Monitoring of cefepime in human serum and plasma by micellar electrokinetic capillary chromatography: Improvement of sample preparation and validation by liquid chromatography coupled to mass spectrometry

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ABSTRACT

Cefepime monitoring in deproteinized human serum and plasma by micellar electrokinetic capillary chromatography and liquid chromatography coupled to mass spectrometry in presence of other drugs is reported. For micellar electrokinetic capillary chromatography, sample preparation comprised dodecylsulfate protein precipitation at pH 4.5 using an increased buffer concentration compared to that of a previous assay and removal of hydrophobic compounds with dichloromethane. This provided robust conditions for cefepime analysis in the presence of sulfamethoxazole and thus enabled its determination in samples of patients that receive co-trimoxazole. The liquid chromatography assay is based upon use of a column with a pentafluorophenyl-propyl modified and multi-endcapped stationary phase and the coupling to electrospray ionization with a single quadrupole detector. The performances of both assays with multi-level internal calibration were assessed with calibration and control samples and both assays were determined to be robust. Cefepime levels monitored by micellar electrokinetic capillary chromatography in samples from patients that were treated with cefepime only and with cefepime and co-

trimoxazole were found to compare well with those obtained by liquid chromatography coupled to mass spectrometry. Cefepime drug levels determined by micellar electrokinetic capillary chromatography could thereby be validated.

1 Introduction

Cefepime (for chemical structure see Fig. 1A) is a parenteral fourth generation cephalosporin that has a broad-spectrum in activity against Gram-positive and Gram–negative bacteria. It is administered for nosocomial infections, infections in immunosuppressed or critically ill patients. The dosing schemes range from 1 g every 12 h to 2 g every 8 h. They also depend on the minimal inhibitory concentration of the causative pathogen and the severity of disease. Cefepime penetrates well into the cerebrospinal fluid and is eliminated mainly by renal excretion. Almost 90% is excreted unchanged (i.e. as active drug) and cefepime clearance correlates well with the creatinine clearance [1]. The half-life of cefepime in the serum of patients with normal renal function is 2 h, whereas that in patients with end-stage renal disease is 18 h. Cefepime overdose may lead to neurotoxic effects, including encephalopathy, neuromuscular excitability, convulsions and coma. To ensure therapeutic levels and avoid neurotoxicity, therapeutic drug monitoring (TDM) of cefepime should be used. Data gathered thus far suggest that TDM can help to minimize the risk of major adverse reactions and to increase drug safety on an individual basis [1–5]. Analytical methods for monitoring cefepime in biosamples include bioassays [6], HPLC with UV detection [7–10], LC–MS [11,12] and MEKC [5,13–16]. They should be capable of accurately monitoring cefepime levels in the range of 1 to 50 μg/mL [5].

An MEKC assay based on protein precipitation using SDS at pH 4.5 and hydrodynamic injection from the collected supernatant was developed and applied in our laboratory for TDM of cefepime [5]. In a 3-year period, TDM with MEKC was applied to more than 1000 patient samples. The monitored cefepime levels ranged between 1.0 and 131.7 µg/mL (989 samples). Furthermore, cefepime concentrations were below the LOQ (<1 µg/mL) in 53 samples and the drug was not detected in 19 samples. Due to an interference with one or more compounds, cefepime levels could not be determined in 50 samples. Many of these sera contained sulfamethoxazole, which is an active ingredient of co-trimoxazole (sulfamethoxazole and trimethoprim). Co-trimoxazole (e.g. Bactrim ®) is an antibiotic formulation that is occasionally used together with cefepime in immunosuppressed patients (e.g. for *Pneumocystis jirovecii* prophylaxis and cefepime for treatment of neutropenic fever). Sulfamethoxazole could not be separated from cefepime under the used MEKC assay conditions. Hence, analyses had to be repeated without co-trimoxazole (i.e. co-trimoxazole treatment was stopped about 24 h before blood collection for TDM of cefepime). This prompted us to change sample preparation to remove hydrophobic compounds such as sulfamethoxazole and to provide conditions that lead to the separation of cefepime and residual amounts of sulfamethoxazole. Furthermore, an LC–MS assay for cefepime was developed, applied to representative sets of patient samples and used to validate the drug levels determined by MEKC. In this paper, modifications of sample preparation for MEKC, the performance of the MEKC assay with modified sample preparation, the development

and characterization of the LC–MS assay and the comparison of cefepime levels obtained by MEKC and LC–MS are presented.

2 Materials and methods

2.1 Chemicals, materials and patient samples

Used reagents were of analytical or research grade. Cefepime dihydrochloride monohydrate was obtained from Sandoz Pharmaceuticals (Cham, Switzerland). Ceftazidime pentahydrate, obtained from Glaxo (Greenford, Middlesex, UK) or deuterated cefepime-d3 sulfate from TRC (Toronto, Canada) were used as internal standards. Sulfamethoxazole was from Helvepharm (Schmitten, Switzerland). Sodium acetate, formic acid (98–100%), acetic acid (100%) and $Na_2HPO_4 \times 2\ H_2O$ were purchased from Merck (Darmstadt, Germany). $Na_2B_4O_7 \times 10\ H_2O$, SDS and ammonium formate were from Fluka (Buchs, Switzerland). Dichloromethane was obtained from VWR (Schlieren, Switzerland). Bovine serum (adult) was purchased from Sigma (St. Louis, MO, USA) and was used for the preparation of calibration and control samples. Patient samples were provided from the University Hospital Bern, Switzerland. The analyses were performed within the context of QC. Analysis of patient samples was approved by the local ethical committee (KEK-BE 035/2014, SNCTP 801).

2.2 MEKC assay with dichloromethane removal of interferences

2.2.1 Solutions, calibrators and controls

The acetate buffer was prepared by dissolving 8.2 g of sodium acetate into 180 mL of water, followed by pH adjustment with acetic acid to 4.5 and filling up with water to a final volume of 200 mL. Stock solutions of cefepime (100 and 500 µg/mL for preparation of calibrators and controls, respectively) and ceftazidime (400 µg/mL, internal standard) were prepared in 10-fold diluted acetate buffer (pH 4.5) and stored as 0.2 mL aliquots at −70°C. The sample preparation reagent was composed of 75 mM SDS in acetate buffer, pH 4.5. For the preparation of calibrators, aliquots of 1.0, 5.0, 10.0, 20.0, 40.0, and 60.0 µL of the 100 µg/mL cefepime stock solution and aliquots of water (49.0, 45.0, 40.0, 30.0, 10.0, and 0 µL, respectively) were added to 100 µL of bovine serum and treated as described in Section 2.2.2. Due to internal calibration with a constant amount of the internal standard, this procedure provided calibrators with 1.0, 5.0, 10.0, 20.0, 40.0, and 60.0 µg/mL cefepime, respectively. Three controls containing 6.0, 25.0, and 45.0 µg/mL cefepime in bovine serum were prepared batchwise. Typically, 3.5 mL of a control was produced by combining an appropriate amount of the 500 µg/mL cefepime stock solution with bovine serum and aliquots of 100 µL were frozen in plastic vials at −70°C. Frozen samples were slowly defrosted and vortex mixed before analysis.

2.2.2 Sample preparation

Sample preparation is based upon deproteination with SDS at pH 4.5. If not stated otherwise, 100 µL of sample (patient sample, control), 20 µL of internal standard solution, 50 µL of sample preparation reagent and 50 µL of water were combined in a 2.0 mL Eppendorf vial. After mixing, 250 µL of dichloromethane was added. The sample was mixed again for about 5 s and centrifuged at 13 000 × g for about 10 min at 20°C using a model

5415 R Eppendorf centrifuge (Hamburg, Germany). Then, 100 μL of the clear supernatant was transferred into a PCR sample vial for analysis. Calibrators were treated the same way except that no water was added before centrifugation.

2.2.3 Instrumentation and running conditions

MEKC analyses were performed in uncoated fused-silica capillaries of 50 μm id (Polymicro Technologies, Phoenix, AZ, USA) and 60 cm (50 cm effective) length using a P/ACE MDQ and a PA800plus CE system (Beckman Coulter, Brea, CA, USA). The BGE (separation medium) was composed of 6 mM $Na_2B_4O_7$, 10 mM Na_2HPO_4, and 75 mM SDS (pH 9.1) and is the same as employed previously [5]. Analyses were carried out with an applied voltage of 15 kV (current: 23.5 μA), analyte detection using the UV detector at 254 nm, and hydrodynamic sample injection during 6 s at 1 psi (1 psi = 6894.8 Pa). To increase the performance, a short water plug was injected after the sample (3 s at 0.5 psi). The temperatures of capillary cartridge and sample holder were maintained at 25 and 25°C, respectively. Quantitation was based upon six-point internal calibration in the range of 1.0 to 60.0 μg/mL of cefepime using corrected peak areas (peak areas divided by detection time) for data evaluation. Other conditions as reported previously [5].

2.3 LC–ESI-MS assay

2.3.1 Solutions, calibrators and controls

Stock solutions of cefepime (500, 10 and 1 μg/mL), deuterated cefepime (10 μg/mL) and ceftazidime (100 μg/mL) were prepared in 50 mM acetic acid and stored as small aliquots

at −70°C. Aliquots of 12.5 and 25.0 µL of the 1 µg/mL cefepime stock standard solution and aliquots of 5.0, 15.0, 25.0, 35.0 and 50.0 µL of the 10 µg/ml cefepime stock standard solution, together with corresponding amounts of 50 mM acetic acid (37.5, 25.0 µL, respectively) and (45.0, 35.0, 25.0, 15.0 and 0 µL, respectively), were added to 50 µL of bovine serum thereby producing calibrators with concentration of 0.25, 0.5, 1.0, 3.0, 5.0, 7.0 and 10.0 µg/mL of cefepime in bovine serum. Three controls containing 0.5, 5.0 and 10.0 µg/mL of cefepime in bovine serum were prepared. Typically, 2 mL of a control, were prepared by combining an appropriate amount of the 500 µg/mL cefepime stock solution with bovine serum. Aliqots of 70 µL were stored at −70°C.

2.3.2 Sample preparation

Sample preparation was based on deproteination with acetonitrile. Fifty microliters of sample (patient sample, control), 10 µL of internal standard solution, 50 µL of 50 mM acetic acid and 125 µL of acetonitrile were combined in a 2.0 mL Eppendorf vial, vortexed for about 15 s and centrifuged at 13 000 x g for 5 min at 20°C. After centrifugation, 125 µL of the clear supernatant was transferred to another Eppendorf vial and combined with 250 µL of water. After mixing for 10 s, 125 µL of dichloromethane was added. Sample was mixed and centrifuged again. Then, 100 µL of the upper aqueous layer was transferred into the LC–MS vial and analyzed. Calibrators were treated the same way, except that no acetic acid was added before centrifugation.

2.3.3 Instrumentation and running conditions

LC–MS analyses were performed with a PFP Nucleodur HPLC column (i.d. 4.0 mm, length 125 mm and particle size 5.0 µm, Macherey–Nagel, Oensingen, Switzerland) using a HP series 1100 (Hewlett Packard, Waldbronn, Germany) coupled to a Agilent 6120 Single Quadrupole LC–MS System with an APCI/ESI interface (Agilent Technologies, Santa Clara, California). A gradient elution was applied with solvent A, containing 5 mM ammonium formate (pH 3.0), and solvent B comprising 100% acetonitrile. The mobile phase flow rate was maintained at 0.5 mL/min. Solvent B was increased from 5 to 20% in the time range of 0–10 min (linear gradient). Thereafter, the column was rinsed for 2 min with 50% of solvent B at a flow rate of 2 mL/min. Re-equilibration was performed during 3 min at the same flow rate with 5% B. The column oven temperature was set to 25°C and the injection volume was 10 µL. Nitrogen, provided by a nitrogen generator N2-Mistral-4 (Schmidlin Labor, Neuheim, Switzerland), was used as nebulizing and desolvation gas. The flow rate of nitrogen was 10 L/min, drying temperature was 300°C and nebulizing pressure was 60 psig. The vaporizer temperature was 250°C, the capillary voltage was set to 4000 V and the charging voltage was 2000 V. Cefepime, deuterated cefepime-d3 and ceftazidime were detected in the positive SIM mode with m/z mass values of 481.1, 484.2 and 547.1, respectively. These values correspond to the $[M+H]^+$ ions of the three molecules. Quantitation was based upon seven-point internal calibration in the range of 0.25 to 10.0 µg/mL of cefepime using peak areas for data evaluation.

2.4 Determination of recovery

To determine the recovery of cefepime and the internal standard, spiked samples in water and bovine serum were analyzed and the recovery was calculated as the mean ratio between the

peak area response of three or four replicates of these samples and the corresponding peak

area response of equivalent samples in water.

2.5 Statistical and graphical data analysis

Data evaluation and presentation was made with SigmaPlot software version 12.5 (Systat

Software, San Jose, CA, USA). The Mann–Whitney rank sum test was used to compare two

groups of data. $P < 0.05$ was considered to be significant.

3 Results and discussion
3.1 MEKC analysis of cefepime in presence of sulfamethoxazole

With our routine MEKC assay, comigration of cefepime with sulfamethoxazole, one of the

two active compounds present in co-trimoxazole (sulfamethoxazole and trimethoprim), was

observed [5]. An example of the interference is presented with the electropherogram

depicted in Fig. 1A. The data represent the analysis of a serum of a patient who received

both cefepime and co-trimoxazole. Initial efforts to separate cefepime and

sulfamethoxazole by modification of the BGE composition were not successful. Thus, efforts

with different sample preparations were investigated to solve this problem. Cefepime and

ceftazidime are hydrophilic compounds (for chemical structures see inserts in Figs. 1A and

1C, respectively; $\log P_{ow}$ values of about –2.7 [17]). Sulfamethoxazole is more hydrophobic

than cefepime and ceftazidime (for chemical structure see insert in Fig. 1E; $\log P_{ow}$ value of

0.89 [18]). Thus, dichloromethane was added to the mixture of serum and sample

preparation reagent (for composition of sample preparation reagent see Ref. [5]). After

mixing and centrifugation, MEKC analysis of the clear aqueous supernatant provided the data presented in Fig. 1B. The magnitudes of two peaks (peaks marked with S+CEF and an asterisk in Fig. 1A) became much smaller compared to the electropherogram obtained without the dichloromethane treatment. Sulfamethoxazole and a second compound which could be a metabolite of sulfamethoxazole became extracted and could be analyzed in the organic phase (insert of Fig. 1B). Cefepime and ceftazidime were not detected in the organic phase. Spiking of the aqueous supernatant with cefepime revealed an electropherogram with an enlarged cefepime peak that exhibited a distinct shoulder at its base which is an indication of comigration of cefepime with another compound. Finally, the extraction of sulfamethoxazole with dichloromethane from the pH 4.5 environment could be confirmed using a sulfamethoxazole standard (data not shown). The data revealed that the extraction efficiency for sulfamethoxazole was about 85%. Thus, the peak in the electropherogram presented in Fig. 1B (marked "CEF?") does contain a residual amount of sulfamethoxazole which falsifies the analytical result. With the calibration for cefepime, a drug level of 17.3 µg/mL was calculated. This is much less compared to the value obtained when the entire peak of Fig. 1A is used for quantification (123.9 µg/mL). These data illustrate that a further modification of the sample preparation was required for proper MEKC analysis of cefepime in such a sample.

A change in the preparation of the acetate buffer for the sample preparation reagent provided the anticipated breakthrough. For the assay described previously [5], the acetate buffer was made from a 0.5 M acetic acid to which 0.5 M sodium acetate solution was

added until pH 4.5 was obtained (estimated buffer composition: 0.500 M acetic acid and

0.177 M NaOH). In the work described here, the acetate buffer was prepared with a 0.500

M sodium acetate solution whose pH was adjusted with acetic acid to 4.5 (Section 2.2.1,

estimated composition of 1.413 M acetic acid and 0.500 M NaOH). Using this buffer with

about 2.8-fold higher concentration in the sample preparation reagent, resolution between

cefepime and sulfamethoxazole became possible. This is illustrated with the analysis of the

same patient sample for which a small cefepime peak of 3.8 µg/mL was now detected

before a large sulfamethoxazole peak (Fig. 1C). Furthermore, the small peak detected before

the internal standard (marked with # in panels A and C of Fig. 1) could not be separated

from the internal standard (Fig. 1C). The small peak is an impurity of ceftazidime used as

internal standard and was not detected under the conditions of Fig. 1A when the internal

standard was omitted. Finally, sample preparation with extraction of sulfamethoxazole with

dichloromethane as described in Section 2.2.2 provided the data of Fig. 1D. Comparison

with panel C reveals the same amount of cefepime (4.1 µg/mL) and a much reduced peak of

sulfamethoxazole and the peak marked with an asterisk were monitored in the aqueous

phase. These data visualize that dichloromethane does not completely remove

sulfamethoxazole. Analysis of the organic phase provided two peaks (insert in Fig. 1D) which

is comparable to the analysis with the previous sample matrix (insert in Fig. 1B).

Furthermore, spiking of the aqueous phase of Fig. 1D with cefepime and reanalysis resulted

in a much enlarged sharp peak for cefepime (Fig. 1E). Thus, the sample matrix change

associated with the acetate buffer in the sample preparation reagent led to the separation

of cefepime and sulfamethoxazole and the use of dichloromethane removed a large portion

of sulfamethoxazole from the sample. Both effects were combined in the modified MEKC

assay and referred to as MEKC-DCM in the remaining part of this paper. Characterization of

the modified assay with data comparison to those obtained with the routine MEKC method

is described below. It is important to note, that BGE composition, sample injection and

applied voltage were kept the same as in the previous assay [5]. No efforts were undertaken

to investigate the reasons for the impact of acetate buffer composition on the MEKC

separation of cefepime and sulfamethoxazole.

3.2 MEKC assay characterization with dichloromethane removal of interferences

With the new sample preparation, internal standard and cefepime were detected around 9

and 11 min, respectively, and the current was about 24 μA, values that are comparable to

those under the previous assay conditions [5]. Electropherograms obtained with the three

control samples comprising 6.0 μg/mL cefepime (panel A), 25.0 μg/mL cefepime (panel B)

and 45.0 μg/mL cefepime (panel C) and a typical patient sample (panel D) are presented in

Fig. 2. Both, cefepime and ceftazidime are depicted to form sharp peaks. Table 1

summarizes the analytical characteristics of the six-level internal calibration for 6

consecutive sets of data. The calibration graphs were found to be linear ($r^2 \geq 0.9991$) with F

values ≥ 4360 ($P < 0.001$). Mean values for the slope and r^2 were 0.0110 mL/μg and 0.9995,

respectively. Furthermore, the mean of the y intercept was –0.0044, a value which is

significantly smaller than the ratio produced by the smallest calibrator. The recovery

assessed for cefepime levels of 5 and 60 µg/mL were determined to be 105.7 and 98.4%, respectively. Corresponding values for ceftazidime were 108.9 and 98.8%, respectively. The limit for quantitation was taken as 1 µg/mL (smallest calibrator) and the detection limit was determined to be 0.5 µg/mL (S/N=3). Typical assay imprecision data for detection times and drug levels obtained for the analysis of the three control samples are summarized in Table 2. Intraday and interday (*n*=6) RSD values for the detection times were < 0.7 and < 1.6%, respectively. Intraday and interday drug level repeatabilities were found to be ≤ 3.1 and ≤ 5.5%, respectively. All these data compare favorably with those obtained previously [5] and illustrate that the MEKC-DCM assay is robust and suitable for TDM. Analysis of human plasma with EDTA as anticoagulant instead of human serum resulted in indistinguishable electropherograms. Thus, plasma patient samples were analyzed with the same assay.

Comparison of cefepime levels determined with the two MEKC methods for 128 patient samples whose electropherograms did not exhibit apparent interferences with cefepime shows excellent agreement between the two assays. Evaluation with the Mann–Whitney rank sum test revealed no statistically significant difference between the two input groups (*P* = 0.701). Linear regression analysis of the 128 data pairs revealed a linear relationship (r^2 = 0.941) described by the equation MEKC = 0.94 x MEKC-DCM + 0.47 which is very close the line of equality (Fig. 3A). Plotting the difference of the two values (MEKC – MEKC-DCM) against the mean shows excellent consistency between the two methods (Fig. 3B). The mean of the differences and SD were –0.80 and 3.25 µg/mL, respectively, indicating that on average the MEKC-DCM method gave slightly higher amounts than the assay without the

dichloromethane treatment. This difference might be a result of inconsistent calibrations. It is, however, insignificant and has no consequences for TDM of cefepime.

3.3 Characterization of the LC–MS assay and data comparison to MEKC

LC–MS chromatograms obtained with a blank (panel A), a calibrator comprising 10.0 μg/mL cefepime (panel B) and a patient sample containing 4.2 μg/mL cefepime (panel C) are presented in Fig. 4. Both, cefepime and ceftazidime are detected as nicely separated peaks. Calibration results of 6 consecutive sets of data are presented in Table 1. The calibration graphs were found to be linear ($r^2 \geq 0.9908$) with F values ≥ 534 ($P < 0.001$). Comparable data were obtained with deuterated cefepime-d3 as internal standard despite that the signal at m/z 484.2 contained a small contribution from cefepime (2.4% of cefepime abundance according to isotope distribution calculated with Isotope Distribution Calculator (www.sisweb.com/mtools/sotope.htm); data not shown). In analogy to the analysis of cefepime by MEKC, all data discussed below are for ceftazidime as internal standard. The recovery assessed for cefepime levels of 1 and 10 μg/mL were determined to be 72.7 and 74.0%, respectively. Corresponding values for ceftazidime were 71.3 and 73.9%, respectively. The matrix effect defined as the ratio of peak area response in presence of matrix and absence of matrix components [19] was 1.1 in all cases. The limit for quantitation was taken as 0.25 μg/mL (smallest calibrator) and the detection limit was determined to be 0.1 μg/mL (S/N=3). Assay imprecision data for detection times and drug levels obtained for the analysis of the three control samples are summarized in Table 2. Intraday and interday

(n=6) RSD levels for the detection times were < 0.5 and < 1.3%, respectively. Intraday and interday drug level repeatabilities were found to be ≤ 4.3 and ≤ 5.4%, respectively. All these data indicate that the LC–MS assay is robust. It is important to note that samples containing more than 10 µg/mL cefepime were diluted before analysis.

The mean ± SD (median, range) of the 128 cefepime levels determined by MEKC-DCM and LC–MS were determined to be 21.42 ± 16.10 (16.26, 2.66 – 71.84) µg/mL and 22.10 ± 16.37 (17.35, 2.39 – 77.49) µg/mL, respectively. Comparison of the two sets of data with the Mann–Whitney rank sum test revealed no statistically significant difference between the two input groups (P = 0.682). Comparison of cefepime levels found in patient serum samples using the two methods shows excellent agreement between the two methods. Linear regression analysis of the 128 data pairs revealed a linear relationship (r^2 = 0.977) described by the equation LC–MS = 1.01 x MEKC-DCM + 0.58 which is very close the line of equality (Figure 5A). Plotting the difference of the two values (LC–MS – MEKC-DCM) against the mean, shown in panel B of Figure 5, again shows excellent consistency between the two methods. The mean of the differences (SD) was –0.68 (2.47) µg/mL, indicating that, on average, the MEKC-DCM method resulted in slightly lower amounts compared to the LC–MS method. This difference, however, is insignificant and has no consequences for TDM of cefepime. In analogy, comparative data analysis of the 128 cefepime levels obtained with MEKC (Fig. 3) and LC–MS revealed a linear relationship with a coefficient of determination r^2 of 0.938, a slope of 1.03 and a y intercept of 0.96 µg/mL. The bias, expressed by the mean of the differences of the corresponding data pairs, and its SD were 1.48 and 4.07 µg/mL,

respectively. Comparison of the two sets of data with the Mann–Whitney rank sum test revealed no statistically significant difference between the two input groups ($P = 0.451$). Thus, for the given set of samples, the data obtained with MEKC-DCM and MEKC could be validated by LC–MS.

3.4 Results with samples of patients receiving co-trimoxazole and other potential interferences

With our previous MEKC assay [5], a number of samples could not be analyzed due to interferences, including those associated with sulfamethoxazole (Fig. 1). If not carefully investigated by peak shape analysis as described previously [5], the use of this assay could lead to unrealistic cefepime levels as is shown with the data of samples 1 to 6 presented in Fig. 6A. The determined cefepime levels (MEKC data of Fig. 6A) are much higher compared to those monitored with the modified sample preparation (MEKC-DCM data). The correctness of the latter cefepime levels could be confirmed by LC–MS (Fig. 6A). MEKC-DCM and LC–MS analysis of samples from patients that received both cefepime and co-trimoxazole revealed cefepime levels that compare well (Fig. 6B). For the 38 samples whose data are presented in Fig. 6B, linear regression analysis of the data pairs revealed a linear relationship with a coefficient of determination r^2 of 0.988, a slope of 1.02 and a y intercept of 0.42 µg/mL. This is very close to a line of equality. Furthermore, comparison of the two sets of data with the Mann–Whitney rank sum test revealed no statistically significant difference between the two input groups ($P = 0.975$). It is interesting to note that the second compound extracted with dichloromethane (panels B and D of Fig. 1) was found in

all 38 samples. This suggests that it represents a metabolite of sulfamethoxazole. These data demonstrate that the MEKC-DCM assay is suitable to properly handle patient samples that contain sulfamethoxazole.

Furthermore, it is important to mention that compounds other than sulfamethoxazole, which are not hydrophobic and are thus not removed by dichloromethane but are detected in proximity of cefepime or the internal standard, may still produce interferences. Analysis of critical samples by MEKC-DCM, including those of Fig. 4 in [5], however, was straightforward. Cefepime levels determined by MEKC-DCM were found to compare well with those obtained with LC–MS. Drug levels of two examples, whose electropherograms are presented in Fig. 6C, are presented as samples 7 and 8 in Fig. 6A. These two samples stem from critically ill patients that received multiple drugs. Sample 7 is the patient serum whose data are presented in Fig. 4B of the previous paper [5] for which cefepime was detected at the front edge of a broad interference of unknown identity (graph a of Fig. 6C). With the MEKC-DCM assay cefepime could be detected interference-free whereas resolution between the internal standard and a broad peak detected shortly thereafter was diminished (Fig. 6C, compare graphs a and b). The cefepime level determined was almost identical to that obtained with LC–MS and somewhat higher than that measured by MEKC (Fig. 6A). Similar results were obtained with sample 8 (Fig. 6A, upper graphs in Fig. 6C). It is important to note that the complexity of these electropherograms is not observed frequently. Typically, very few peaks other than those of cefepime and the internal standard are monitored (Fig. 2D; examples given in [5]). Thus, to avoid interferences with

sulfamethoxazole and other neutral compounds, the previously described MEKC assay should be replaced with the MEKC-DCM method described in this paper. With MEKC-DCM, analysis of both the sample and the same sample fortified with a small amount of cefepime is also recommended to verify peak assignment (for examples see Ref. [5]).

4 Concluding remarks

Sample preparation for MEKC analysis of cefepime in serum and plasma was modified to provide resolution between cefepime and sulfamethoxazole and to remove a large part of sulfamethoxazole from the sample with dichloromethane. With these changes, MEKC can be used to analyze cefepime in samples of patients that receive cefepime and co-trimoxazole. It provided also good data with other samples of critically ill patients that received multiple drugs. The performance of the improved MEKC assay with multi-level internal calibration was assessed and determined to be robust. Cefepime levels of representative sets of samples stemming from patients that were treated with cefepime only and with cefepime and co-trimoxazole were found to compare well with those of a laboratory developed LC–MS assay. Cefepime drug levels determined by MEKC could thereby be validated. The data presented suggest that MEKC can be efficiently used for TDM of cefepime. The MEKC method is not better than LC–MS. However, it represents an attractive alternative to LC–MS for laboratories that do not have LC–MS instrumentation. The costs of the assay and the amounts of chemicals required for analysis are lower compared to those associated with LC–MS.

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Conflicts of interest

The authors have declared no conflict of interest.

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Legends:

Figure 1: MEKC electropherograms obtained for a patient sample comprising

sulfomethoxazole by analysis (A) with sample matrix of the routine method [5], (B) of the

aqueous phase after extraction with dichloromethane from the same sample matrix, (C)

with the new sample matrix, (D) of the aqueous phase after extraction with

dichloromethane from the new sample matrix, and (E) of sample D spiked with cefepime.

The inserts in panels B and D depict corresponding electropherograms obtained for the

analysis of the organic phases. The chemical structures of cefepime, ceftazidime and

sulfomethoxazole are given as inserts in panels A, C and E, respectively. Key: CEF, cefepime;

IST, internal standard (ceftazidime); S, sulfamethoxazole; * coextracting compound; #
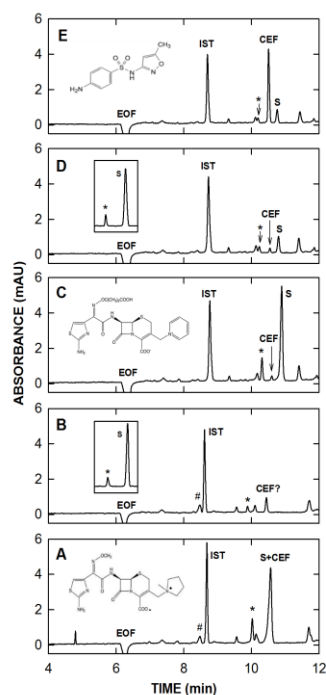
impurity of internal standard.

Figure 1

Figure 2: Electropherograms obtained with the MEKC-DCM assay for bovine serum controls comprising (A) 6.0 µg/mL cefepime, (B) 25.0 µg/mL cefepime and (C) 45.0 µg/mL cefepime together with 80.0 µg/mL ceftazidime, and (D) a patient sample whose cefepime level was determined to be 42.8 µg/mL. Key: CEF, cefepime; IST, internal standard (ceftazidime).

Figure 2
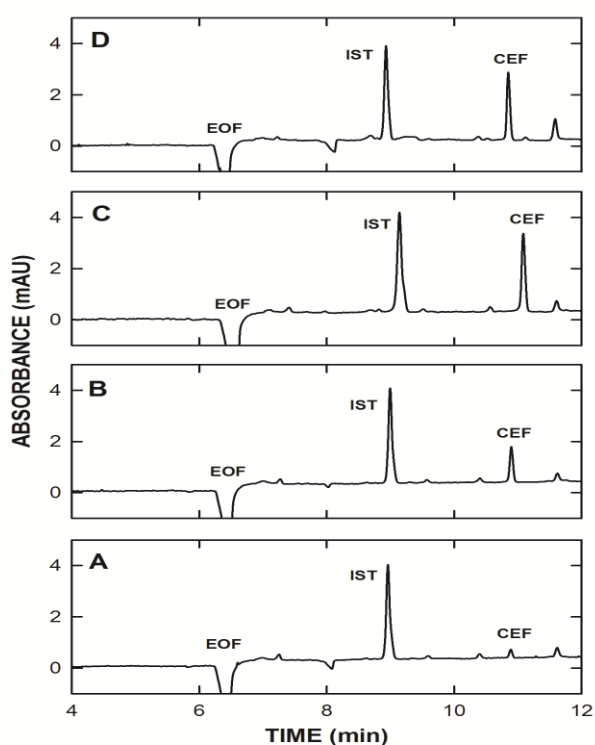
Figure 3. (A) Comparative cefepime levels and (B) bias analysis data for 128 patient samples determined by MEKC and MEKC-DCM. In panel A, the solid line represents a correlation graph determined by linear regression analysis, the broken lines describe the 95% prediction interval around the regression line and the dotted lines are the 95% confidence interval for the regression line. The data of panel B represent difference vs. mean of each data pair. The solid lines represent mean and mean $\pm$ 2 SD of the data. The broken line is a graph determined by linear regression analysis.
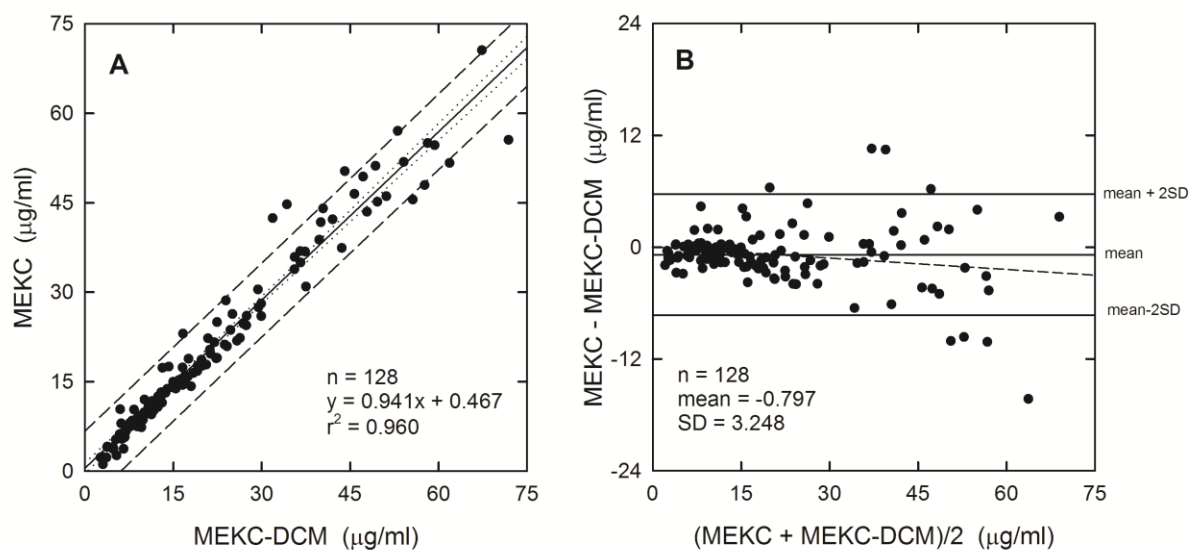
Figure 3

Figure 4. LC–MS chromatograms (combined mass traces of 481.1 and 547.1) obtained with

(A) a blank, (B) a calibrator comprising 10.0 μg/mL cefepime and (C) a patient sample whose

cefepime level was determined to be 4.2 μg/mL. Key: CEF, cefepime; IST, internal standard
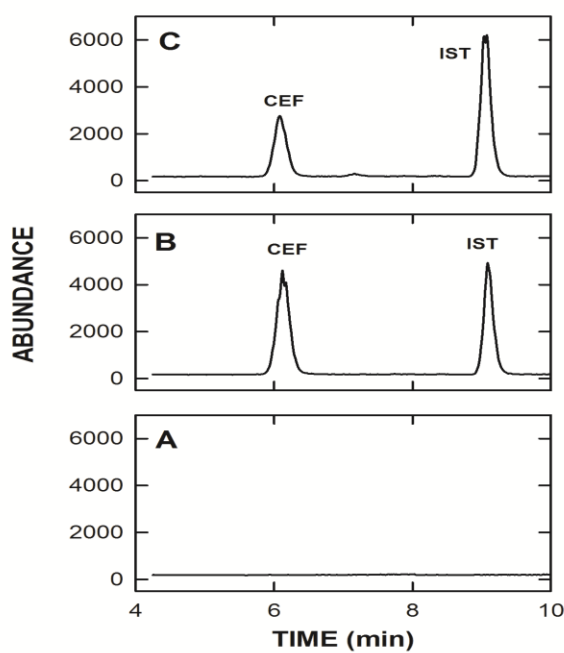
(ceftazidime).

Figure 4

Figure 5. (A) Comparative cefepime levels and (B) bias analysis data for 128 patient samples

determined by MEKC-DCM and LC–MS. In panel A, the solid line represents a correlation

graph determined by linear regression analysis, the broken lines describe the 95% prediction

interval around the regression line and the dotted lines are the 95% confidence interval for

the regression line. The data of panel B represent difference vs. mean of each data pair. The

solid lines represent mean and mean $\pm$ 2 SD of the data. The broken line is a graph

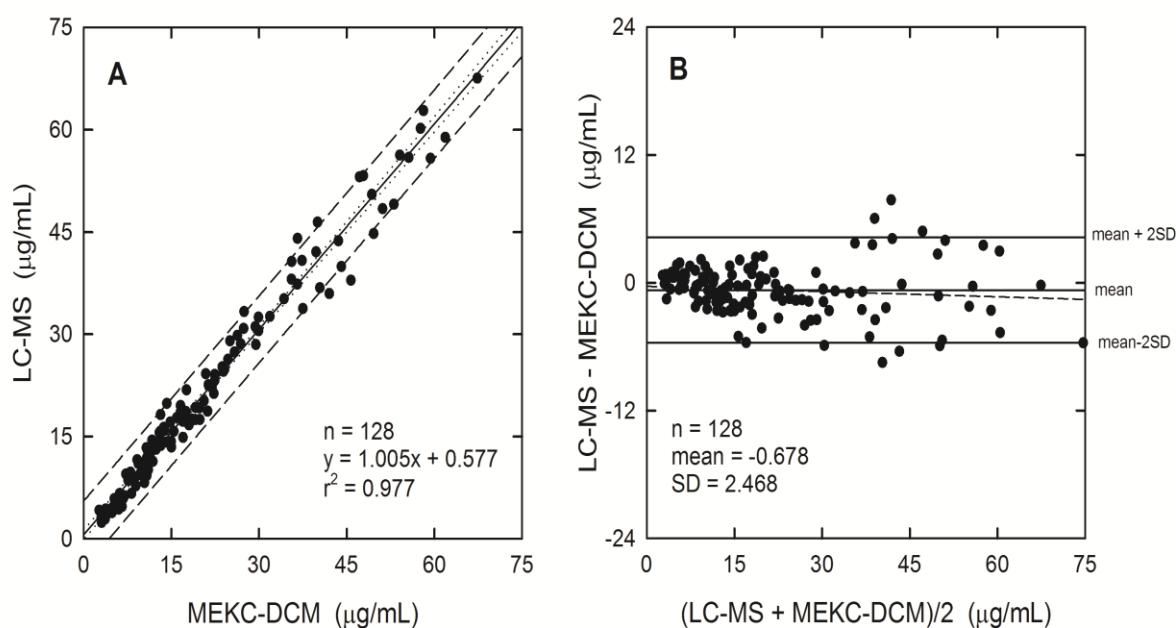determined by linear regression analysis.

Figure 5

Figure 6. (A) Comparison of cefepime levels of 8 patient samples determined by MEKC, MEKC-DCM and LC–MS. (B) Comparative cefepime levels determined by MEKC-DCM and LC–MS of samples from 38 patients that were under pharmacotherapy with cefepime and co-trimoxazole (explanations as in Fig. 3). (C) MEKC and MEKC-DCM electropherograms of sample 7 of Fig. 6A (graphs a and b, respectively) and sample 8 of Fig. 6A (graphs c and d, respectively).
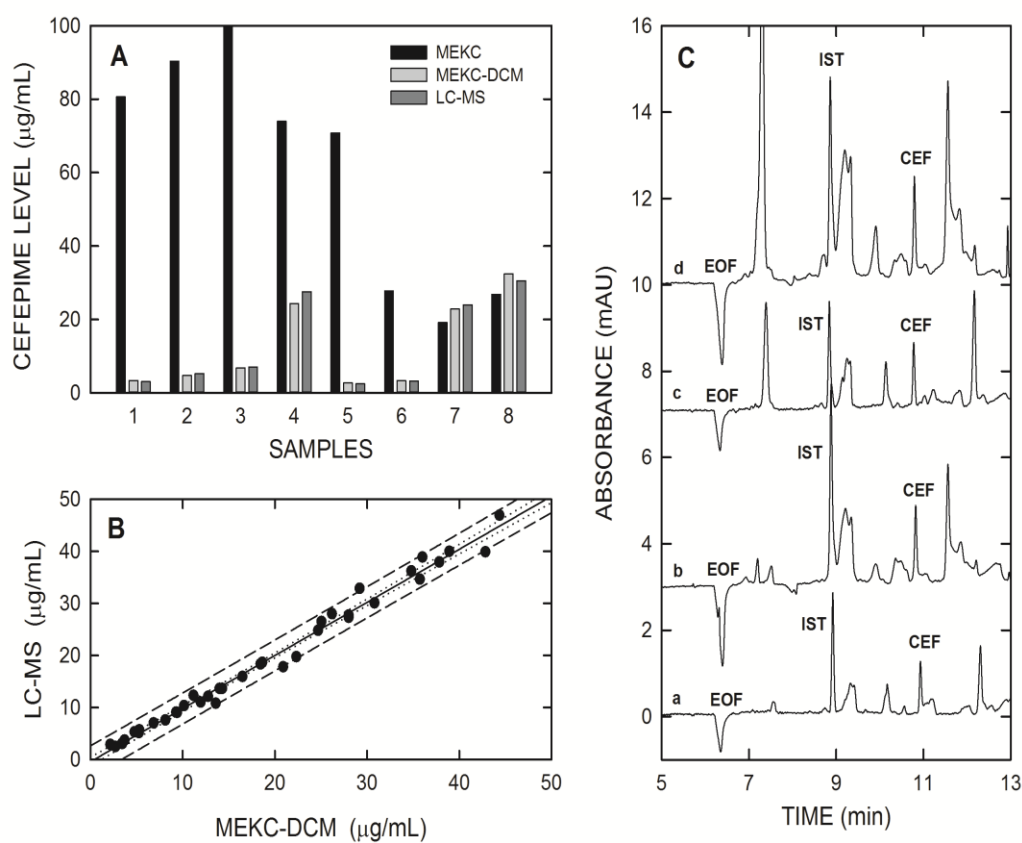

Figure 6

Table 1: Statistical evaluation of calibration data [a]

Assay	Slope		y-intercept		r^2		F value	
	mean [mL/µg]	RSD [%]	range	mean	range	mean	F	P
MEKC [b]	0.0110	2.43	-0.0092– -0.0009	-0.0044	0.9991–1.0000	0.9995	≥ 4360.3	< 0.0001
LC-MS [b]	0.1508	8.76	-0.0167–0.0112	-0.0039	0.9908–0.9998	0.9963	≥ 534.2	< 0.0001

a) The assigned concentration values and ratios of corrected peak areas (cefepime/IST) were taken as x-axis and y-axis values, respectively.

b) Interday calibration from 6 consecutive sets of data.

a) Table 2: **Typical imprecision data for cefepime assessed with control samples**

Assay	target level [µg/mL]	Intraday imprecision [a]			Interday imprecision [a]		
		IST time [min]	Cefepime time [min]	Drug level [µg/mL]	IST time [min]	Cefepime time [min]	Drug level [µg/mL]
MEKC	6.0	8.96 (0.62)	10.90 (0.55)	5.63 (3.14)	8.92 (1.34)	10.81 (1.48)	5.9 (5.51)
	25.0	8.96 (0.61)	10.89 (0.47)	24.78 (1.55)	8.93 (1.42)	10.82 (1.51)	23.7 (3.35)
	45.0	8.96 (0.51)	10.88 (0.41)	47.24 (2.71)	8.94 (1.39)	10.83 (1.48)	43.7 (3.74)
LC-MS	0.5	9.13 (0.35)	6.08 (0.16)	0.52 (1.90)	9.11 (0.53)	6.09 (0.86)	0.49 (4.48)
	5.0	9.12 (0.22)	6.07 (0.19)	5.30 (4.29)	9.08 (0.60)	6.07 (1.34)	5.10 (5.42)
	10.0	9.09 (0.34)	6.09 (0.44)	9.75 (1.68)	9.10 (0.80)	6.09 (1.16)	10.27 (4.43)

a)   mean values (RSD, %) for n=6 (number of repeats)