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Journal of Chromatography A, 1081 (2005) 55–59
JOURNAL OF CHROMATOGRAPHY A
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Determination of fosfomycin in pus by capillary zone electrophoresis

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Available online 11 February 2005

Abstract

A method is described for the determination of fosfomycin in pus by capillary zone electrophoresis with reversed electroosmotic flow, and indirect UV absorbance detection. Sample pre-treatment is limited to removal of proteins and cell debris by adding the double volume of methanol, followed by vortexing for few seconds, and centrifugation at 15,000 × *g* for 2 min. The supernatant is directly injected into the instrument. Fosfomycin is separated from sample constituents with a background electrolyte at pH 7.25 (25 mM benzoate buffer with 0.5 mM hexadecyltrimethylammonium bromide added, adjusted to pH with tris(hydroxymethyl)-aminomethane (TRIS)). Separation is carried out in a capillary with 50 μm I.D., 64.5 cm total length, 56.0 cm to the detector, at 25 °C with −25 kV voltage applied. Due to the low absorbance of the analyte, indirect UV detection was performed at 254 nm using a bubble cell capillary. Sample was injected by pressure (450 mbar s). Repeatability for fosfomycin in spiked pus (from 8 or 10 consecutive injections of three different series at concentrations of 100 μg/mL of the antibiotic) was between 2.4 and 8.2% relative standard deviation (RSD). Accuracy (expressed as recovery of fosfomycin determined by three independent analysis at 10, 100 and 300 μg/mL fosfomycin added to plain pus) was between 75 and 102%. Intermediate reproducibility ($n = 9$ at three different days) was between 2 and 12% RSD. Limit of detection and limit of quantitation were 4.5 and 15 μg/mL, respectively. The concentration of fosfomycin in pus of patients treated with the antibiotic ranged up to 240 μg/mL. The concentration of other anionic pus constituents identified beside chloride (acetate, succinate, lactate, phosphate) ranged between 20 and 7800 μg/mL.

Keywords: Fosfomycin; Antibiotics; Clinical analysis; Body fluid; Pus; Capillary electrophoresis

1. Introduction

The antibiotic treatment of purulent bacterial infections is a complex matter. Generally, the requirements of an antibiotic are an adequate antibacterial spectrum and sufficient concentrations at the site of infection [1–4]. Particularly in infections of deep or encapsulated compartments, the penetration of antimicrobial agents to the target site is considered to be significantly impaired. In addition to a low pH [2,5,6], lack of oxygen [3,6] and the presence of enzymes that can deactivate the antimicrobial agent [3,6]. High protein concentrations in the range of 54 mg/mL [5–7] and the presence of cations and short-chain organic acids additionally hamper the antimicrobial activity of antibiotics. A clinical study was performed in order to gain information about the capability of an antibiotic to penetrate into the abscess cavity in human patients.

Different methods have been described so far for the analysis of different antibiotics in pus, e.g. for the determination of cephalosporins, fluoroquinolones, and penicillins. The most common method is high-performance liquid chromatography (HPLC) [1,8–12], but microbial culture experiments like agar diffusion assay [4], paper-disk methods [13] or bioassays [6] were used for the analysis of antibiotics as well.

Fosfomycin, (−)-(1*R*,2*S*)-(1,2-epoxypropyl) phosphonic acid (Fig. 1) is an antibiotic commonly used for the treatment of urinary tract infections and other infections that are caused by Gram-positive and Gram-negative bacterial species. Fosfomycin inhibits the synthesis of the bacterial cell walls and due to its different structure no cross-resistance with other

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doi:10.1016/j.chroma.2005.01.085

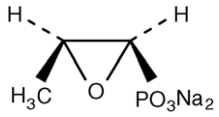

Fig. 1. Structural formula of fosfomycin (as disodium salt).

antibiotics is observed [14]. Fosfomycin concentrations in plasma have been determined by different analytical methods like gas chromatography [15–17], ion exchange chromatography [18] and capillary electrophoresis (CE) [19–21]. One paper reported the analysis of fosfomycin from pus obtained from cattle and sheep by standard bacteriological methods [22] and another showed the eligibility of fosfomycin for the treatment of abscesses in patients [23], also by bacteriological methods.

We have previously developed a CE method based on indirect UV and conductivity detection for this low-UV absorbing analyte in plasma and microdialysates [21] for clinical use. It is the aim of the present paper to adapt this method for the determination of the antibiotic in pus obtained from patients. The method should be feasible, including a simple procedure for sample pre-treatment. As it was found that the pus samples obtained from patients differ strongly in terms of their quantitative composition of matrix anions, these constituents were identified in order to select the appropriate separation conditions for fosfomycin.

2. Experimental

2.1. Chemicals

Fosfomycin disodium salt ("Sandoz 8 g Trockenstechampulle") was kindly provided by Sandoz GmbH (Kundl, Austria). Tris(hydroxymethyl)-aminomethane (TRIS) and benzoic acid (used for the background electrolyte (BGE)), phosphoric acid, acetic acid, lactic acid, methanol (HPLC grade) were purchased from E. Merck (Darmstadt, Germany); succinic acid was from Sigma (Milwaukee, WI, USA). All these chemicals were analytical grade. Hexadecyltrimethylammonium bromide (CTAB, >99.0% purity) was from Fluka Chemie (Buchs, Switzerland). Ringer's solution ÖAB (154 mM Na, 2.7 mM Ca, 4 mM K, 163.4 mM Cl) was from Mayrhofer Pharmazeutika (Linz, Austria). As solvent of the BGE, ultrapure water was used with resistance >18 MΩ cm, prepared by a Millipore Milli-Q apparatus (Bedford, MA, USA).

2.2. Instrumentation

Capillary zone electrophoresis was carried out with a ^{3D}CE apparatus (Agilent Technologies, Palo Alto, CA, USA), equipped with an uncoated fused silica capillary (50 μm I.D., total length 64.5 cm, effective length 56.0 cm; Agilent Technologies) and with a bubble cell (optical path length 150 μm) for indirect detection with a diode array detector at 254 nm. Determination of fosfomycin was performed using a 25 mM benzoate buffer solution with 0.5 mM CTAB added, adjusted to pH of 7.25 with 1 M TRIS for pus analysis. The short-chain organic acids were quantified in a BGE consisting of 25 mM benzoate solution with 0.5 mM CTAB added, but adjusted to pH 4.75 with TRIS. The buffer solutions were degassed by ultrasonication prior to use. Samples were hydrodynamically injected (450 mbar s pressure, applied at the cathodic end of the capillary). Voltage was −25 kV. The capillary cassette was thermostated to 25 °C.

New capillaries were conditioned before use with 1 and 0.1 M sodium hydroxide at 45 °C for 15 min each, followed by rinsing with water and buffer solution for 15 min each at 25 °C. Before each run, the capillary was flushed with 0.1 M sodium hydroxide (4.5 min), water (2 min) and buffer solution (6 min). To diminish buffer depletion during a sequence, the inlet and outlet buffer vial and the conditioning buffer vial were refilled before each run by the built-in replenishment system. For storage overnight, the capillary was flushed with water, 0.1 M sodium hydroxide followed again with water (flushing 3 or 2.5 min, respectively) as described for CTAB-treated capillaries in [24].

2.3. Procedures

A stock solution of fosfomycin (concentration 10 mg/mL) was prepared in Ringer's solution. This solution was stable for 4 months when kept at −80 °C. Pus samples from patients were stored at −80 °C directly after collection. Concentrations in patients' pus were determined by external calibration. Drug-free pus was spiked with concentrations of fosfomycin in the range of 10–1000 μg/mL and incubated at 37 °C for 20 min. Forty microlitres of pus from patients were mixed with 80 μL methanol, vortexed for few seconds and centrifuged at 15,000 × *g* for 2 min at room temperature to remove proteins and cell debris. Twenty-five microlitres of the supernatant were filled into an autosampler vial and injected into the CE system.

Evaluation of accuracy and precision of the method was performed at three different concentrations of fosfomycin spiked to drug-free pus, injected in triplicate on three different days.

3. Results and discussion

3.1. Separation of fosfomycin from matrix components

The selection of the conditions for separation of the anionic analyte from the anionic matrix components is less arbitrary when the kind of these components is known. In this case, one can derive information about a possible free migration window into which the analyte could be placed by varying the pH of the BGE. The typical electropherograms of blank pus obtained at two different pH values are shown in Fig. 2. pH 7.25 is chosen, because at this relatively high pH

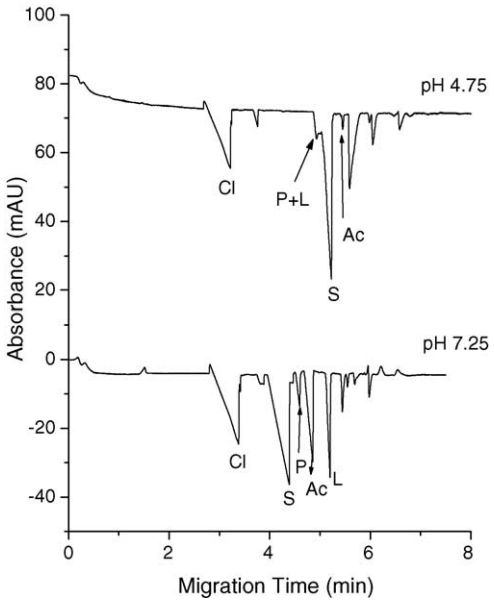

Fig. 2. Electropherograms of blank pus (after precipitation with double volume methanol) at two different pH of the BGE. Abbreviations: Cl, chloride; S, succinate; P, phosphate; Ac, acetate; L, lactate. Experimental conditions: BGE 25 mM benzoate buffer with 0.5 mM CTAB added, adjusted with TRIS to pH 7.25 or 4.75, respectively. Uncoated capillary (50 μm I.D., 375 μm O.D., 64.5 cm total length, 56.0 cm to the detector), temperature of 25 °C; voltage −25 kV; UV detection at 254 nm (bubble cell); injection by pressure, 450 mbar s.

most acidic analytes are fully deprotonated, and are migrating as anions according to their actual mobility. At this pH, about seven main peaks can be differentiated. Note, however, that with indirect UV detection the individual analytes could have different detector responses, and the analyte peaks with the largest area are not necessarily present in highest concentrations.

Identification of the matrix anions can be based on which compounds are probable. Overall, proteins possibly present in the sample were separated by precipitation, and can thus not occur in the electropherograms. According to the literature [25–34], a number of small aliphatic acids are constituents of pus. Short-chain organic acids are the outcomes of pyruvate formed in the glycolytic pathway of the bacterial metabolism [31]. If the purulent infection is caused by aerobic bacteria, only acetic acid and lactic acid are formed [25,26]. If anaerobic bacteria cause the infection succinic acid, lactic acid, acetic acid, propionic acid, (iso)butyric acid and (iso)valeric acid can be present, as determined by gas or liquid chromatography [25–30,35–37]. Garg et al. [31] evaluated the constituents of 40 pus samples including short-chain acids by ^{1}H NMR, and characterized additionally different amino acids, which are products of the hydrolysis of proteins and cells in the pus cavity by enzymes [31].

For closer inspection of the kind of the analytes, we have thus selected a second, lower pH (4.75), because it is in the range of the pK_a of the carboxylic group (note that both BGEs have pH values in the isoelectric range of the amino acids).

Table 1
Concentration range of acids evaluated by standard addition method in nine pus samples

	Concentration range (μg/mL)
Succinic acid	20–7800
Phosphoric acid	130–920
Acetic acid	118–3750
Lactic acid	600–4300

Therefore, the effective mobility of these anions should be sensitively affected. The resulting electropherogram at pH 4.75 is shown in Fig. 2 (upper trace). Interestingly, the number of differentiable peaks decreased, as some analytes are co-migrating under these conditions.

Running electropherograms at both pH values after addition of reference compounds (at concentrations of 50 and 1000 μg/mL, respectively) allowed the identification of the following matrix components: chloride, phosphate, lactate, acetate, and succinate. Quantitation of these matrix ions from the peak areas showed a broad concentration range in different pus samples (Table 1). As from these compounds, the actual mobilities and the pK_a values are known, the course of their effective mobilities as function of the pH can be depicted. It follows from the resulting plots (not shown) that pH values around 3.5, on the one hand, and pH values higher than 7, at the other hand, are favorable for the separation of the matrix constituents, and deliver an appropriate mobility window into which fosfomycin could be positioned. Taking into account the reversed EOF moving in the same direction as the separands, we have chosen the pH leading to higher actual mobilities, as under these conditions resolution of the analytes should be favored [38,39].

In Fig. 3, the electropherograms of pus at pH 7.25 before and after addition of fosfomycin are shown. Fosfomycin migrates in the window between acetate and lactate, and is well separated from all matrix compounds. Therefore, this BGE was applied for the quantitative determination of fosfomycin in clinical samples.

3.2. Determination of fosfomycin in patient's pus

Single or multiple doses of fosfomycin were administered intravenously to 14 patients, pus samples were collected at different time points and the drug concentrations were determined. Pus samples were taken from differently located lesions, e.g. from perianal, subcutaneous, pancreas, leg or jaw abscesses. A difference between the samples was not only visible in the diverse electropherograms, but also in the consistency of the samples: some were liquid, others more gel-like. They also differed in colour [7]—from brown, cream to grey.

It should be pointed out that the ionic matrix components influenced the electrophoretic migration behavior of the antibiotic and caused a severe shift of its migration time. Therefore, fosfomycin was not identified by its migration time, but

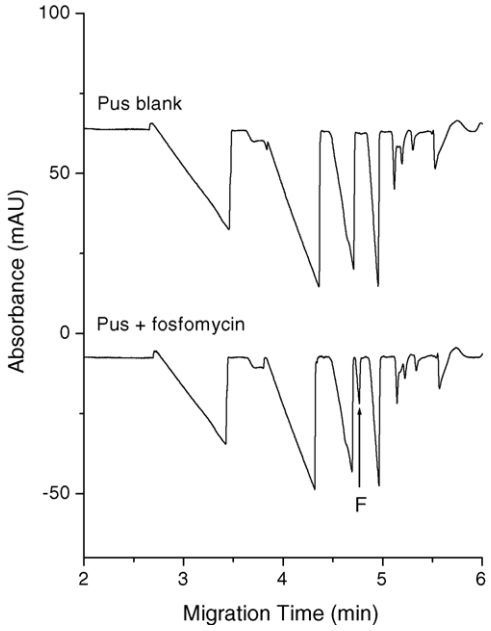

Fig. 3. Electropherograms of blank patient pus (top); patient pus spiked with fosfomycin (indicated by "F") at concentration 200 μg/mL (bottom). Protein precipitated with double volume methanol prior to injection. Experimental conditions: 25 mM benzoate buffer with 0.5 mM CTAB added, adjusted to pH 7.25 with TRIS. Other conditions as in Fig. 2.

by comparison of the electropherograms obtained from the initial sample and that recorded after fosfomycin standard addition. Electropherograms of two patients' pus are shown in Fig. 4. It can be seen that the mutual concentration of the matrix anions differs significantly (see also Table 2). However, fosfomycin can be resolved from the matrix anions, especially from acetate, enabling its quantitation in these real samples.

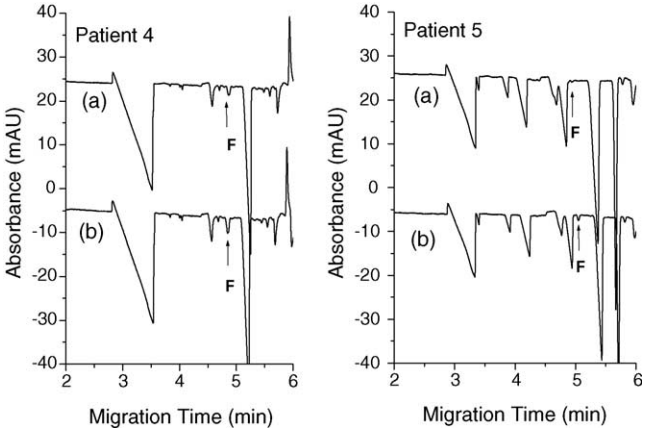

Fig. 4. Electropherograms of pus of two patients before and after spiking with fosfomycin (50 μg/mL). Fosfomycin peak is marked with "F". Protein precipitation with double volume of methanol prior to injection. Experimental conditions: 25 mM benzoate buffer with 0.5 mM CTAB added, adjusted to pH 7.2 with TRIS. Other conditions as in Fig. 2.

Table 2
Figures of merit of the determination of fosfomycin in pus[a]

LOD (μg/mL)[b]	4.5		
LOQ (μg/mL)[c]	15.0		
Percent recovery (RSD, %)			
c = 10 μg/mL	98 (8)	81 (21)	77 (5)
c = 100 μg/mL	102 (9)	98.3 (4.9)	92.5 (1.6)
c = 300 μg/mL	86.6 (12.1)	90.0 (2.5)	75.4 (9.4)

[a] From 10 repetitive injections of sample aliquots of pus with fosfomycin added at a concentration of 100 μg/mL.
[b] S/N = 3.
[c] S/N = 10.

3.3. Quantitation, figures of merit

Repeatability, accuracy, limit of detection (LOD) and limit of quantitation (LOQ) were determined according to ICH guidelines [40,41]. The repeatability of the determination of the peak area of fosfomycin (described by the relative standard deviation (RSD), %) was derived from 8 to 10 replicate injections of sample aliquots at a concentration level of the analyte of 100 μg/mL spiked to plain pus. The measurements were performed on three different days and led to the following results: 5.4% (n = 10); 2.7% (n = 8); 8.2% (n = 8). The resulting RSD is acceptable for the present goal.

Accuracy was determined by measuring the recovery of the analyte after addition to plain pus [40–42]. The according calibration line (expressed as $y = a + bx$, where x is the concentration of fosfomycin in μg/mL, and y is peak area; a is the intercept, both in mAU s) was constructed by external calibration by the aid of five concentrations of fosfomycin added to plain pus in the range between 4.5 and 1000 μg/mL. The resulting equation is $y = 0.51(\pm 1.83) + 0.0708(\pm 0.0085)x$ with the linear correlation coefficient (R) of 0.9956. Accordingly, the LOD (for a signal to noise ratio (S/N) of 3) is 4.5 μg/mL, and the LOQ (signal to noise ratio 10) is 15.0 μg/mL fosfomycin.

The recovery of the analyte was measured in triplicate, with three independent measurements at three different concentration levels. The recoveries for the concentration levels of 100 μg/mL are between 92.5 and 100.2%, those at 300 μg/mL are lower; they range from 75.4 to 90.0%. For all data, the null hypothesis can be accepted at the 95% probability level, meaning that no significant difference is observed. The third concentration level selected was 10 μg fosfomycin/mL, which was between the LOD and the LOQ, and should therefore result in a lower accuracy. The recovery was here between 77 and 98%. Judging the difference of these two data indeed leads to the result that the null hypothesis must be rejected at the 95% probability level, which means that the difference is larger than caused only by the indeterminate error. This is not surprising, because the concentration of 10 μg/mL is below the LOQ (which is 15 μg/mL, see above). However, this recovery at all concentration levels is acceptably high for the present goal. The intermediate precision (determined as relative standard deviation of the yield determined from three independently processed sample aliquots

Table 3
Concentrations (μg/mL) of fosfomycin in pus of 14 patients

Patient no.	Concentration (span)	Patient no.	Concentration (span)
1	<LOD	9	238 (3)
2	7.1 (2.4)[a]	10	155 (1)
3	10.6 (12.3)[a]	11-A	102 (3)
4	68.7 (18.6)	11	168 (8)
5	14.4 (5.1)[a]	12	<LOD
6	<LOD	12-A	11.8 (3.3)
7	<LOD	13	47.3 (2.1)
8	34.3 (4.6)	14	<LOD

Concentrations were evaluated by external calibration. Span of measurements in duplicate (%) are given in parentheses.
[a] Between LOD and LOQ.

at three different days, three measurements each; see Table 2) is acceptable as well: it ranges between 1.6 and 12.1%.

In the course of the present study, the concentration of fosfomycin was determined in pus which was collected from 14 patients at different time points after a single or multiple dose of 8 g of the antibiotic. The analyte concentrations found in patients' pus are given in Table 3, with the span of the determinations carried out in duplicate. In five samples, the concentration is below the LOD; in four samples, it was between the LOD and LOQ; and seven samples contained fosfomycin above the LOQ, with 238 μg/mL fosfomycin being the highest concentration found.

4. Conclusions

By the capillary electrophoretic method with indirect UV detection (this detection mode is needed because of the optical properties of the analyte), which requires only a minimum sample pre-treatment, fosfomycin can be determined in abscess fluid. The quantitative results demonstrate that fosfomycin may be a useful option for the therapy of deep compartment infections, which are commonly considered to be hardly accessible for antibiotics. However, the high inter-individual differences of the fosfomycin concentrations presented requires careful evaluation of the results. In order to assess whether efficient levels of fosfomycin are reached in the abscess fluid, the varying time intervals between drug administration and pus sampling, individual pharmaco-kinetics, plasma levels and the size and location of the abscess have to be considered for each patient. These data will be published elsewhere.

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