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Determination of meropenem in plasma and filtrate-dialysate from patients under continuous veno-venous haemodiafiltration by SPE-LC

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Abstract

Meropenem, a carbapenem antibiotic displaying a broad spectrum of antibacterial activity, is administered in Medical Intensive Care Unit to critically ill patients undergoing continuous veno-venous haemodiafiltration (CVVHDF). However, there are limited data available to substantial rational dosing decisions in this condition. In an attempt to refine our knowledge and propose a rationally designed dosage regimen, we have developed a HPLC method to determine meropenem after solid-phase extraction (SPE) of plasma and dialysate fluids obtained from patients under CVVHDF. The assay comprises the simultaneous measurement of meropenem's open-ring metabolite UK-1a, whose fate has never been studied in CVVHDF patients. The clean-up procedure involved a SPE on C18 cartridge. Matrix components were eliminated with phosphate buffer pH 7.4 followed by 15:85 MeOH–phosphate buffer pH 7.4. Meropenem and UK-1a were subsequently desorbed with MeOH. The eluates were evaporated under nitrogen at room temperature (RT) and reconstituted in phosphate buffer pH 7.4. Separation was performed at RT on a Nucleosil 100–5 μm C18 AB cartridge column (125 × 4 mm I.D.) equipped with a guard column (8 × 4 mm I.D.) with UV–DAD detection set at 208 nm. The mobile phase was 1 ml min^{-1}, using a step-wise gradient elution program: %MeOH/0.005 M tetrabutylammonium chloride pH 7.4; 10/90–50/50 in 27 min. Over the range of 5–100 μg ml^{-1}, the regression coefficient of the calibration curves (plasma and dialysate) were >0.998. The absolute extraction recoveries of meropenem and UK-1a in plasma and filtrate-dialysate were stable and ranged from 88–93 to 72–77% for meropenem, and from 95–104 to 75–82% for UK-1a. In plasma and filtrate-dialysate, respectively, the mean intra-assay precision was 4.1 and 2.6% for meropenem and 4.2 and 3.7% for UK-1a. The inter-assay variability was 2.8 and 3.6% for meropenem and 2.3 and 2.8% for UK-1a. The accuracy was satisfactory for both meropenem and UK-1a with deviation never exceeding 9.0% of the nominal concentrations. The stability of meropenem, studied

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in biological samples left at RT and at +4 °C, was satisfactory with < 5% degradation after 1.5 h in blood but reached 22% in filtrate-dialysate samples stored at RT for 8 h, precluding accurate measurements of meropenem excreted unchanged in the filtrate-dialysate left at RT during the CVVHDF procedure. The method reported here enables accurate measurements of meropenem in critically ill patients under CVVHDF, making dosage individualisation possible in such patients. The levels of the metabolite UK-1a encountered in this population of patients were higher than those observed in healthy volunteers but was similar to those observed in patients with renal impairment under hemodialysis.

Keywords: Meropenem; Solid-phase extraction; Carbapenem stability; Continuous haemodiafiltration; CVVHDF

1. Introduction

Meropenem (1 in Fig. 1), a new carbapenem antibiotic with broad spectrum of antibacterial activity and good stability to β-lactamases, is effective in the treatment of a wide range of infections including gram-positive and -negative bacteria [1]. Unlike imipenem, meropenem is stable against renal dehydropeptidase I (DHP-I) [2] and does not need to be administered with a DHP-I enzyme inhibitor such as cilastatin [3]. Meropenem has therefore, advantages in Intensive Care Unit (ICU), notably in critically ill patients with renal failure, a population at risk for accumulating co-administered drugs or metabolites.

Meropenem shows a good tolerability at high doses and a low incidence of seizures that makes it particularly useful in treating serious infections where large doses of antibiotics are required [4].

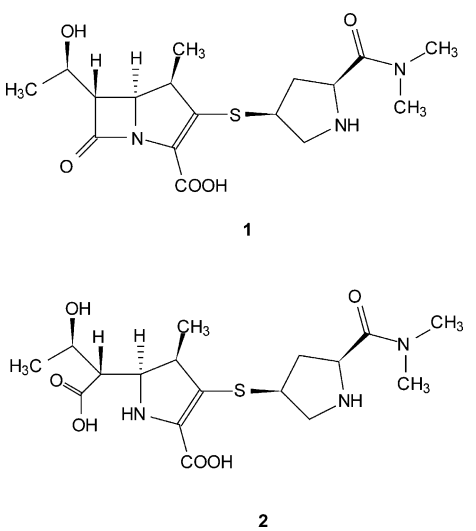

Fig. 1. Structure of meropenem (1) and its open-ring metabolite UK-1a (2).

Meropenem is metabolised into the microbiologically inactive open ring metabolite UK-1a, (2 in Fig. 1) and cleared mainly through renal excretion [5]. In healthy volunteers, 70% of the administered dose is excreted unchanged in urine and 20% as the metabolite UK-1a [6]. The pharmacokinetics of meropenem are similar to other parenteral carbapenems with low protein binding and predominant renal excretion.

In critically ill patients under continuous veno-venous haemodiafiltration (CVVHDF), most antibiotics, included meropenem, are given at a standard fixed mg kg^{-1} dosage, determined on an empirical basis. The patients' pathophysiological status (organs blood flow, residual renal function) and the filter performance characteristics are generally not considered. To propose a rational and individualised dosage regimen, an accurate assay of meropenem in biological fluids was therefore required. In addition, even though a few reports have appeared on the fate of the open-ring metabolite of meropenem in patients with end-stage renal disease under haemodiafiltration [7,16,26], no information were available on the extent of circulating UK-1a in the special population of CVVHDF patients. The high concentration of circulating metabolite reported in subjects with renal insufficiency suggest indeed that hydrolysis is higher in such patients and that the renal excretion of the metabolite is an important albeit *slow* process [21,26,7,16].

Several techniques have been reported for the determination of meropenem and its main metabolite in biological fluids, using either HPLC or microbiological methods for meropenem, and radioimmunoassay (RIA) or HPLC for UK-1a [7–10]. In these studies, samples were either di-

rectly injected after dilution and filtration [6–8], or were subjected to a preliminary protein precipitation step with acetonitrile [9–11,31], or with acetonitrile followed by a clean-up with dichloromethane [12,30], or after protein precipitation with trichloroacetic acid [13,32]. Other alternate methods for the assay of meropenem imply either the solid phase extraction [14–21,24] or column switching [22,23] approaches. The open-ring metabolite can be analysed separately in plasma by HPLC at 215 [26] or at 220 nm in urine [17] but is generally measured in plasma by RIA [6,7,16,17], notably using a highly specific ^{125}I-radiotracer [27]. This UK-1a radiotracer was not available to us however, but since the occurrence of relatively high level of UK-1a had been previously reported in patients with renal insufficiency, with or without hemofiltration [7,16,21,25,26], the simultaneous determination of the open-ring metabolite in biological fluids from CVVHDF patients was considered. In fact, the assay of meropenem in the complex matrix samples encountered with patients under CVVHDF has been only occasionally reported, involving either a bioassay [26], or the sample dilution and filtration [8], or after a protein precipitation step by acetonitrile [11], or after solid-phase extraction (SPE) [16,21], but the analytical method validation is generally not detailed. In addition, the measurement of UK-1a by HPLC has been reported only once in samples from patients with end-stage renal disease [26].

The aim of our work was therefore, to develop a new HPLC method to determine simultaneously meropenem and its metabolite UK-1a, in plasma and dialysate fluids of ICU patients under CVVHDF.

2. Experimental

2.1. Chemicals

Meropenem, (4*R*,5*S*,6*S*)-3-[[(3*S*,5*S*)-5-(dimethylcarbamoyl)-3-pyrrolidinyl]thio]-6-[(1*R*)-1-hydroxyethyl]-4-methyl-7-oxo-1-azabicyclo[3,2,0]hept-2-ene-2-carboxylic acid was generously supplied by Zeneca Pharmaceutical (Wilmington, DE, USA) and its open-ring metabolite UK-1a, also designated ICI-213689 [7,8], or ZM-213689 [27], by Analytical Science Group (Osaka, Japan). Tetrabutylammonium chloride (TBA) and 8-chlorotheophylline (used as internal standard, I.S.) were purchased from Sigma Chemie (Buchs, Switzerland). Potassium dihydrogenphosphate p.a., dinatrium hydrogenphosphate dihydrate p.a. and methanol (MeOH) for chromatography LiChrosolv® were from E. Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q® UF-Plus apparatus (Millipore).

2.2. Chromatographic system

The chromatographic system consisted of a Hewlett-Packard 1050 (Hewlett-Packard, Germany) connected to an HP 1050 online degasser, an HP 1050 autosampler and an HP 1050 diode array detector set at UV 208 nm. The software HPChemStation B.02.04 loaded on an HP Vectra 486/33N was used to pilot the HPLC instrument and to process the data (area integration, calculation and plotting of chromatograms) throughout the method validation and samples analysis. Baselines were visually inspected and were manually adjusted using peak start and end features of the software HPChemStation.

In the optimised series of analysis, the mobile phase was delivered at 1 ml min^{-1}, with the following step-wise gradient elution program: % MeOH/0.005 M TBA pH 7.4; 10/90 at 0 min → 10/90 at 10 min, → 50/50 at 27 min, → 10/90 at 28 min, → 10/90 at 35 min (the 28–35 min elution corresponds to the re-equilibration step).

The separations were performed on a ChromCart® cartridge column (125 × 4 mm I.D.) Nucleosil 100–5 μm C18 AB (Macherey-Nagel, Düren, Germany) equipped with a guard column (8 × 4 mm I.D.) filled with the same packing material. The injection volume was 30 μl in this study.

2.3. Solutions

The phosphate buffer solution was prepared by mixing 197 ml of a 1/15 M potassium dihydrogenphosphate solution (9.07 g dissolved in 1000.0 ml

of ultrapure water) and 803 ml of a 1/15 M di-natrium hydrogenphosphate solution (11.87 g dissolved in 1000.0 ml of ultrapure water). The pH was adjusted to 7.4 with HCl 2 M.

The buffered TBA 0.005 M solution was prepared by adding 1.39 g of TBA to 1000.0 ml of the stock solution of phosphate buffer and the pH adjusted to 7.4 with HCl 2 M.

2.4. Stock solution, standard and control samples

2.4.1. Calibration and control standard in plasma

Stock standard solutions were prepared by dissolving meropenem (20 mg) and UK-1a (20 mg) in 10 ml of ultrapure water to get a 2.0 mg ml^{-1} concentration for both components. Plasma calibration standards at 5–100 μg ml^{-1} concentrations of meropenem and UK-1a, along with plasma control samples at 15, 30, 60 μg ml^{-1} were prepared in batches of 10 ml by adding appropriate volumes of the respective stock solutions to blank plasma from outdated transfusion bags (total added volume $\leq 10\%$ of the biological sample volume, in accordance with the literature, [11]). Calibration standards and control samples were stored as 500 μl aliquots in polypropylene Eppendorf tubes at -80 °C until use and thawed the day of analysis.

The compound 8-chloro-theophylline has previously been used as internal standard for the HPLC analysis of the β-lactam ceftazidime in biological fluids [29] and was used in this study at the concentration of 125 μg ml^{-1} in ultrapure water.

2.4.2. Calibration and control standard in dialysate

Dialysate calibration standards at 5–100 μg ml^{-1} concentration of meropenem and UK-1a, along with control samples at 15, 30 and 60 μg ml^{-1}, were prepared in batches of 10 ml by adding an appropriate volume of the meropenem and UK-1a stock solutions to a pool blank dialysate obtained from one patient under CVVHDF who was allergic to β-lactams antibiotics and therefore, had not received meropenem nor structurally related drugs. These standards were stored similarly to plasma calibration standards.

2.5. Plasma and dialysate collection

Blood and dialysate samples were obtained from patients undergoing CVVHDF in the medical or surgical ICU, according to a clinical protocol approved by the Ethics Committee of the University Hospital.

To prevent blood clogging into the haemodiafiltration machine (PRISMA CFM™, Hospal, Belgium), sodium heparinate (Liquemin®, Roche, Switzerland) was continuously infused into the incoming (arterial) blood stream. Blood samples were collected from the haemodiafiltration device incoming (arterial) and outcoming (venous) blood stream into 4 ml serum Monovettes (Sarstedt, Nümbrecht, Germany) and stored at $+4$ °C before being centrifuged within 1.5 h at $900 \times g$ for 10 min at $+4$ °C. The plasma was immediately frozen at -80 °C in 2 ml microtubes (Sarstedt, Nümbrecht, Germany) until analysis.

Aliquots of dialysate were collected and stored at $+4$ °C before being frozen, within 1.5 h, at -80 °C. At the end of the 12 h period following the administration of the meropenem dose, an aliquot was taken from the whole filtrate-dialysate collected.

2.6. Sample preparation

The clean up procedure of biological samples (plasma and dialysates) was performed by SPE using the 24 tubes vacuum manifold Macherey-Nagel (Düren, Germany). The C18 Sep-Pak cartridges (Waters, Milford, MA, USA) were conditioned with 3×1 ml MeOH followed by 2×1 ml phosphate buffer pH 7.4. An aliquot (400 μl) of plasma or dialysate sample was mixed in a polypropylene Eppendorf vial with 50 μl of I.S. solution. A 200 μl volume of the resulting solution was loaded on the cartridges in duplicate and drawn through completely under light vacuum (typically 4 mm Hg). The cartridge was washed four times with 200 μl of phosphate buffer pH 7.4 solution and twice with 200 μl of 15% MeOH in phosphate buffer pH 7.4 solution. Meropenem and UK-1a were subsequently desorbed with four times 500 μl of MeOH. The eluted solutions were evaporated under a nitrogen

steam at room temperature and the residue was reconstituted in 100 μl of phosphate buffer pH 7.4.

The samples were introduced into 200 μl HPLC microvials (Hewlett-Packard, Germany) and a volume of 30 μl was used for HPLC analysis.

2.7. Calibration curves

Quantitative analysis of meropenem and UK-1a was performed using the internal standard (I.S.) method. Calibration curves were obtained by unweighted least-squares linear regression analysis of the peak ratio of meropenem and UK-1a to I.S., versus the ratio of the injected amount of meropenem, respectively UK-1a to I.S. in each standard solution.

2.8. Validation of the method

The calibration curve was determined at each level with two sets of calibration standards: one set at the beginning and the second at the end of the HPLC run. Throughout clinical sample analysis, control samples at the three concentration levels (15, 30 and 60 μg ml^{-1} of each compound for plasma and dialysate) were assayed every five samples. All samples were analysed in duplicate, with the duplication process starting at the clean-up procedure to detect variability associated with the SPE procedure.

The control samples were used for determination of precision and accuracy of the method, precision being calculated as the coefficient of variation (C.V.%) within a single run and between different assays, and accuracy as the percentage of deviation between nominal and experimental concentration calculated with the established calibration curves.

The absolute recovery (expressed in %) was calculated as the ratio of the peak area of meropenem and UK-1a in spiked plasma and dialysate samples subjected to SPE, to the peak area of the same amount of meropenem and UK-1a in aqueous solution directly injected onto the HPLC system.

The limit of quantitation (LOQ) and limit of detection (LOD) of the method—expressed in micrograms/injection—(30 μl injection volume) was experimentally determined by analysing dialysate and plasma samples ($n = 3$) spiked with meropenem and UK-1a at 5–0.64 μg ml^{-1} concentrations. The lower LOQ was chosen as the concentrations which provided measurements with a precision and accuracy within the recommended $\pm 20\%$ from their nominal values, in accordance with the guidelines of the Washington Conference [28].

2.9. Stability

The blood samples collected during the clinical study were stored temporarily—but no more than 1.5 h—in the fridge at $+4$ °C prior to their centrifugation. The stability of meropenem in blood was therefore, assessed in vitro at $+4$ °C as follows: heparinised blood samples spiked with an isotonic pharmaceutical iv formulation of meropenem (15 and 85 μg ml^{-1} in 0.9% NaCl) were stored in a fridge at $+4$ °C for 8 h. Aliquots (2 ml) were taken at 0, 1, 2, 4 and 8 h and immediately centrifuged at $900 \times g$ for 10 min at $+4$ °C. The plasma was frozen at -80 °C until the day of analysis.

Since dialysate samples were drawn simultaneously from the CVVHDF device, the same procedure—except for the centrifugation step—was used for aliquots of dialysate spiked with meropenem. Since the whole filtrate-dialysate was collected in a container left at RT during the continuous haemodiafiltration procedure, the stability of meropenem in this biological matrix was also assessed at RT over 8 h.

2.10. Clinical applications of the HPLC method

At the time at which this study was initiated, there was only limited information available on the dose adjustment of meropenem for patients with renal failure under CVVHDF [8,11,30–32]. A clinical study was therefore, initiated aiming at determining the pharmacokinetics of meropenem in this population of critically ill patients. This study aimed also to evaluate the fate of meropenem within the haemodiafiltration filter, and the UK-1a levels in this population of pa-

tients. A detailed report of this study will be published elsewhere. Briefly, 15 patients (62 ± 8 years, 72 ± 16 kg, 6 F), receiving meropenem at a 0.5–1.0 g dose every 8–12 h were included in the study. Blood samples were simultaneously drawn at timed intervals over the dosing period, from the incoming and outcoming blood lines of the filter, together with ultrafiltrate (UF) samples.

2.11. Graphics

Meropenem concentration profiles in venous and arterial plasma as well as in dialysate samples were plotted using GraphPad Prism 3.0.

3. Results

3.1. Chromatograms

The proposed method enables the simultaneous quantitation of meropenem and its open-ring metabolite UK-1a in plasma samples on the same HPLC run, as shown in the chromatograms of Fig. 2a–c. The detection at UV 208 nm provided a satisfactory selectivity and adequate sensitivity (down to 2.5 μg ml^{-1}) for meropenem. The metabolite UK-1a was however, eluted relatively early, even in the presence of the ion pairing agent TBA. Its signal, especially at low concentrations, could not always be totally resolved from minor peaks present in plasma, without however precluding an acceptable assessment of its concentration in patients (see infra).

The Fig. 2c shows the chromatographic profile of a plasma sample (onto which I.S. has been added) taken from a patient under CVVHDF, 40 min after starting a 30 min iv infusion of meropenem (22 mg kg^{-1}). The retention time for UK-1a, meropenem and I.S is 4.9, 13.8 and 20.2 min, respectively. The corresponding concentrations of meropenem and UK-1a in this sample are 46.9 and 6.9 μg ml^{-1}, respectively. As shown in the chromatogram of a blank plasma (Fig. 2a), no interfering peaks were observed at the retention time of meropenem and UK-1a.

The chromatographic profiles of a filtrate-dialysate sample, blank or spiked with meropenem and UK-1a, as well as a filtrate-dialysate sample taken from a patient at the end of the infusion, are shown in Fig. 3a–c, respectively. The concentrations of UK-1a and meropenem were 50 μg ml^{-1} for the spiked sample (b), and 4.7 and 33.1 μg ml^{-1}, respectively, in the patient's filtrate-dialysate (c).

3.2. Calibration curves

The standard curves for meropenem and UK-1a were satisfactorily described by unweighted least-squares linear regression analysis over the concentration range 5–100 μg ml^{-1} in plasma and filtrate-dialysate samples. Mean standard curve parameters obtained throughout the analyses of these series of samples are reported in Table 1. The parameters of the curves and the intercepts were stable. The mean regression coefficients r^2 were always ≥ 0.998 either for the plasma or for the filtrate-dialysate calibration curves.

3.3. SPE procedure

One of the critical issues during the method development was the *complexity* of the biological samples requiring preliminary sample purification by SPE. Several SPE cartridges were evaluated, among them, Supelclean LC18 (Supelco), and C18 Sep-Pak (Waters). The latter gave the best results in term of recovery (reliable adsorption of meropenem/UK-1a in aqueous buffers, and predictable elution of analytes with organic solvent) and clean up (elimination of undesirable matrix compounds perturbing the HPLC analy-

Fig. 2. (a) Chromatographic profile of a blank plasma. (b) Chromatographic profile of a plasma control at 50 μg ml^{-1} of UK-1a (I) and meropenem (II), spiked with I.S. (III, chlorotheophylline at 125 μg ml^{-1}). (c) Chromatographic profile of a plasma from a patient under CVVHD, 40 min after having been given meropenem 22 mg kg^{-1} iv over 30 min. Concentrations of UK-1a (I) and meropenem (II) were 6.9 and 46.9 μg ml^{-1}, respectively.

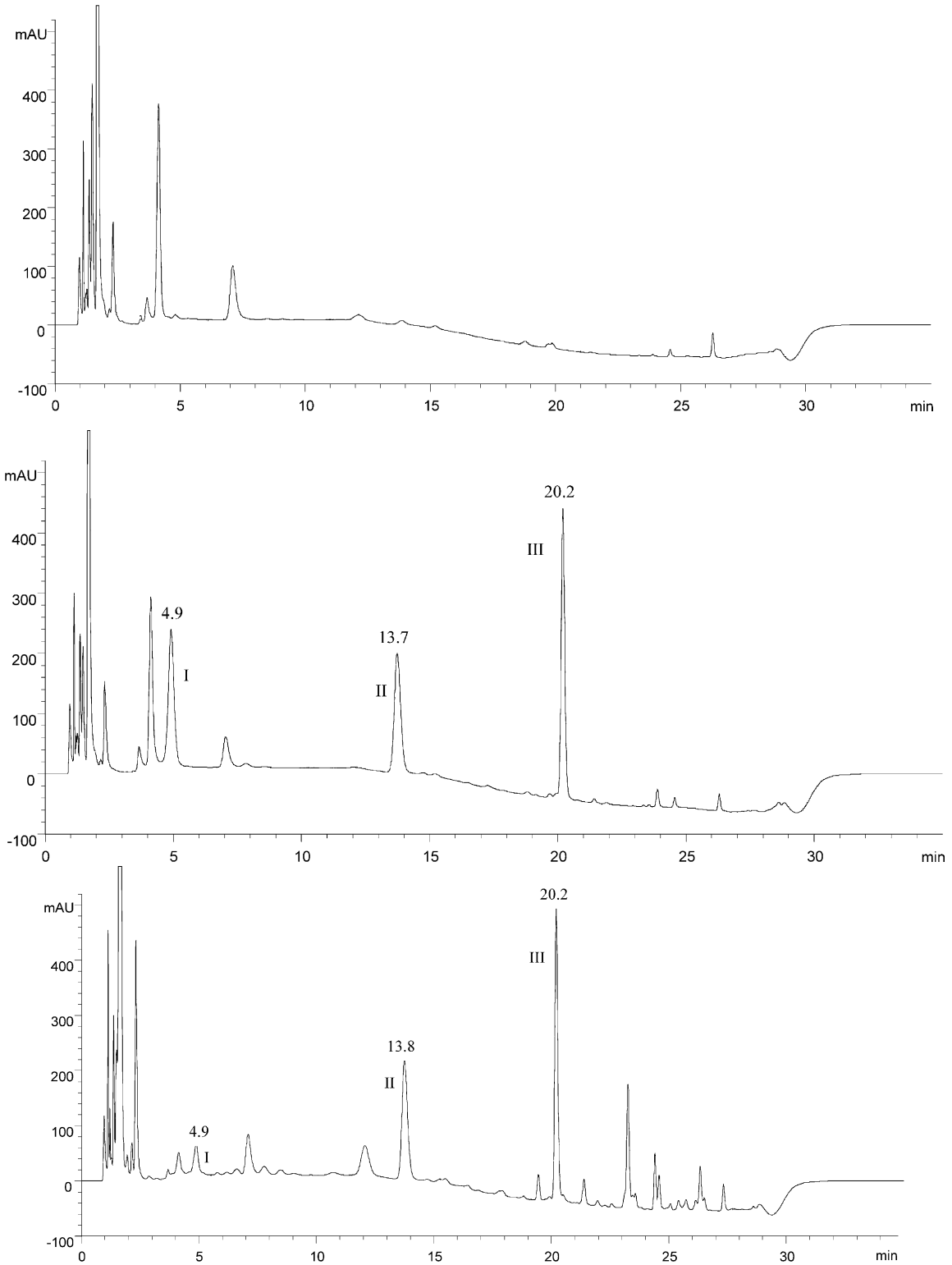
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Fig. 2.

sis). The clean-up procedure by SPE, applied to plasma and filtrate-dialysate samples, eliminated reliably most of the components from the relatively complex biological matrixes encountered in such patients. With an appropriate vacuum manifold, 24 samples could be processed simultaneously. After loading the sample on the cartridge, most of the components of the matrix were efficiently removed by washing the cartridges four times with 200 μl of phosphate buffer pH 7.4 and twice with 200 μl of 15% MeOH in phosphate buffer pH 7.4. Applying a larger washing volume caused some loss of meropenem and UK-1a due to premature elution.

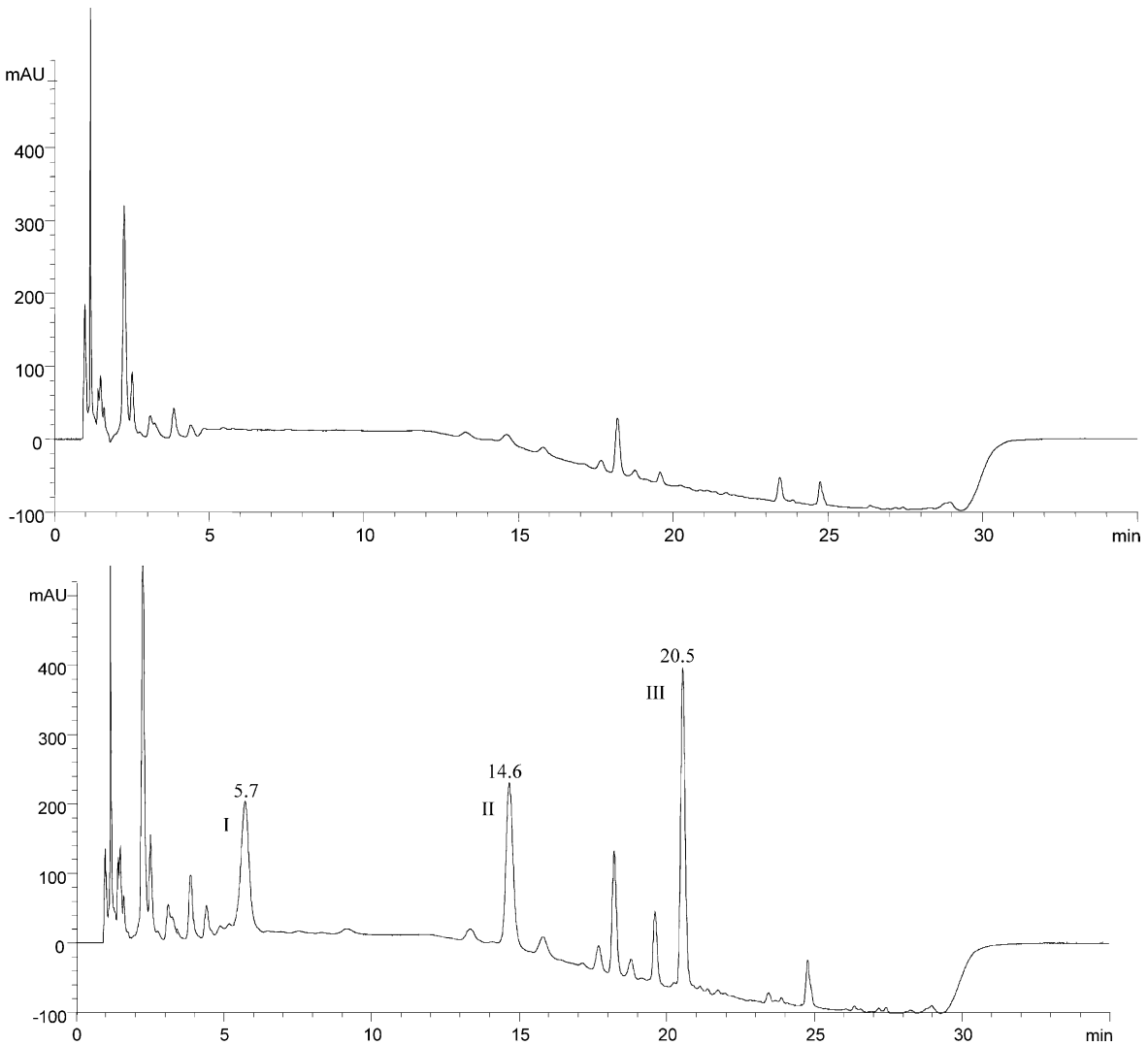

Fig. 3. (a) Chromatographic profile of a blank filtrate-dialysate sample. (b) Chromatographic profile of a filtrate-dialysate control sample at 50 μg ml^{-1} of UK-1a (1) and meropenem (II), spiked with I.S. (III, chlorotheophylline at 125 μg ml^{-1}). (c) Chromatographic profile of a filtrate-dialysate from a patient under CVVHD, at the end of the meropenem infusion. Concentrations of UK-1a (I) and meropenem (II) were 4.7 and 33.1 μg ml^{-1}, respectively.

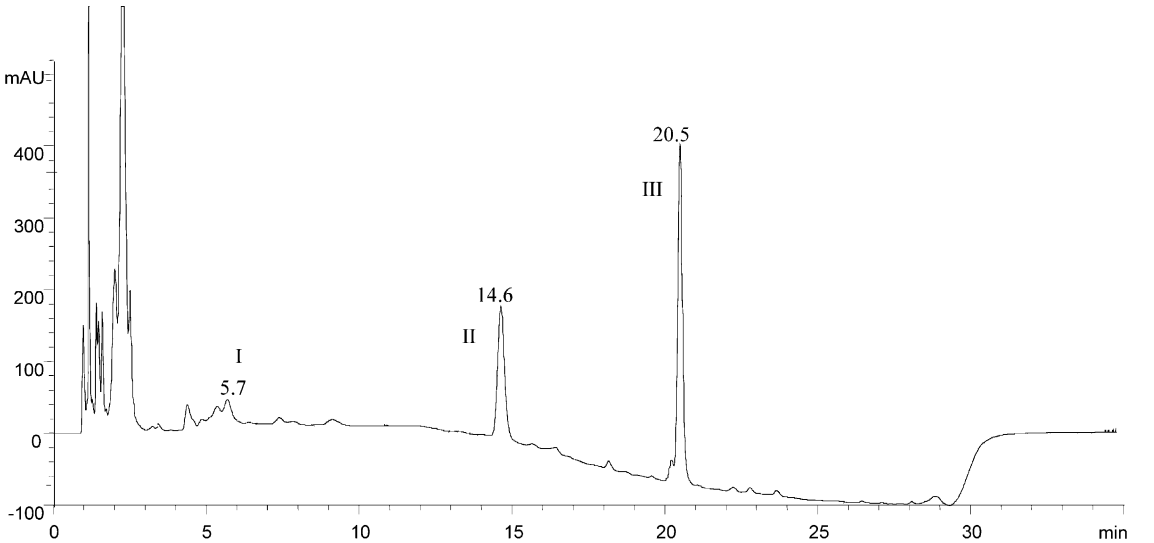

Fig. 3. (*Continued*)

3.4. Validation of the HPLC method: precision, accuracy, LOQ and LOD

The precision and accuracy of the control samples are given in Tables 2 and 3. The concentrations of meropenem control samples were selected at 15, 30 and 60 μg ml^{-1} to encompass the range of concentrations presumably present in plasma from the patients included in the study. The same range of concentrations were used for the filtrate-dialysate control samples, presuming similar concentration in this biological fluid.

Throughout this concentration range, the mean intra-assay ($n = 6$ analysed in duplicate) precision was 4.1 and 2.6% for meropenem, and 4.2 and 3.7% for UK-1a, in plasma and filtrate-dialysate, respectively. Similar precision values were found for the inter-assay ($n = 6$) variability, with a mean values of 2.8 and 3.6% for meropenem and 2.3 and 2.8% for UK-1a in plasma and filtrate-dialysate, respectively.

The accuracy was satisfactory for both meropenem and UK-1a in plasma and filtrate-dialysate, the experimental values never departing more than 9.0% from the nominal concentrations of the spiked samples.

The efficiency of the SPE was determined for plasma and filtrate-dialysate. The absolute extraction recoveries for meropenem and UK-1a (at 15, 30 and 60 μg ml^{-1}) were satisfactorily reproducible, ranging from 88–93% and 72–77% for meropenem and from 95–104% and 75–82% for UK-1a, in plasma and filtrate-dialysate, respectively (Table 4).

The LOQ for meropenem in plasma is 2.5 μg ml^{-1}, a concentration below the threshold of 4 μg ml^{-1} corresponding to the Minimum Inhibitory Concentration (MIC) of sensitive bacteria [1].

LOQ of UK-1a was however, difficult to assess accurately at concentrations ranging between 2.5 and 5 μg ml^{-1}, because of the presence of near-by peaks, as already stated. Determining this value accurately is of limited interest, since in our study UK-1a was measured essentially to ascertain that it would not significantly *accumulate* in this population of patients.

The LOD was 1.25 and 2.5 μg ml^{-1} for meropenem and UK-1a, respectively, both in plasma and in filtrate-dialysate. A lower LOD could be obtained by loading a larger sample volume on the cartridges and/or by injecting a larger volume of sample onto the HPLC column. This was not necessary in our clinical study, the meropenem concentrations being expected to remain above MIC of sensitive bacteria [1].

3.5. Samples stability

Meropenem stability, and the appearance of the open-ring form UK-1a, was evaluated to ascertain that any spontaneous degradation of meropenem would not occur ex vivo in biological fluids from the time between blood collection and final processing (centrifugation and freezing) prior to storage at −80 °C. The in vitro stability of isotonic meropenem (i.e. pharmaceutical formulation) in human blood was measured at +4 °C for 8 h, and in the filtrate-dialysate medium both at +4 °C and at room temperature for 8 h. The Figs. 4 and 5 show the stability profile of meropenem in blood at +4 °C and in filtrate-dialysate samples at room temperature (+22 °C, RT), respectively.

The open-ring form of meropenem has been detected in pharmaceutical meropenem iv formulations after immediate reconstitution of the drug in NaCl 0.9%. This could explain in part the presence of UK-1a at the beginning of the stability study (Figs. 4 and 5 at 0 h). The presence of residual UK-1a in the bulk meropenem has been previously reported [27].

Plasma and dialysate samples were stored as 400 μl duplicates in Eppendorf vials at −80 °C, temperature at which meropenem has been reported to be stable for at least 3 months with no significant decrease of concentration and after repeated freezing–thawing cycles on 4 consecutive days [13].

The stability of meropenem in blood was acceptable at +4 °C at both 15 (=39 μM) and 85 μg ml^{-1} (=222 μM) (Fig. 4), with a mean decrease of 11% from the starting concentration after 8 h. Blood samples collected from the patients enrolled in the clinical study were not allowed to stand more than 1.5 h in the fridge (+4 °C) of the ICU prior to their processing at the laboratory, indicating less than 5% meropenem degradation in vitro during this time period (Fig. 4).

Meropenem was also reasonably stable in spiked filtrate-dialysate sample left at +4 °C, with a mean loss of meropenem after 8 h corresponding to 11% of the starting concentration. Since the aliquots of filtrate-dialysate were collected directly from the machine and processed simultaneously with the blood samples, the degradation after 1.5 h appears acceptable.

However, in vitro determination of the stability of meropenem in filtrate-dialysate revealed a substantial decomposition of meropenem at room temperature. At concentrations of 15 (39 μM) (Fig. 5) and 85 μg ml^{-1} (222 μM), the loss of meropenem after 8 h was 19 and 10%, respectively (mean 15%), corresponding to a degradation of approximately 22% after 12 h. The poor stability of meropenem in the filtrate-dialysate stored at room temperature (RT) precluded the attempt—originally planned—to measure accurately the total amount of meropenem excreted unchanged in the filtrate-dialysate collected in a container left at RT during the 12 h following the administration of meropenem to CVVHDF patients.

Interestingly, the decrease in meropenem level (expressed in μM) over time is not accompanied by the simultaneous equal appearance of the

Table 1
Mean parameters of the calibration curves for meropenem and UK-1a (internal standard method)

	$y = mx + b$		Regression coefficient (r^2)
	m	b	
Plasma ($n = 6$)			
Meropenem	2.41E−01 ± 0.037	−3.64E−03 ± 0.0048	0.999 ± 0.0004
UK-1a	2.51E−01 ± 0.034	−1.22E−03 ± 0.0178	0.998 ± 0.0030
Filtrate-dialysate ($n = 6$)			
Meropenem	1.86E−01 ± 0.014	4.90E−03 ± 0.0104	0.999 ± 0.000
UK-1a	1.95E−01 ± 0.007	1.56E−02 ± 0.0100	0.999 ± 0.000

Table 2
Precision and accuracy of the HPLC assay for meropenem and its open ring metabolite UK-1a in plasma

Nominal concentration (μg ml^{-1})	Concentration found (μg ml^{-1})	Precision C.V.%	Accuracy deviation* (%)
Meropenem			
Intra-assay ($n = 6$)			
60.0	63.3 ± 3.7	5.9	5.5
30.0	29.6 ± 0.9	3.0	−1.4
15.0	14.7 ± 0.5	3.5	−1.9
Inter-assay ($n = 6$)			
60.0	62.2 ± 1.5	2.5	3.7
30.0	29.3 ± 0.7	2.3	−2.3
15.0	14.1 ± 0.5	3.6	−5.8
UK-1a			
Intra-assay ($n = 6$)			
60.0	61.3 ± 3.3	5.3	2.2
30.0	28.8 ± 1.1	3.8	−4.2
15.0	14.3 ± 0.5	3.5	−4.8
Inter-assay ($n = 6$)			
60.0	61.8 ± 1.5	2.4	3.1
30.0	28.8 ± 0.8	2.6	−4.0
15.0	14.0 ± 0.3	2.0	−6.4

open-ring form, suggesting that UK-1a is only one among several degradation products of meropenem. The determination of such other biotransformation products of meropenem was outside the scope of the present investigation.

3.6. Clinical applications

This method was applied to the analysis of plasma and filtrate-dialysate samples from 15 ICU patients included in the study mentionned above. The Fig. 6 shows the concentration–time curve of meropenem in one patient receiving meropenem 1000 mg tid (every 8 h), in the plasma from the arterial and venous lines, and in the filtrate-dialysate outlet. As expected, the concentrations of meropenem measured in the arterial plasma line are always higher than those observed in the venous plasma and filtrate-dialysate, which are both essentially identical. The Fig. 7a–c show the concentration–time curve of meropenem in nine patients receiving meropenem 1000 mg bid (every 12 h), in the arterial, venous lines, and in the filtrate-dialysate outlet, respectively.

The detailed pharmacokinetic assessment will be reported elsewhere. Briefly, meropenem clearance, distribution volume and elimination half-life ($t_{\frac{1}{2}}$) were in our study 4.5 l h^{-1} (41%), 0.5 l kg^{-1} (28%) and 5.1 h (35%), respectively (average, (CV)). The CVVHDF clearance, assessed by the extraction through the machine was 2.3 l h^{-1} (31%). The sieving coefficient (Sc) and ultrafiltration clearance (CL_{UF}) were 0.65 (39%) and 1.0 l h^{-1} (39%), respectively. The $CL_{UF,}$ determined by the recovery of meropenem from the ultrafiltrate, corresponded surprisingly to only less than half the CVVHDF clearance, suggesting a presumable 'catalysator effect' of the filter, that may possibly explain in part the disappearance of meropenem. Meropenem dose adjustment recommendation are outside the scope of this present report and will be reported in detail elsewhere (Robatel et al, in preparation).

The peak concentration (C_{max}) of UK-1a encountered in the plasma of this population of patients ranged from 3.7 to 12.3 μg ml^{-1} at the end of the infusion, after a 1000 mg-dose of meropenem. Interestingly, UK-1a was shown not to significantly accumulate in patients under

CVVHDF, and appears therefore to be dialysable, in accordance with previous observations in patients on hemodialysis [7,16,21].

4. Discussion and conclusion

The proposed method enables the quantitation of meropenem and the simultaneous assessment of its open-ring metabolite UK-1a in biological fluids in a single HPLC run.

Careful control of the buffer solution pH at 7.4, and of the composition and gradient elution program of the mobile phase is mandatory for standardising the peak shape and retention time of meropenem, and obtaining satisfactory separation of UK-1a from the nearby peaks eluted at 5.0 min (Fig. 2c).

In contrast to previous reports wherein meropenem was detected at approximately 300 nm, our proposed method implies the measurement of meropenem using UV diode-array detection at 208 nm in order to detect in the same HPLC run the weakly absorbing open-ring metabolite, whose fate had never been studied in CVVHDF patients. At such a short wavelength, the detection is indeed very sensitive for meropenem but renders the HPLC assay certainly less specific, requiring a preliminary clean-up by SPE followed by a HPLC gradient program elution. Peak identity could also be confirmed by examining the UV spectra recorded on-line at both the retention time of meropenem or UK-1a. Indeed, during the analysis of more than 460 clinical samples from CVVHDF patients, no interfering peaks were detected at the retention time of meropenem, as exemplified in Fig. 2a–c. In some plasma samples of patients however, the baseline separation of small levels of UK-1a (RT = 5.4 min) could not always be achieved from an unidentified peak eluted just before (RT = 5.0 min), without precluding however, an acceptable determination of UK-1a levels.

If our method enabled to confirm that there was no risk of metabolite accumulation in this population of patients, it was found a posteriori, that the proposed method may not be optimal for the measurement of the rather low levels of UK-1a found in the complex matrices such as occasionally encountered in this study.

Table 3
Precision and accuracy of the HPLC assay for meropenem and its open ring metabolite UK-1a in filtrate-dialysate

Nominal concentration (μg ml^{-1})	Concentration found (μg ml^{-1})	Precision C.V.%	Accuracy deviation* (%)
Meropenem			
Intra-assay ($n = 6$)			
60.0	64.3 ± 1.9	2.9	7.2
30.0	30.0 ± 0.6	2.0	−0.1
15.0	14.8 ± 0.4	2.9	−1.3
Inter-assay ($n = 6$)			
60.0	65.4 ± 1.1	1.7	9.0
30.0	29.9 ± 0.7	2.3	−0.2
15.0	14.0 ± 1.0	6.8	−6.8
UK-1a			
Intra-assay ($n = 6$)			
60.0	63.4 ± 2.8	4.4	6.5
30.0	29.6 ± 0.8	2.5	−1.5
15.0	15.0 ± 0.6	4.2	−0.2
Inter-assay ($n = 6$)			
60.0	61.8 ± 2.4	3.9	3.0
30.0	30.1 ± 0.8	2.5	0.4
15.0	15.0 ± 0.3	1.9	0.0

Table 4
Absolute recovery of meropenem and UK-1a

Nominal concentration (μg ml^{-1})	Recovery (mean ± S.D.) (%)
Meropenem	
Plasma	
60.0	88.1 ± 1.2
30.0	87.2 ± 2.7
15.0	93.4 ± 5.1
Filtrate-dialysate	
60.0	72.3 ± 3.8
30.0	75.6 ± 6.7
15.0	76.5 ± 3.3
UK-1a	
Plasma	
60.0	103.9 ± 2.5
30.0	94.6 ± 10.9
15.0	98.4 ± 1.8
Filtrate-dialysate	
60.0	78.9 ± 9
30.0	82.0 ± 10.7
15.0	75.0 ± 1.9

There was some concern that the calibration samples prepared with a plasma collected from healthy volunteers (blood from outdated transfusion bag) might not fully reflect the complexity of the plasma matrix from CVVHDF patients. Getting blood from such patients solely for the purpose of calibration samples preparation would not

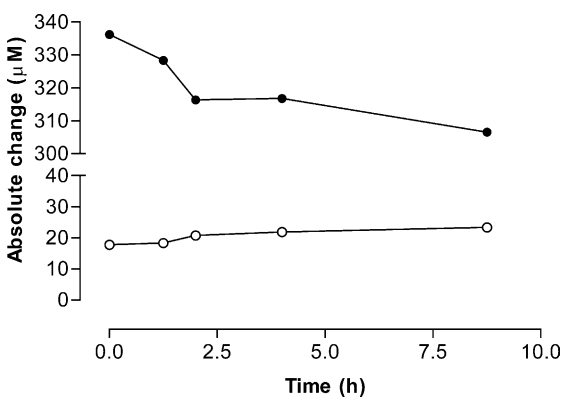

Fig. 4. Stability profile of meropenem at 85 μg ml^{-1} (222 μM) in blood at +4 °C, (absolute change, μM): closed circle, meropenem; open circle, UK-1a.

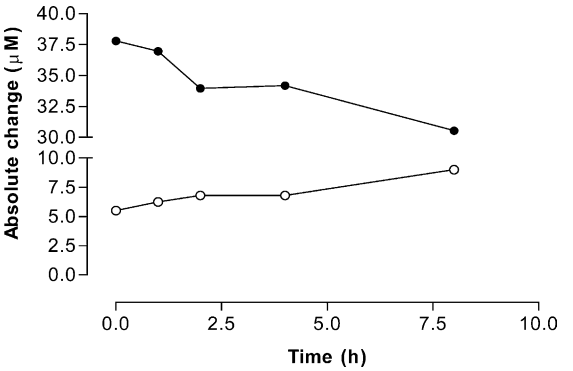

Fig. 5. Stability profile of meropenem at 15 μg ml^{-1} (39 μM) in filtrate-dialysate at room temperature (+22 °C), (absolute change, μM): closed circle, meropenem; open circle, UK-1a.

be ethically acceptable. Moreover, we observed a large inter-individual variability in the appearance (i.e. color, turbidity) of plasma samples collected from these patients. The difficulty of selecting one suitable matrix representative for all samples encountered in the study led us to use plasma from healthy volunteers as an suitable source for the preparation of calibration samples.

Though the accuracy was satisfactory for meropenem in both plasma and filtrate-dialysate samples at the concentrations encountered in our study, there was a slight tendency to overestimate (mean + 6.6%) the actual concentrations at the upper range of concentrations, suggesting that samples at concentrations higher than 100 μg ml^{-1} should best be diluted before their processing by SPE to achieve optimal accuracy.

The HPLC run is lengthy (more than 35 min for the analysis of both meropenem and UK-1a). If the assay of UK-1a is not required, which is probably the case in most studies where the active meropenem only is of interest, the gradient program elution may be accelerated and UK-1a can be 'buried' in the solvent front, and the retention time of meropenem can be decreased to allow enhanced throughput.

The poor stability of betalactams in biological samples, at room temperature or even frozen at −20 °C, is well known. Precautions should therefore, be taken to prevent meropenem decomposition in processed samples (i.e. reconstituted extracts in HPLC vials) let at room temperature

in the auto sampler rack. The time during which HPLC vials are stored in the auto sampler rack at room temperature should therefore be minimised and the samples placed in the temperature-controlled autosampler just prior to the analysis.

The proposed HPLC method was applied to the analysis of samples collected during a study aimed at characterizing meropenem pharmacokinetics in CVVHDF patients. The biological matrix is highly complex in this population, both in plasma—incomplete epuration of endogenous metabolic breakdown substances by the dialyser filter—and in the filtrate-dialysate. This ICU population receives various drugs potentially capable of interfering with the assay, making mandatory an adequate clean-up by SPE to minimise or at least to standardise at best the influence of the matrix effect. Even so, regular changes of HPLC columns and precolumns were necessary in the semi-routine analysis to prevent a gradual pressure increase, affecting over time the retention time ($> \pm 1$ min) of the analytes. However, the batch-to-batch variability was low and the column-to-column ruggedness satisfactory.

The method can not therefore, be considered robust, but this recognised limitation is mostly a function of the type of samples analysed in this study. Enhanced robustness is expected for the analysis of samples with less complex matrices.

Our stability experiments demonstrate that the spontaneous degradation of meropenem is not a matter of concern at low temperature ($\leq +4$ °C) in aqueous solution. However, it becomes important in a biological matrix at room temperature ($\geq +20$ °C). UK-1a seem to be only one of the metabolite of meropenem through spontaneous degradation, and other products are expected to exist. These in vitro observations are in line with the finding of a possible 'catalysator effect' of CVVHD filters, where circulating meropenem is exposed to a wide surface of a complex biological plasma-filter interface maintained at $+37$ °C. In this condition as well, the disappearance of meropenem was not compensated by an equivalent formation of UK-1a in the filter.

Since the haemodiafiltration procedure does not compensate for all the mechanisms involved in the renal clearance of β-lactams (i.e. proximal tubular

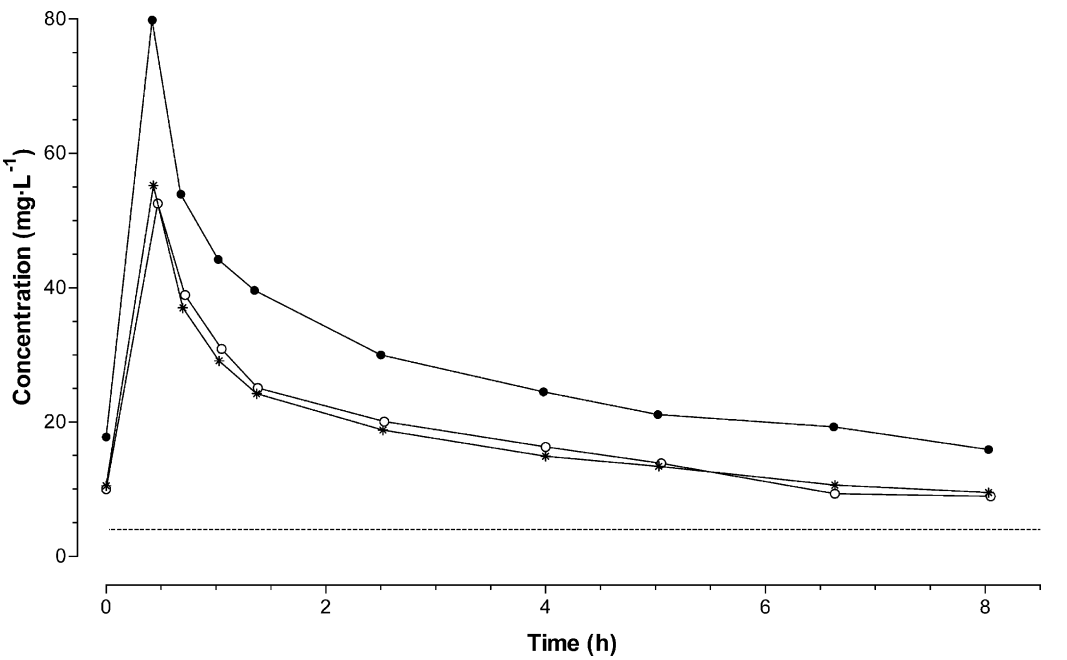

Fig. 6. Concentration–time curve of meropenem in one patient (patient # 2) receiving meropenem 1000 mg tid (every 8 h) in arterial plasma (●), in venous plasma (○), and in filtrate-dialysate (*); dashed line, MIC of susceptible germs = 4 mg l^{-1}.

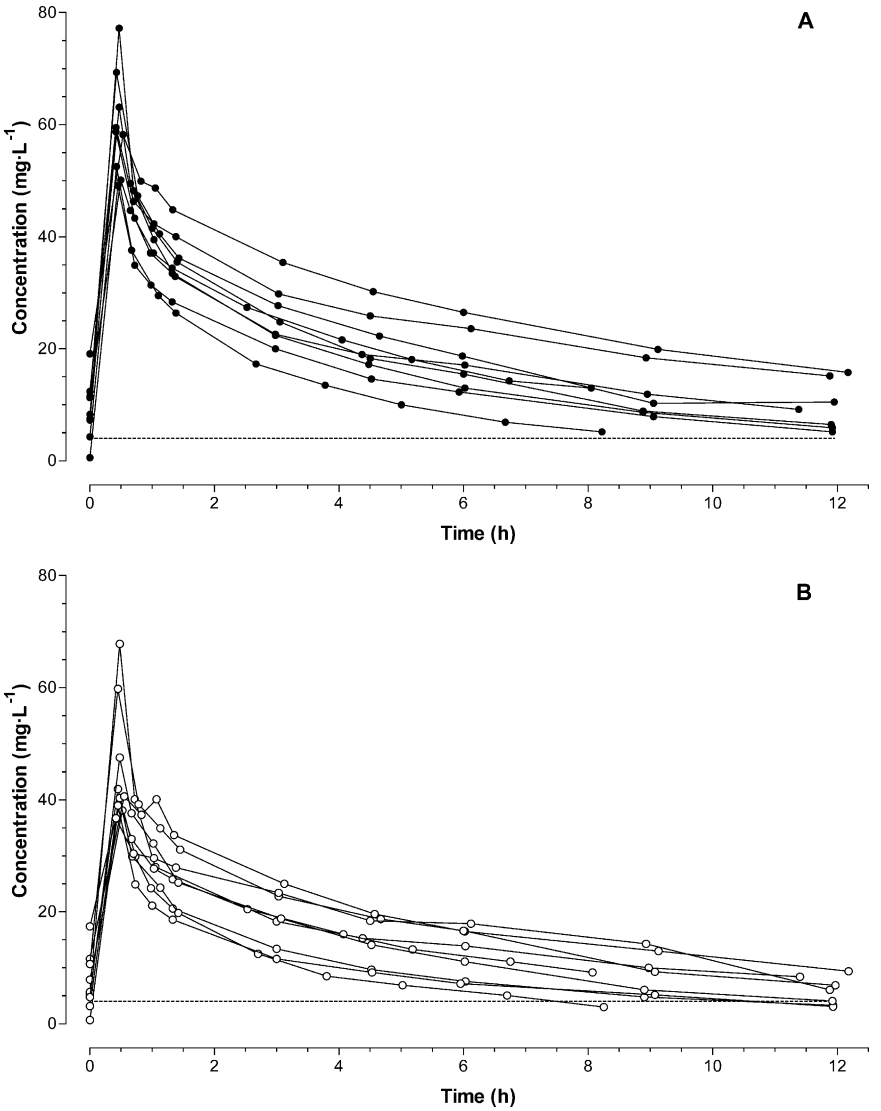

Fig. 7. Concentration–time curve of meropenem in nine patients receiving meropenem 1000 mg bid (every 12 h) in arterial plasma (●) (A), in venous plasma (○) (B), and in filtrate-dialysate (*) (C), dashed line: MIC of susceptible germs = 4 mg l^{-1}.

secretion in addition to glomerular filtration), it was of interest to ascertain the fate of meropenem and UK-1a in acute renal failure patients undergoing CVVHDF. The meropenem concentration ranges found in our study were in accordance with the predicted levels (Robatel et al., in preparation), and UK-1a concentrations ranged from 3.7 to 12.3 μg ml^{-1} at the end of the infusion similar to those encountered in haemodialysed patients [7,16], reaching up to 10.8 ± 1.4 μg ml^{-1} after a 500 mg meropenem's single dose. The reported concentration of UK-1a in patients with type III renal failure ($CL_{CR} \leq 30$ ml min^{-1}) was 4 μg ml^{-1} 0.5 h after the administration of a 500 mg meropenem's single dose [26]. By comparison, the concentrations of UK-1a observed in healthy volunteers were between 1 and 1.6 ± 0.6 μg ml^{-1} [6,16,17] after a 500 mg meropenem dose, and < 5 μg ml^{-1} after a 2000 mg dose [25]. Nevertheless, the ranges of UK-1a concentrations chosen for

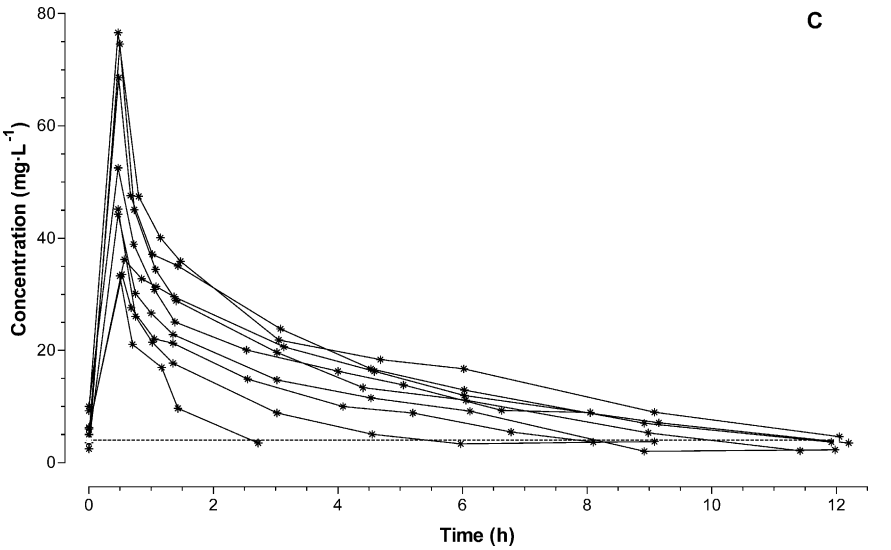

Fig. 7. (*Continued*)

the calibration curves were found a posteriori much higher than those actually found in our biological samples. The measurement of UK-1a was nevertheless found of interest for assessing the possible decomposition of meropenem into UK-1a and to evaluate the rate of meropenem transformation in in vitro studies.

In conclusion, the proposed method enables the accurate measurements of meropenem in biological fluids and is applicable to critically-ill patients under CVVHDF. Its open ring metabolite levels could also be assessed accurately enough in the same run for excluding a possible accumulation. The fate of meropenem can thus be better evaluated and dosage individualisation and recommendations established.

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