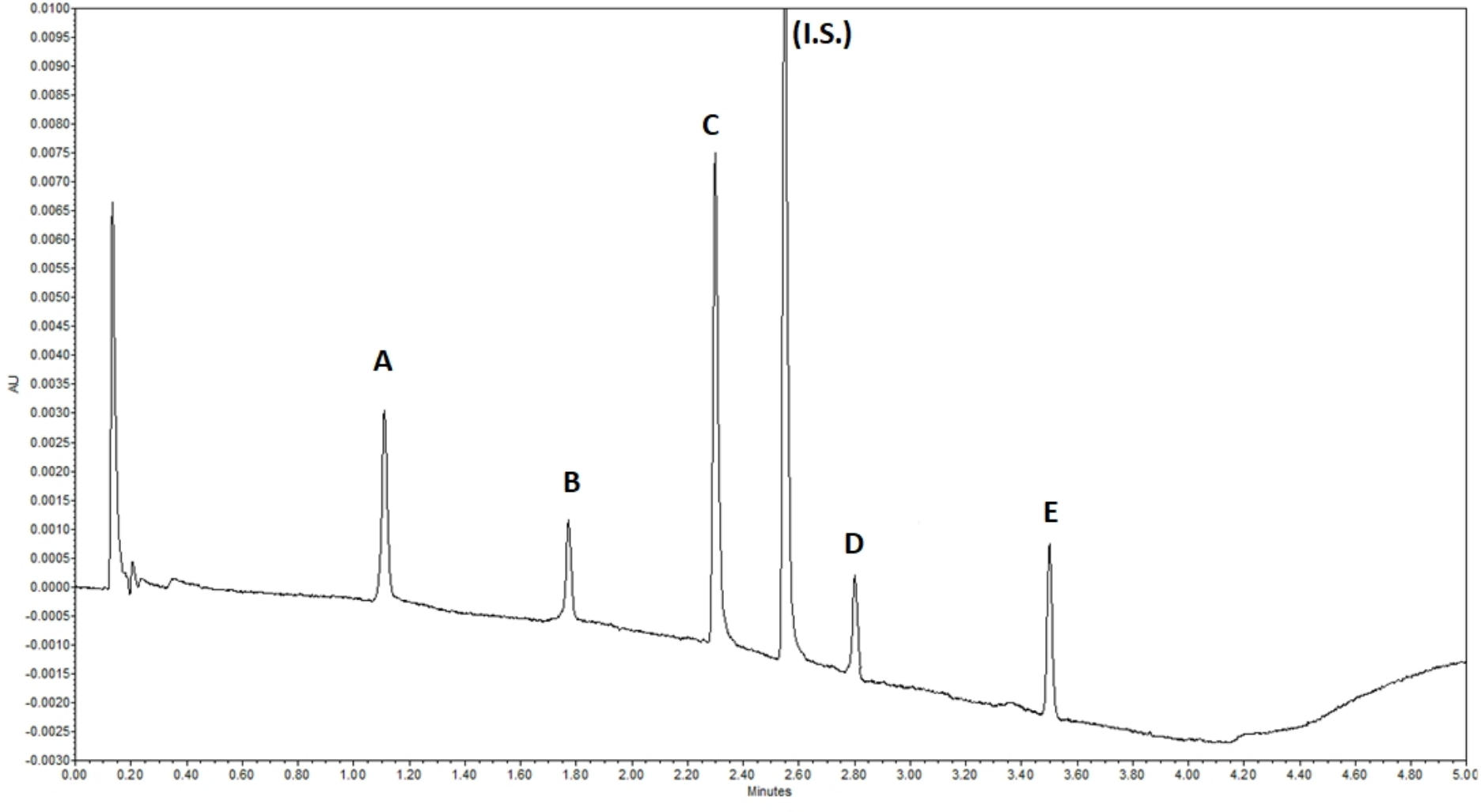
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Table 1: Analytical data of the AA-DLLME-SFO-UHPLC-PDA method

	Meropenem	Metronidazole	Ciprofloxacin	Linezolid	Piperacillin
λ_{max}[a] (nm)	300	315	277	251	235
Conc. Range (µg mL^{-1})	0.025 – 35.0	0.020 – 30.0	0.005 - 10	0.025 - 30	0.25 - 50
Intercept (± SD)	-0.0021 (± 0.0004)	-0.0037 (± 0.0007)	0.0010 (± 0.0001)	0.0013 (± 0.0004)	-0.0041 (± 0.0009)
Slope (± SD)	0.0396 (± 0.0008)	0.0549 (± 0.0008)	0.341 (± 0.0008)	0.0987 (± 0.0008)	0.0244 (± 0.0008)
r^2	0.9997	0.9997	0.9998	0.9996	0.9998
LOQ (µg mL^{-1})	0.025	0.020	0.005	0.025	0.25
LOD (µg mL^{-1})	0.008	0.006	0.001	0.008	0.08

[a] wavelenght of the PDA detection

Table 2: Precision and accuracy assays

Analyte	Concentration (µg mL^{-1})	Accuracy (BIAS%)		Precision (RSD%)	
		Intra-day	Inter-day	Intra-day	Inter-day
MER	LLOQ (0.025)	+5.36 ± (0.45)	-7.54 ± (0.75)	8.45	9.87
	QCL (0.050)	-4.25 ± (0.31)	-5.53 ± (0.19)	7.32	3.47
	QCM (1.00)	+1.12 ± (0.45)	+4.12 ± (0.45)	1.15	2.57
	QCH (30.0)	+0.74 ± (0.02)	+1.98 ± (0.11)	3.34	5.57
MET	LLOQ (0.020)	-4.17 ± (0.13)	+8.98 ± (0.61)	3.21	6.78
	QCL (0.050)	+7.13 ± (0.31)	+9.34 ± (0.67)	4.48	7.14
	QCM (1.00)	-3.13 ± (0.10)	-5.55 ± (0.25)	3.15	4.57
	QCH (25.0)	-1.45 ± (0.03)	+3.21 ± (0.10)	2.10	3.19
CIP	LLOQ (0.005)	-3.14 ± (0.24)	+2.54 ± (0.22)	7.71	8.86
	QCL (0.010)	-2.15 ± (0.09)	+6.28 ± (0.45)	4.13	7.10
	QCM (0.75)	-4.68 ± (0.10)	-1.48 ± (0.10)	2.13	6.89
	QCH (7.50)	+3.13 ± (0.02)	+3.18 ± (0.10)	0.47	3.12
LIN	LLOQ (0.025)	-8.14 ± (0.50)	+6.47 ± (0.61)	6.14	9.47
	QCL (0.050)	+7.71 ± (0.16)	-4.54 ± (0.15)	2.14	3.14
	QCM (1.00)	+1.57 ± (0.05)	+2.98 ± (0.10)	3.14	3.39
	QCH (25.0)	-3.54 ± (0.06)	+4.54 ± (0.29)	1.78	6.41
PIP	LLOQ (0.25)	+1.15 ± (0.05)	+2.34 ± (0.13)	4.14	5.54
	QCL (0.50)	+0.69 ± (0.01)	-2.14 ± (0.10)	1.36	4.79
	QCM (5.0)	-1.58 ± (0.08)	+2.45 ± (0.11)	5.47	4.17
	QCH (45.0)	+2.54 ± (0.08)	+3.45 ± (0.07)	3.17	2.10

Table 3: Summary of the system suitability test

Analyte	t_R[a]	α[b]	*k*[c]	Rs[d]	As[e]	N[f]
Metronidazole	1.13	1.56	5.28	3.94	1.07	5670
Meropenem	1.76	1.31	8.78	3.73	1.12	8100
Ciprofloxacin	2.32	1.10	11.89	2.16	1.13	8610
Ulifloxacin (I.S.)	2.56	1.10	13.22	2.21	1.13	16380
Linezolid	2.79	1.24	14.50	5.75	1.07	19460
Piperacillin	3.48	-	18.33	-	1.09	30270

[a] Retention time (min), [b] Selectivity, [c] Retention factor, [d] Resolution, [e] Asymmetry

[f] Theoretical plates

Table 4. Comparison of the proposed method with other methods.

Method	Analytes	Matrix	Extraction time (min)	LOD (µg mL^{-1})	Instrumentation	Run time	Reference
MEPS	CIP,LIN	Human plasma	10	0.01,0.02	UHPLC-DAD	3	[17]
P.P.	MER, LIN, PIP	urine, serum, CSF [a], Bronchial aspiration	10	0.50,0.25,0.15	UHPLC-MS	5	[18]
P.P	MER,MET	Plasma	25	0.1,0.05	LC-MS/MS	11	[19]
P.P. with evaporation	MER, LIN	bacterial growth media	50	0.5, 0.5	HPLC-DAD	15	[20]
SPE	CIP,PIP	Human plasma	70	0.5, 2.5	HPLC-DAD	20	[21]
P.P.	PIP,MER,LIN	PLASMA	20	1.5	UHPLC-MS/MS	5	[22]
P.P.	PIP,MER,CIP, LIN	SERUM	ND	ND	UHPLC-MS/MS	5	[23]
MEPS	MERO, LIN, LEVO	Plasma	10	0.02, 0.01, 0.01	UHPLC-DAD	5	[34]
AA-DLLME-SFO	MER,MET, CIP,LIN,PIP	Human plasma	8	0.025,0.020,0.005,0.025,0.25	UHPLC- DAD	5	Current paper

[a]Cerebro-spinal fluid

Supplementary table 1: Log P, pKa, trade names and therapeutic index of the investigated drugs

Analyte	Trade name	Log P	pKa	Therapeutic range(µg/mL)
Meropenem	Merrem	-0.69	3.47	2-35
Metronidazole	Deflamon	-0.15	2.6	15-25
Ciprofloxacin	Ciproxin	-0.57	5.9	0.2-4
Linezolid	Zyvox	0.61	14.45	2-10
Piperacillin	Tazocin	0.67	3.49	18-60