ELSEVIER

Journal of Chromatography B, 751 (2001) 357–363

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Rapid antibiotic drug monitoring: Meropenem and ceftazidime determination in serum and bronchial secretions by high-performance liquid chromatography–integrated sample preparation

M. Ehrlich, F.D. Daschner, K. Kümmerer*

Institute of Environmental Medicine and Hospital Epidemiology, Hugstetter Strasse 55, D 79106 Freiburg i. Br., Germany

Received 25 May 1999; received in revised form 8 September 2000; accepted 11 September 2000

Abstract

A sensitive and rapid HPLC assay for the determination of the beta-lactam antibiotics ceftazidime and meropenem in serum and bronchial secretions is described. HPLC–integrated sample preparation allows direct injection of serum samples without any pretreatment. Sputum samples need only a simple homogenisation and volume measurement but no liquefying reagents are necessary. The inline extraction technique is realized by automatically switching from the extraction column to the analytical column. After the matrix passed the extraction column, the retained analyte is quantitatively transferred to the analytical column where separation by isocratic HPLC is performed. Ceftazidime and meropenem are detected according to their absorption maxima at 258 and 296 nm, respectively. The detection limit of both antibiotics is estimated to be better than 0.5 μg/ml in serum as well as in sputum samples. The described procedure allows determination of the antibiotics within 30–45 min, thereby facilitating drug monitoring in clinical routine.

Keywords: Meropenem; Ceftazidime; Sputum; Bronchial secretion; Monitoring

1. Introduction

Meropenem (Meronem®, Merrem®) is a broad-spectrum parenteral antibiotic of the carbapenem class. It is more active than imipenem against many gram-negative pathogens and it has shown activity against strains of *Enterobacteriaceae* and *Pseudomonas aeruginosa* resistant to imipenem, aminoglycosides, quinolones, and third-generation cephalosporins [1]. Meropenem plays an important role in the treatment of multi-resistant respiratory infections of cystic-fibrosis (CF) patients [2].

Ceftazidime (Fortum®) is a third-generation cephalosporin agent for parenteral administration, its activity against *Pseudomonas aeruginosa* is greater than that of other cephalosporins [1]. Ceftazidime is one of the first line antibiotics for the therapy of respiratory infections of CF-patients. The chemical structure of meropenem and ceftazidime is shown in Figs. 1 and 2.

Although several reports describe the determi-

*Corresponding author. Tel.: +49-761-270-5464; fax: +49-761-270-5485.

E-mail address: kkuemmer@iuk1.ukl.uni-freiburg.de (K. Kümmerer).

0378-4347/01/$ – see front matter
PII: S0378-4347(00)00504-1

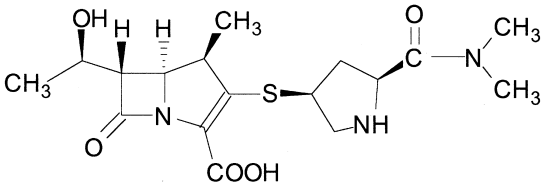

Fig. 1. Chemical structure of meropenem.

nation of ceftazidime and meropenem in the serum of healthy volunteers [3–6] only few pharmacokinetic data are available for the antimicrobial therapy of particular diseases. Furthermore, the antibiotic concentration at the site of an infection is in most cases not deducible from the serum concentration and the antimicrobial effective quantity of the administrated dose remains unknown.

The aim of the present study was to provide a rapid, accurate and sensitive analytical method to determine ceftazidime and meropenem in serum and bronchial-secretions, which allows fast drug-monitoring of respiratory infections in clinical routine. The HPLC-integrated extraction overcomes time-consuming sample pretreatment, such as liquid–liquid or solid-phase extraction prior to HPLC determination. Sputum samples are usually difficult to handle for analysis because of their inhomogeneous and high viscous consistency.

HPLC–integrated sample preparation reported here uses special column packing materials which allow direct and repetitive injection of untreated biofluids. LiChrospher® RP-ADS belongs to the unique family of restricted access materials. It possesses two chemically different surfaces. At the outer surface of the spherical particles with a diameter of 25 μm, hydrophilic, electroneutral diol-groups are

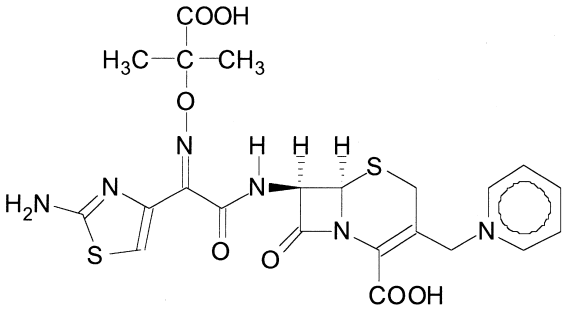

Fig. 2. Chemical structure of ceftazidime.

bound. This chemically inert layer prevents the column against an unwanted contamination caused by interactions with the protein matrix, even when used repetitively. The inner surface of the porous particles is exclusively covered with a hydrophobic dispersion phase (C_4, C_8, C_{18} alkyl-chains). These adsorption centres are accessible for low molecular analytes such as β-lactam antibiotics [7,8].

2. Material and methods

2.1. Reagents and chemicals

Meropenem was a gift of Zeneca (Schwetzingen, Germany), ceftazidime was kindly supplied by Glaxo Wellcome (Bad Oldesloe, Germany). Acetonitrile (LiChrosolv®) and sodium dihydrogenphosphate monohydrate were purchased from Merck KG (Darmstadt, Germany). Formic acid sodium salt was obtained from Sigma (Deisenhofen, Germany). 1-heptanesulfonic acid sodium salt monohydrate was a product of Fluka Chemie AG (Buchs, Switzerland).

All reagents were at least analytical-grade, except for acetonitrile which was gradient grade. HPLC-grade water was generated using a Milli-Q water-purification system from Millipore S.A. (Molsheim, France).

Pooled drug-free sputum samples were obtained from the cystic fibrosis out-patient clinic Freiburg, Germany, and were stored at −80°C until analysis. Pooled blank sera were purchased from Plasmadienst GmbH (Offenbach, Germany).

2.2. Apparatus

Homogenizing procedures were performed with Ultra-Turrax® (Typ TP18/10) from Janke & Kunkel KG (Staufen, Germany). A Biofuge 15 from Heraeus Sepatech GmbH (Hanau, Germany) was used for centrifugation.

The in-line-extraction system was constructed with LiChroCART® cartridge (25×4 mm), packed with LiChrospher® ADS C_8 or C_{18}, cartridge holder manu-CART® and in-line filter (2-μm sieve), Merck KG (Darmstadt, Germany).

The HPLC-system consisted of two LC-10AT HPLC-pumps, CTO-10AC column oven, SPD-10A

UV–Vis detector and FCV-12AH 6-Port valve, controlled by a CBM-10A Module (all items obtained from Shimadzu, Duisburg, Germany). Samples were injected via Rheodyne 7125 manual injection valve equipped with a 20-μl or 100-μl sample loop. Chromatography was performed on a Nucleosil C_{18}-250 mm×4.6 mm, 5 μm analytical column preceeded by a guardcolumn CC 8/4 Nucleosil 100-5 C_{18} (Macherey & Nagel, Düren, Germany).

2.3. Analytical procedure

2.3.1. Serum samples

The direct injection of the serum samples is the only manual step in the analytical procedure. Extraction and chromatography is executed automatically by the HPLC-integrated-extraction system.

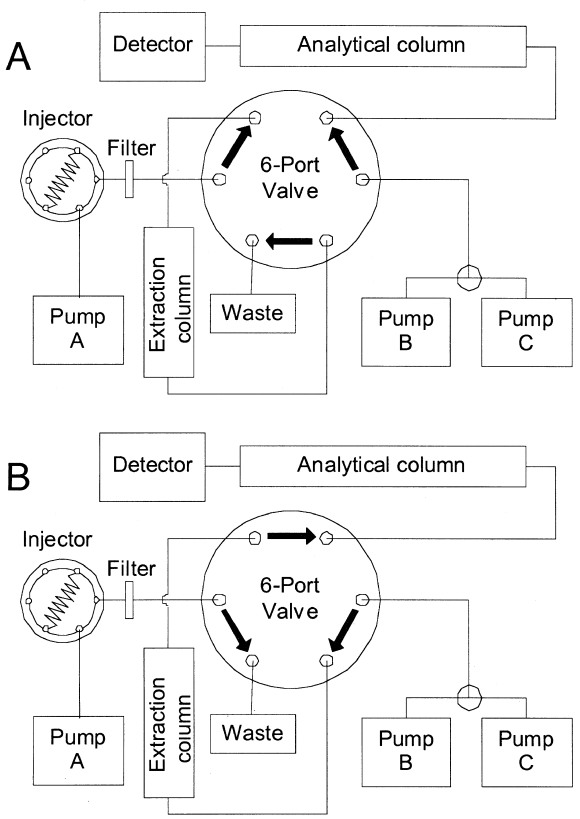

Fig. 3. Scheme of the HPLC–integrated sample preparation. (A) Shows the system in initial position, ready for sample injection: HPLC circulation is isolated from extraction side. (B) displays the transfer step: the extraction column is connected with the analytical column. See Section 2.3 for details.

The block diagram of the HPLC-integrated sample preparation is shown in Fig. 3.

2.3.2. Sample pretreatment of sputum samples

The sputum sample was transferred to a tared 10-ml polyethylene-tube. For each mg 1 μl of 50 m*M* sodium dihydrogenphosphate buffer (pH 5.0) was added and homogenized for 30 s at 2000 rpm with the Ultra-turrax. Occasionally appearing foam was disposed of by ultrasonic treatment. The total volume was measured with a graduated pipette. The sample volume was calculated by substraction of the buffer-volume from the total volume. After centrifugation for 10 min at 10 000 rpm, corresponding to 8832 *g*, an aliquot of the supernatant was injected in the HPLC-integrated extraction system.

2.3.3. HPLC-integrated extraction

The operation of the HPLC-integrated extraction consists of three main components: sample application and fractionation, transfer of the analyte fraction and chromatographical separation.

The mobile phase for the extraction operation contained 12.5 m*M* sodium formiate buffer and 5 m*M* *n*-heptanesulfonic acid ion-pair reagent and was adjusted to pH 3.0 (reservoir of pump A).

An aliquot of the biological fluid was injected into the manual injector. The mobile phase (pump A set to 0.8 ml/min) carried the sample to the extraction column. The antibiotic analyte was retained on this column, while higher molecular weight matrix compounds were discharged to waste with the eluent (Fig. 3A).

After 8 min the matrix had been washed out from the extraction column. The software time-schedule automatically switched the high-pressure valve into transfer position (Fig. 3B) thereby coupling the extraction column with the HPLC-circulation.

Reservoir of pump B contained acetonitrile and reservoir of pump C consisted of 50 m*M* sodium dihydrogenphosphate buffer (pH 5.0). In this valve position the analytical mobile phase delivered from pumps B and C in a ratio of 5:95 v/v and 4:96 v/v for assaying meropenem and ceftazidime, respectively, passed the extraction column in an inverse direction at a flow-rate of 1.0 ml/min. The higher elution power of the analytical mobile phase desorbed the analyte from the extraction column and

transfered it to the analytical column. After 13 min the transfer was complete and the valve was switched to the initial position (Fig. 3A).

The extraction column was re-equilibrated by pump A while simultaneously the disconnected HPLC-circulation performed the conventional chromatography. The settings of pumps B and C remained as described above and the column oven was set to 35°C. The eluent was monitored at 296 and 258 nm for meropenem and ceftazidime, respectively.

The extraction column was washed each day after a number of analyses with methanol–water (50:50, v/v) at 1.0 ml/min for at least 15 min.

2.4. Quantification and statistics

2.4.1. Quantification

Standards for the linearity study were made by diluting an aqueous stock solution of the respective antibiotic in a range of 0.5–40 μg/ml.

For each sample-loop (20 μl or 100 μl) a separate calibration was performed. All standards were injected into the in-line extraction system. The calibration curves were based on the peak areas of each standard plotted versus the nominal antibiotic concentration using least-squares linear regression.

2.4.2. Recovery, accuracy and precision

Serum: Pooled blank serum samples were spiked with standard solutions of meropenem or ceftazidime to yield concentrations between 0.5 and 20 μg/ml.

Bronchial secretions: 50–100 μl of standard solution were added to approximately 0.5 g of the sputum samples. The resulting concentrations depended on the sample volumes which were measured during the sample pretreatment.

Chromatogram peaks for meropenem and ceftazidime were identified by their retention times and quantified by their peak areas.

Accuracy of the meropenem and ceftazidim serum assays were determined by calculating the mean percent differences between nominal and measured concentrations. The assay precision was characterized by mean value and coefficient of variation (C.V.).

The average quotient of measured concentrations and nominal concentrations indicated the ratios of the recovered antibiotics. The limit of quantification was estimated at a signal-to-noise ratio of 10:1.

For assays of sputum samples the volume of each individual weighted sample was measured, therefore errors resulting from the sample pretreatment were considered.

3. Results and discussion

The analytical method described for meropenem and ceftazidime overcomes the time-consuming procedures of previously reported methods [4–7]. The presented serum assay is fully automated and allows determination of antibiotic levels within 30 min after sampling.

The sample pretreatment for sputum samples requires only a simple homogenizing step, volume measurement and centrifugation.

3.1. Sample pretreatment

The inhomogeneous and high viscous structure of the sputum samples generated a couple of problems. Prior to the liquefying procedure (see Section 2.3) only the weight of the samples is determinable but the analytical system requires a known volume aliquot. Indirect evaluation of the sample volume after the dilution and homogenizing procedures solved this problem.

A lot of methods and agents for liquefying sputum samples are described [10]. Most of them involve incubation and/or heating of the samples which leads to degradation of β-lactam antibiotics. Some sputum liquefying agents are chemically incompatible with β-lactams. In our own preliminary experiments meropenem has shown increased degradation in the presence of the frequently used mucolytic agent *N*-acetylcysteine.

The pretreatment procedure for sputum samples described in this paper dispenses with sputum liquefying agents. After centrifugation the supernatant is separated from solid components and can be injected into the in-line extraction system.

3.2. HPLC-integrated extraction

For quantitative separation the matrix components must already have been flushed to waste while the analyte is still retained on the column. The elution profile of the protein peak is shown in Fig. 4. After 5

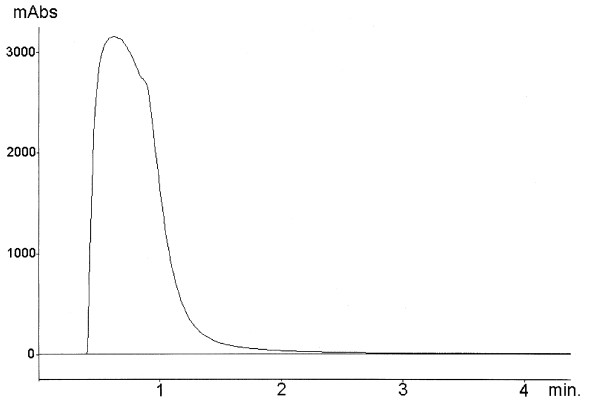

Fig. 4. Example of matrix elution: 100 μl of pooled blank serum was injected into the extraction column. UV-detector was set to 296 nm and coupled directly with the extraction column (LiChrospher® RP-ADS C_{18}).

min the matrix peak returned to the baseline, so the retention time of the antibiotics had to be longer.

Retention time of the antibiotics at the extraction column increased with decreasing pH of the extraction mobile phase but the pH adjustment was limited by the column specification (pH 2–7.5) and potential serum precipitation. The buffer molarity also affected the interaction of analyte and extraction columns. Reduction of the molarity reduced the elution strength of the mobile phase, however, buffer capacity had to be considered.

Without ion-pair agents it was impossible to achieve good separation of the relatively hydrophilic β-lactam antibiotics on the extraction column. The 12.5 m*M* formiate buffer, pH 3.0 with 5 m*M* *n*-heptanesulfonic acid as ion-pair agent fulfilled all mentioned requirements.

Three extraction columns were tested: ADS-RP C_4, C_8, C_{18}. Best retention on the extraction column was achieved using ADS-RP C_8 for the ceftazidime and ADS-RP C_{18} for the meropenem assay. Injection of 50 μg/ml meropenem or ceftazidime standard solution showed no break-through of the antibiotics during the matrix elution period of 8 min.

The used phosphate buffer (pH 5.0) for the pretreatment procedure and the HPLC-analysis provided maximum stability of the analytes in aqueous solution [11,12]. A typical chromatogram of ceftazidime or meropenem in pooled serum compared with a drug free serum sample is shown in Fig. 5(1) and (3), respectively. Chromatograms of blank and spiked pool sputum are shown in Fig. 5(2) and (4).

Both antibiotics are well separated from other detectable components in serum and sputum at the selected wavelength. The contrasting peak areas of the matrix elution at the extraction step (Fig. 4) and the remaining matrix components in the resulting chromatogram (Fig. 5) demonstrate the clean-up efficiency.

3.3. Quantification and statistics

Calibration curves were determined with aqueous standard solutions of the antibiotics in concentration ranges from 0.5 to 40 μg/ml.

The 20-μl sample loop was used for serum concentrations above 10 μg/ml to prolong the lifetime of the extraction column. Sputum samples and less concentrated serum samples were injected in a 100-μl loop to increase the detection limit.

The equations of the regression lines of meropenem were $y=36\,561x-3139.5$ ($r^2=0.9993$) and $y=156\,209x-17\,034$ ($r^2=0.9980$) for 100-μl and 20-μl sample loops, respectively, where x represents the analyte concentration in μg/ml and y the corresponding peak area. Linear regression analysis for ceftazidime yielded the following equations: $y=189\,459x+50\,395$ ($r^2=0.9997$) and $y=44\,608x-605.94$ ($r^2=0.9997$) for 100-μl and 20-μl sample loops, respectively.

The recovery, precision and accuracy of the meroponem and ceftazidime assay are summarized in Table 1.

The recovery of spiked sputum samples were (mean recovery in percent±SD, nominal concentration in brackets): 56.7±7.7 (1.05–1.77 μg/ml, $N=5$); 73.4±5.7 (3.99–4.63 μg/ml, $N=5$) for the meropenem assay and 49.3±7.4 (1.59–2.22 μg/ml, $N=6$); 56.9±4.9 (3.15–3.51 μg/ml, $N=6$) for the ceftazidime assay. The quantification limits of the assays were less then 0.5 μg/ml for both antibiotics.

The supposed reason for the lower recoveries of spiked sputum samples is the adsorption of the antibiotics at macromolecules (e.g., proteins and polysaccharides). The higher standard deviations may be an effect of the inhomogeneous structure of sputum samples. The measured antibiotic concentration therefore represents the effective antibiotic concentration in bronchial secretions because anti-

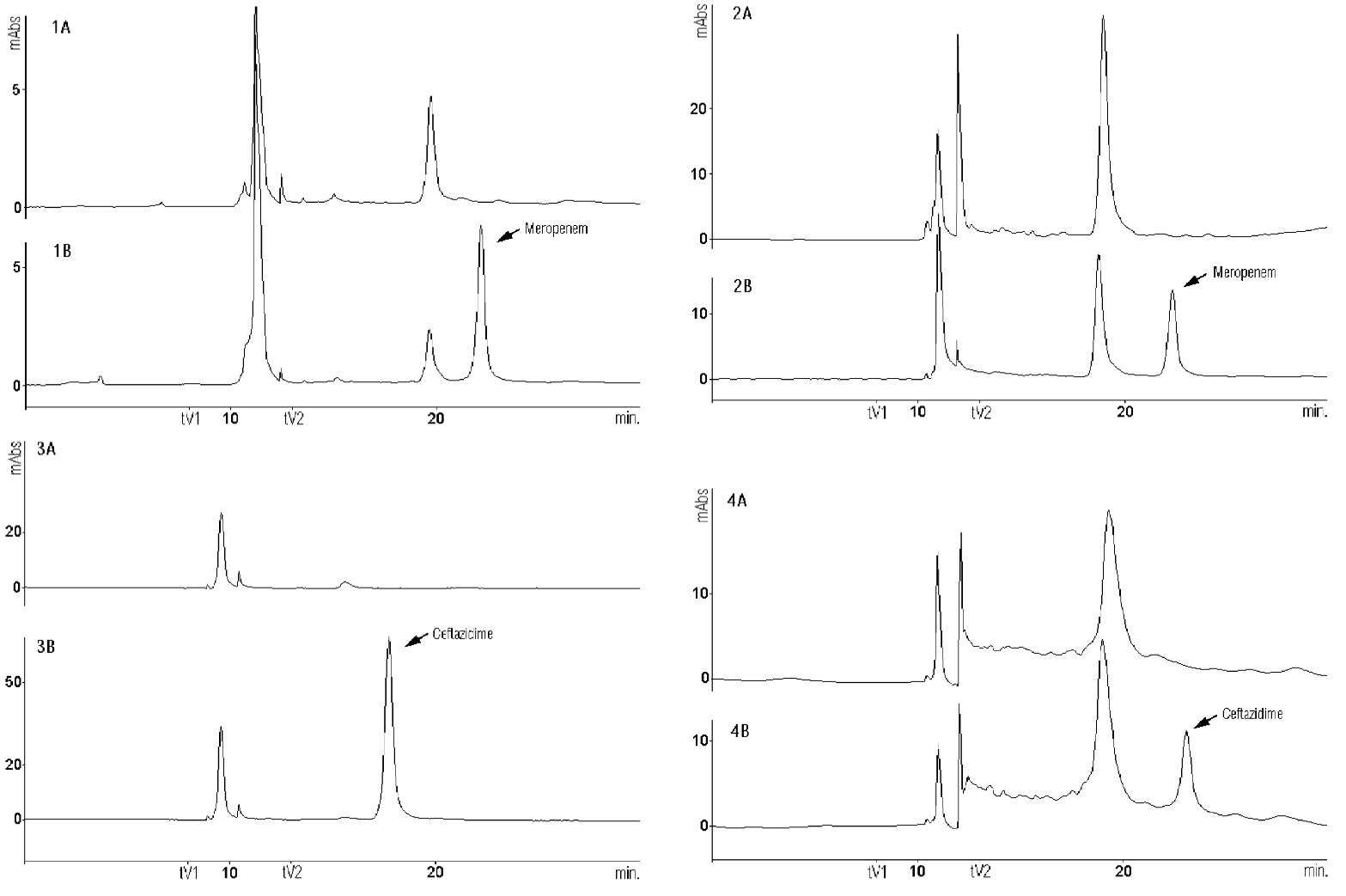

Fig. 5. Chromatograms of spiked matrix versus blank matrix. tV1 labels the valve switching time to transfer position, tV2 indicates shift to initial position of the valve. (1A) Blank serum, (1B) serum spiked with 4.88 μg/ml meropenem, (2A) blank sputum, (2B) spiked sputum with 4.40 μg/ml meropenem, (3A) blank serum, (3B) serum spiked with 12.03 μg/ml ceftazidime, (4A) blank sputum and (4B) spiked sputum with 3.27 μg/ml ceftazidime.

Table 1
Statistics of spiked serum samples for meropenem and ceftazidime

Nominal concentration (μg/ml)	N	Precision		Accuracy		Recovery	
		Mean (μg/ml)	C.V. (%)	Mean difference from nominal (μg/ml)	Mean difference from nominal (%)	Mean recovery from nominal (%)	SD
Ceftazidime							
19.54	5	21.32	1.9	1.78	9.1	109.1	0.02
8.32	4	8.25	2.4	0.17	2.0	99.2	0.02
1.95	6	1.87	6.6	0.10	5.0	95.8	0.06
Meropenem							
28.12	5	29.58	6.0	1.54	5.5	105.2	0.06
9.76	5	9.66	2.2	0.21	2.2	98.9	0.02
4.88	5	4.65	3.3	0.23	4.8	95.2	0.03
0.98	5	0.87	5.1	0.10	10.6	89.4	0.05

biotic molecules adsorbed at macromolecules are considered not to interact with pathogens.

Sputum samples of CF-patients were measured along with spiked pool sputum samples to validate the method. The correlation between nominal concentration and the obtained peak area was good (r^2=0.9855, N=12).

4. Conclusion

The presented assay for the determination of ceftazidime and meropenem in serum and sputum samples is sufficiently fast, sensitive and reliable for drug monitoring of these antimicrobial agents in clinical routine. It is also suitable for pharmacokinetic studies in these biological fluids. The detection limits of the assays allow the determination of antibiotic levels down to the MIC_{90} of the most susceptible pathogens.

The method has shown its clinical suitability for controlling the antimicrobial therapy of CF patients. The evaluation of serum and sputum antibiotic levels was helpful to avoid subinhibitoric concentrations at the site of infection.

5. Uncited reference

[9]

Acknowledgements

This work was supported by Zeneca and Glaxo Wellcome.

References

[1] Micromedex DrugDex, Micromedex Inc, 1999.
[2] B.A. Christenson, B. Ljungberg, L. Eriksson, I. Nilsson-Ehle, Eur. J. Clin. Microbiol. Infect. Dis. 17 (1998) 873.
[3] S. Bompadre, L. Ferrante, F.P. Alò, L. Leone, J. Chromatogr. B 669 (1995) 265.
[4] H. Elkhaïli, S. Niedergang, D. Pompei, L. Linger, D. Leveque, F. Jehl, J. Chromatogr. B 686 (1996) 19.
[5] M.A. Al-Meshal, M.A. Ramadan, K.M. Lofti, A.M. Shibl, Clin. Pharm. Ther. 20 (1995) 159.
[6] J.S. Leeder, M. Spino, A.M. Tesoro, S.M. MacLeod, Antimicrob. Agents Chemother. 24 (1983) 720.
[7] K.-S. Boos, A. Rudolphi, S. Vielhauer, A. Walfort, D. Lubda, F. Eisenbeiβ, Fresenius J. Anal. Chem. 352 (1995) 684.
[8] LiChrospher® ADS Manual, Merck KGaA, Darmstadt, Germany, 1996.
[9] E. Bergogne-Bérézin, C. Muller-Serieys, M. Aubier, M.C. Dombret, Eur. J. Clin. Pharmacol. 46 (1994) 87.
[10] R. Hirsch, J.E. Zastrow, R.C. Kory, J. Lab. Clin. Med. 74/72 (1969), August.
[11] Y. Takeuchi, M. Sunagawa, Y. Isobe, Y. Hamazume, T. Noguchi, Chem. Pharm. Bull. 43 (1995) 689.
[12] M. Zhou, R.E. Notari, J. Pharm. Sci. 84 (1995) 534.