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Determination of Meropenem, Ceftazidime and Piperacillin Levels in Serum and Meropenem in Cerebrospinal Fluid by Liquid Chromatography for Routine Quanti ication

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Abstract

With this in mind, -lactam antibiotics are a frequent target of therapeutic drug monitoring (TDM) in the intensive care unit (ICU) setting. In this work, we developed a rapid and easy-to-use high-performance liquid chromatography (HPLC) assay for the detection of meropenem, ceftazidime, and piperacillin in human blood and meopenem in cerebrospinal fluid (CSF).

Methods: In this procedure, a stationary phase of 5.0 m Atlantis® T3 was employed. The composition of the A mobile phase was 99.4% water and 0.6% formic acid (pH 2.30). Acetonitrile (93.6% m/m), water (6% m/m), and formic acid (0.4%) made up mobile phase B. Meropenem, ceftazidime, and piperacillin were all determined using a gradient elution technique. The wavelengths of 309nm, 258nm, 235nm, and 260nm were employed for UV absorbance detection. An internal standard was added during sample preparation, and acetonitrile/methanol was used to precipitate the proteins.

Results The linearity, specificity, accuracy, and precision of the approach were all studied. Antibiotic compounds and the internal standard were tested for their stability. Meropenem had a retention time of 7.222 minutes and a single run duration of 23 minutes. Quantification of meropenem was performed between 0.1mg/l (the minimum detectable concentration in serum and CSF) and 100mg/l (the maximum detectable concentration in serum and CSF). The mean ratio of meropenem concentrations in the cerebrospinal fluid to those in the blood was 0.129, and this ratio varied widely across individuals. Meropenem, ceftazidime, and piperacillin all passed an external validation using the proposed technique with a score of 0.092.Results show that the established test permits investigation of relationships among administered dose, serum concentration, and CSF concentration of meropenem. Serum from humans may also be tested for ceftazidime and piperacillin. Research investigating how much meropenem makes it into cerebrospinal fluid may be carried out in future with a larger sample size. Serum and cerebrospinal fluid (CSF) substance measurements using the proposed procedure are suggested.

Keywords: Meropenem, Ceftazidime, Piperacillin, Therapeutic drug monitoring, HPLC, validation, human serum, cerebrospinal fluid

Background : Meropenem ((4R,5S,6S)-3-[[(3S,5S)-5-[(Dimethylamino)carbonyl]-3-pyrrolidinyl]thio]-6-[(1R)-1-hydroxyethyl]4-Methyl-7-Oxa-1-Azabicyclo[3.2.0]: Chemical Structure (Scenario 1[1]) In the case of bacteria, hept-2-ene-2-carboxylic acid, a member of the carbapenem family, is effective against a broad range of gram-positive and gram-negative organisms. It is a beta-lactam antibiotic, therefore it penetrates the bacterial cell and prevents the formation of the cell wall [2]. Due to its remarkable stability against beta-lactamases [3, 4], it is used as a last-resort antibiotic in intensive care units. Ventriculitis is a frequent complication of external ventricular drains (EVD) used to treat acute subarachnoid hemorrhage, intraventricular bleedings, and other acute cerebral diseases [5]. Treatment of such nosocomial infections with meropenem is often recommended. Due to the rising prevalence of antimicrobial resistance and the scarcity of new antimicrobials suitable for clinical use, optimizing doses for existing treatment regimens is becoming more important to guarantee maximum therapeutic efficacy [3, 6, 7]. Therapeutic drug monitoring (TDM) is often used to optimize treatment with several antibiotics, especially -lactam antibiotics. TDM may be particularly useful in ICUs for treating critically ill patients, who often overdose or underdose due to altered pharmacokinetics caused by varying degrees of organ failure [8-10]. However, the greatest challenge for critical care physicians is still determining how to access, keep, and control antibiotic concentrations in specific tissues. In cases of

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ventriculitis, meropenem is ineffective because it cannot cross the blood-cerebrospinal fluid (CSF) barrier [11]. Recent investigations [12, 13] reveal wide variation in the amount of meropenem that makes it into CSF from patients with ventriculitis. Further data shows that the standard dosage of meropenem (3 2g as intermittent infusion) is insufficient to generate CSF concentrations below the minimum inhibitory concentration (MIC) [13]. Treatment failure may be avoided with the continuous infusion of beta-lactam antibiotics by maintaining concentrations between doses [14]. Furthermore, the maximum recommended dosage of meropenem (up to 6g/24h [15]) may not be effective, requiring dosing regimens greater than the maximum recommended dose [16]. In all patients within 48 hours [16], utilizing median beginning dosages of 8.8g/24h and TDM-guided dosage modification, adequate CSF concentrations were obtained. To meet the needs of intensive care units, we plan to

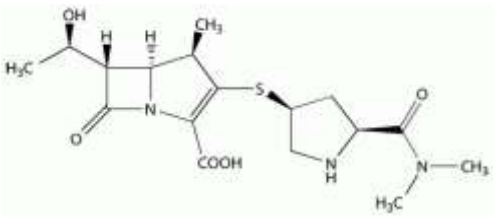

Figure 1: Structure of meropenem

describe a simple method to determinate meropenem in human serum and cerebrospinal fluid. The aim of this study is to demonstrate the development, validation and routine use of internal standard high-performance liquid chromatography assay for meropenem in human serum and cerebrospinal fluid. Additionally this method is able to determine ceftazidime andpiperacillin in human serum.

Materials and Methods

Antibacterial agents and other substances

We used meropenem powder for solution for injection/ infusion, commercially available from Dr. Friedrich Eberth Arzneimittel (Ursensollen, Germany), piperacillin/ tazobactam powder for solution for injection/infusion, commercially available from Fresenius Kabi Deutschland (Bad Homburg, Germany) and ceftazidime powder for solution for injection/infusion, commercially available from Dr. Friedrich Eberth Arzneimittel (Ursensollen, Germany). Also cefotaxime powder for solution for injection/infusion, commercially available from Fresenius Kabi Deutschland (Bad Homburg, Germany), cefazolin powder for solution for injection/infusion, commercially available from MIP Pharma GmbH (Blieskastel, Germany) and porcine serum from bio&sell GmbH (Feucht, Germany). Patient serum and patient cerebrospinal fluid were received from ICUs for TDM.

Solvents

We purchased formic acid, sodium hydroxide, methanol (HPLC grade) and acetonitrile (HPLC grade) from Th. GeyerGmbH & Co. KG (Renningen, Germany). Purified water was purchased from Fresenius Kabi Deutschland GmbH (Bad Homburg, Germany).

High-performance liquid chromatography (HPLC)

We employed a Shimadzu HPLC system, which included a column oven, UV-Vis detector, and temperature-controlled autosampler. The chromatographic apparatus for the double internal standard technique was controlled using Labsolution (Shimadzu, Germany) software. Atlantis® T3 5m, 15cm x 4,6mm Column (Waters Corpotation, Milford, MA, USA) was used as the stationary phase.

The pH of mobile phase A (99.4 m/m water, 0.6% m/m formic acid) was adjusted to 2.30 with 1M sodium hydroxide. Acetonitrile (93.6% m/m), water (6% m/m), and formic acid (0.4%) made up mobile phase B. As can be seen in table 1, we used a gradient elution technique using mobile phases A and B.

There was a 1.0ml/min flow rate from the pump. A 309nm (meropenem) to 235nm (piperacillin) to 258nm (ceftazidime) to 260nm (cefotaxime) to 270nm (cefazolin) UV absorbance detector was used to determine the presence of each antibiotic. The temperature in the column oven was

routinely set at 20 degrees Celsius. Median retention times for meropenem were 7.222 minutes, piperacillin was 17.541 minutes, ceftazidime was 9.861 minutes, cefotaxime was 12.105 minutes, and cefazolin was 12.105 minutes at 20 degrees Celsius while the technique was running.

Reference standards

To determine the content of the commercially available powders for solution for injection/infusion we used chemical reference substances (CRS). Meropenem trihydrate CRS (content 86.9%), piperacillin CRS (content 95.2%), ceftazidime CRS (content 85.5%), cefotaxime acid CRS (content 90.6%) and cefazolin (content 99.2%) were purchased from Sigma-Aldrich Chemie GmbH (Tauf kirchen,Germany).

Sample preparation

We prepared samples by mixing 250µl patient serum or CSF with 50µl internal standard (cefotaxime 125mg/l and cefazolin 125mg/l) and 500µl

acetonitrile/methanol (1:1) for precipitation. The samples were mixed for 10s and centrifuged at 10 000 RPM for 10min. 200µl of the supernatant were diluted with 460µl water and 50µl of this mixture was injected.

Results

Selectivity

Selectivity of the analytical method was proven using six individual sources of the appropriate blank matrix (human serum), which were individually analyzed and evaluated for interference. No relevant interference was detected but to prevent interference with the internal standard we decided to use a mixture of two internal standards. If there is an interference with cefotaxime we can use cefazolin to analyze the patient sample. Interference may occur in patients who received cefotaxime or cefazolin in earlier therapy regimes.

Carry-over

To prevent carry-over we injected blank samples after high concentration samples [17]. There was no carry-over detected in the blank samples.

Table 1: Gradient time program for HPLC

Time (min)	Solvent B concentration (%)
0.01	10
1	10
11	35
15	35
16	10
23	10

Lower limit of quantification

The lower limit of quantification is defined as the lowest concentration of analyte in a sample, which can reliably be quantified, with an acceptable accuracy and precision. LLOQis aimed to be at least 5 times the signal of a blank sample [17]. For this analytical method, the LLOQ for meropenem is 0.1mg/l in serum and CSF, 0.2mg/l for ceftazidime in serum and 10mg/l for piperacillin in serum.

Calibration curve

For time-dependent drugs, the main parameter associated with therapeutic success is the percentage of time that the levels of antibiotics at the infection site exceed the minimum inhibitory concentration (%*f* T > MIC) of the pathogen [18]. Due to the clinically sensible breakpoint against the pathogenic *Pseudomonas spp*. at 2 mg/l [19] we defined the target concentration in CSF > 2mg/l. For meropenem levels in serum we defined target concentrations of 8 – 16mg/l (100% *f*T > 4x MIC - 100% *f*T > 8x MIC). Ceftazidime serum target concentrations 32 – 48mg/l (100% *f*T > 4x MIC - 100% *f*T
> 6x MIC) and piperacillin serum target concentrations 64 – 96mg/l (100% *f* T > 4x MIC - 100% *f*T > 6x MIC) due to their MIC breakpoints against *Pseudomonas spp* [19].

According to the target concentration range a minimum of six calibration concentration levels were used for each method [17]. The LLOQ is defined being the lowest calibration standard and the highest calibration standard defines the upper limit of quantification (ULOQ) as seen in table 2 [17]. LLOQ is 0.1mg/l for meropenem in serum and liquor, the ULOQ is 100mg/l in serum and 25mg/l in CSF. LLOQ is 0.2mg/l for ceftazidime in serum; the ULOQ is 75mg/l in serum. LLOQ is 10mg/l for piperacillin in serum; the ULOQis 200mg/l in serum.

For the calibration standards, we used porcine serum and residual material of human CSF. To prepare the calibration standards we spiked 200µl matrix with 50µl antibiotic solution (target concentration level x5 mg/l). The following steps were performed analog the sample preparation. All calibration curves analysis used freshly spiked samples. The correlation between mean area ratio and concentration ratio was strong for all calibration curves (R^2 >0.9999).

Accuracy

The accuracy describes the closeness of the determined value obtained by the method to the nominal concentration of the analyte. Accuracy was assessed on samples spiked with known amounts of the analyte. These samples were spiked independently from the calibration standards and were analyzed against the calibration curve. For the validation of the accuracy, we analyzed LLOQ, low, medium and high concentration samples. The mean concentration within a value of 15% from the nominal values is commonly considered acceptable, except for the LLOQ, which is acceptable within 20% of the nominal value [17]. The accuracy was demonstrated with all mean concentrations between 88.56% and 100.82% of the nominal value.

Precision

The precision of the analytical method describes the closeness of repeated individual measures of analyte in the same sample. Precision can be expressed as the relative standard deviation (RSD). Precision of the analytical method should be demonstrated for the LLOQ, low, medium and high sample concentrations. The RSD value should not exceed 15% for the low, medium and high concentration samples, except for the LLOQ, which should not exceed 20% [17]. Precision was demonstrated for every antibiotic substance with all RSD values ranging between 0.64% and 12.95%.

Stability

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The low stability of meropenem in aqueous solutions or biological fluids is often reported in literature [20-22]. Even transport between clinic and laboratory is difficult due tothe limited stability [21]. To detect stability, we analyzedthe degradation of meropenem, ceftazidime and piperacillin under relevant conditions. Therefore, we evaluated the stability of meropenem in spiked porcine serum and CSF after sample preparation at 5°C. This simulates the conditionsin our autosampler and no relevant degradation was detectedover 15h as seen in figure 2. Additionally we analyzed the stability of meropenem, ceftazidime and piperacillin in biological matrix.

We spiked human serum with a mixture of all three antibiotics and measured the concentrations at the beginning, after 24h and 48h. One sample was stored at 5°C, one in the freezer at -32°C and one at ambient temperature 25°C. The concentrations at 25°C decreased very fast comparedto the samples at 5°C and -32°C as shown for meropenem

Table: 2 Antibiotic concentration levels used for the calibration curves. Every calibration sample contains 25mg/l internal standard (cefotaxime and cefazolin).

Conc. Level	Meropenem serum	Meropenem CSF	Ceftazidime serum	Piperacillin serum
#1	0.1 mg/l	0.1 mg/l	0.2 mg/l	10 mg/l
#2	0.2 mg/l	0.2 mg/l	0.5 mg/l	25 mg/l
#3	0.5 mg/l	0.5 mg/l	1 mg/l	50 mg/l
#4	1 mg/l	1 mg/l	2.5 mg/l	100 mg/l
#5	5 mg/l	2.5 mg/l	5 mg/l	160 mg/l
#6	12.5 mg/l	5 mg/l	10 mg/l	200 mg/l
#7	25 mg/l	10 mg/l	20 mg/l	not used
#8	50 mg/l	25 mg/l	30 mg/l	not used
#9	100 mg/l	not used	50 mg/l	not used
#10	not used	not used	75 mg/l	not used

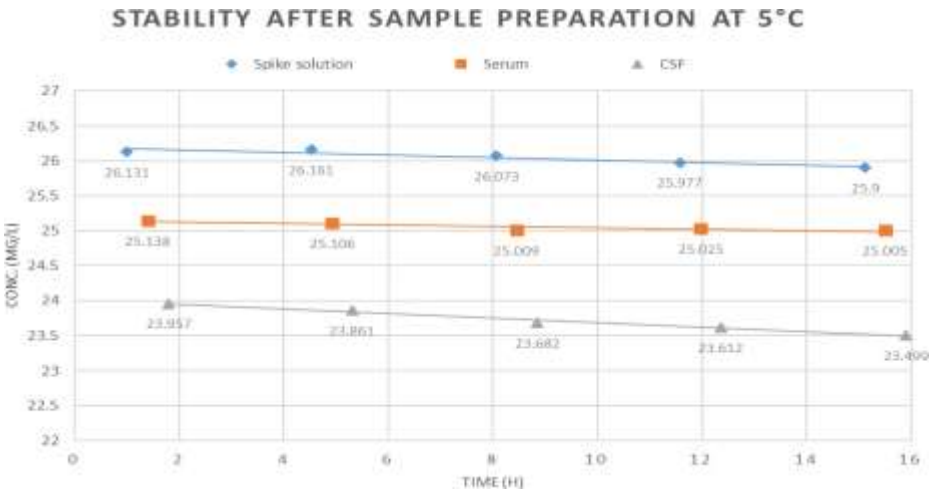

Figure 2: Stability of meropenem in porcine serum and CSF after sample preparation at 5°C.

(figure 3). The same degradation progress was detected for ceftazidime and piperacillin. At 25°C the value 90% of start concentration was passed within the first 24h and at 5°C after 48h. At -32°C the value 90% of start concentration was not passed within 48h. Consequently, we concluded to freeze the collected patient samples and analyze them within 24h after collection.

Quality control samples

We performed quality control samples to show our system und methods work as we expect on days with analysis of unknown samples. Therefore, high and low concentration samples were prepared out of antibiotic (meropenem + ceftazidime + piperacillin) and internal standard (cefotaxime + cefazolin) stock solution with porcine serum. The low concentration sample was spiked with 4mg/l meropenem, 16mg/l ceftazidime and 34mg/l piperacillin. The high concentration sample was spiked with 16mg/l meropenem, 65mg/l ceftazidime and 137mg/l piperacillin. We defined the acceptable concentration range of the measured antibiotics with ±7% and the acceptable area range of internal standard with ±7.5% due to the recommendation of the EMA guidelineon bioanalytical method validation[17].

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They recommend ranges of ±15% but we decided to define closer limits with
±7% and ±7.5%.

External validation

To verify the performance of the method an external validation assay was passed. This assay was offered by INSTAND (Gesellschaft zur Förderung der Qualitätssicherung in medizinischen Laboratorien e.V., Düsseldorf). The achieved certificate is valid for 12 months and proves that two samples with unknown concentration of meropenem, ceftazidime and piperacillin were analyzed correctly within acceptable limits. The results are shown in table 3.

Routine analysis

The method we described here is routinely used in our laboratory to determine meropenem levels in human sera and CSF. Within the setting described above, we measured 64 pairs of simultaneous collected human serum and CSF samples from critically ill patients on intensive care units. The serum levels of meropenem ranged between 5.4mg/l up to 49.3mg/l (mean 18.6mg/l ± 7.6mg/l, median 16.4mg/l). The CSF levels of meropenem ranged between 0.3mg/l up to 17.9mg/l (mean 2.6mg/l ± 2.6mg/l, median 1.9mg/l). For our measurements, the mean CSF/serum ratio was 0.129 ± 0.092. Patient characteristics and dosage regimes are collected in table 4.

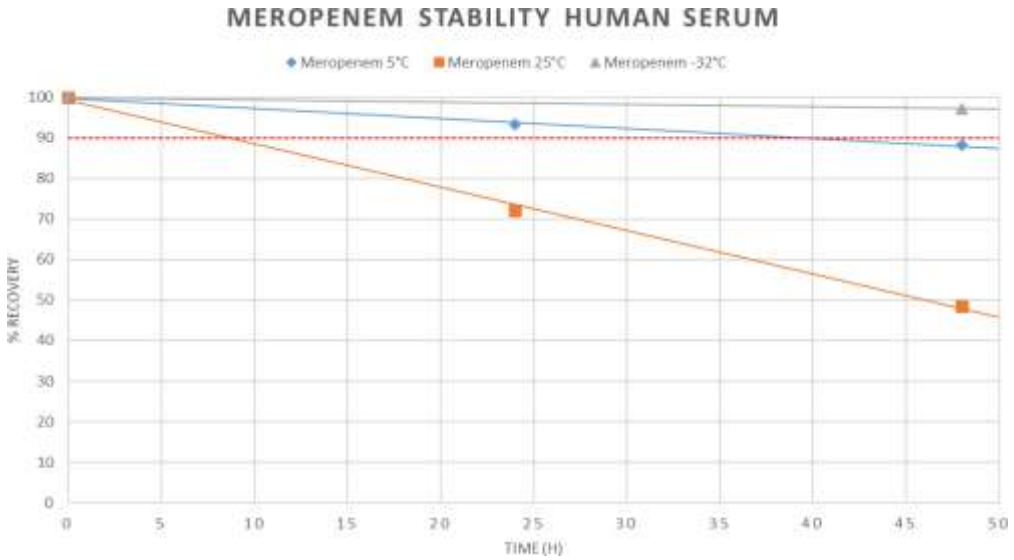

Figure 3: Degradation of meropenem in human serum at 5°C, 25°C and -32°C over 48h

Table 3: Results of the external validation by INSTAND

Substance	Sample	Unit	Measured conc.	Target conc.	Lower limit	Upper limit	Deviation	Result +/-
Ceftazidime	1	mg/l	0	0	0	2.50		+
	2	mg/l	3.80	4.08	2.86	5.30	-6.9%	+
Meropenem	1	mg/l	58.9	63.2	44.2	82.2	-6.8%	+
	2	mg/l	46.1	48.3	33.8	62.8	-4.6%	+
Piperacillin	1	mg/l	14.6	17.4	12.2	22.6	-16.1%	+
	2	mg/l	120	132	92.4	172	-9.1%	+

Table 4: Patient characteristics and dosage regimes

No. of patients	30
Gender (No. M/no. F)	19/11
Mean age (yr)	53.0
Mean body weight (kg)	80.3
No. of serum/CSF pair	64
Mean serum conc. (mg/l)	18.7 ± 7.6
Mean CSF conc. (mg/l)	2.6 ± 2.6
Mean CSF/serum ratio	0.129 ± 0.092

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Dose continuous 8000mg/24h	14
Dose continuous 6000mg/24h	46
Dose continuous 4000mg/24h	2
Dose continuous 3000mg/24h	1
Dose continuous 2000mg/24h	1
Mean dose continuous /24h (mg)	6.3 ± 1.1
Indication ventriculitis	14
Indication meningitis	2
Indication subarachnoid hemorrhage	1
Indication brainstem abscess	2
Indication shunt infection	3
Indication unknown	8

Discussion

The developed assay is reproducible, accurate, precise, and linear across the range of the calibration curves. The preparation of our samples is quick and simple. The HPLC assay time of 23min is acceptable for the processing ofsamples for routine TDM.

Previous studies described large interindividual variabilityin the concentrations of meropenem in plasma and CSF[13]. A reduced distribution into CSF has been documented for β-lactams, especially carbapenems, due to their hydrophilic nature [23, 24]. Additionally results were published on whether meropenem plasma concentrations simply used as a surrogate parameter of CSF concentrations may lead to under dosing [25]. These results are in line with Blassmann et al. who is reporting a median CSF/plasma penetration of 9% in 21 neurocritical care patients with ventriculitis [13]. We measured serum and CSF levels at the same time during continuous infusion of meropenem; this gave us also the chance to calculate the CSF/serum ratio and for our 64 pairs of serum and CSF levels the mean ratio was 0.129 ± 0.092. Compared to the non β-lactam antibiotic linezolid with a reported CSF/serum ratio of 0.71 ± 0.16 [26], meropenem has a very poor CNS penetration. Our data from routine analysiswith a mean CSF/serum ratio of 12.9% support the data from Blassmann et al. and suggest a high interindividual variability of serum levels, CSF levels and the CSF/serum ratio due to large standard deviations. If these analysis are compared with

reported CSF penetration of between 21 and 39% in patients with bacterial meningitis [27, 28], it is suspected that dosing regimens for meropenem in patients with meningitis cannot be extrapolated to patients with ventriculitis.

Our data contains two patients with proven or suspected meningitis in which the measured CSF/serum ratio are higher than the mean CSF/serum ratio (13.7% and 23.3% compared with mean 12.9%). It is likely, that drug penetration in inflamed meninges is greater than in patients with non- inflamed. Consequently, in critically ill patients with CNS infections, the standard dosing regimen of meropenem with 6g daily does not predictively achieve optimal plasma and CSF concentrations in all patients. Results like these push the need for TDM of meropenem in plasma and in CSF to avoid either the risk of dose-dependent toxicity or that of treatment failure. The development of meropenem-induced toxicity is significantly affected in patients with a high serum meropenem concentration. The threshold concentrations for which there is 50% risk of developing a neurotoxicity event is described with meropenem c_{min} = 64.2mg/l and a nephrotoxicity event with c_{min} = 44.45mg/l [29].

Furthermore, optimized dosing strategies like administration of higher than standard dosages or administration by continuous infusion should be taken into consideration. Continuous infusion has been demonstrated to improve PK/PD target attainment in various further studies of time-dependent antibiotics [30-32]. Recommended daily doses for meropenem are 6g in adults [5]. High initial meropenem doses (median 8.8g/24h by continuous infusion) together with dose adjustments according to TDM ensured sufficient CSF concentrations in all patients according to Tiede et al [16] Consistent evidence is now available showing that therapeutic drug monitoring and guided individual dose optimization of meropenem is justified and feasible in clinical practice to reduce underexposure, improve tolerability and possibly response to therapy [16].

We have demonstrated meropenem, ceftazidime and piperacillin to be stable in human serum up to 48h in frozen condition at -32°C. This is important because it was shown that meropenem was unstable when stored at temperatures above 4°C [20-22]. Furthermore, meropenem, ceftazidime and piperacillin were stable after treatment with acetonitrile/ methanol. Accordingly, the prepared samples can be assayed under storage conditions of 5°C within 24h period and no relevant loss of meropenem, ceftazidime or piperacillin was detected.

Conclusion

In the present study, we developed a simple method for the quantification of meropenem in human serum and CSF. The developed method could be easily and quickly performed and enabled the quantification of meropenem in patient samples

for routine TDM. In the future, this method can be used to evaluate the serum and CSF concentrations of meropenem in critically ill patients. Consequently, meropenem dosage regimes should be tailored to individual patients. This is essential because our data suggests that there is a high variability in serum concentrations, CSF concentrations and CSF/serum ratios.

Furthermore, the developed method creates the chance to study CSF penetration of meropenem because the simplest way to study the entry of drugs into the CNS is to measure drug concentrations in the CSF during a continuous drug infusion[33]. Additionally, this method enables to quantify ceftazidime and piperacillin concentrations in human serum. Our investigation was limited due to the lack of information about clinical outcomes of the patients and the lack of microbiological analysis. In conclusion, our results are in line with other studies that showed a high variability of serum and CSF levels of meropenem, and future studies can be performed using the method described above.

Ethics approval

Ethics Committee of "Ärztekammer Baden-Württemberg"in Stuttgart, Germany (authorization number: F-2020-057).

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Competing interests

None declared.

Contributors

SG and HV: development and validation of analytical assays. SG, SS, GG, HV and AR: collection of routine data. SG, AS and HD: analysis and interpretation of data. SG, HV, AR, SS, LE, GG, AS and HD: revision for intellectual contentand approval of the final version.

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