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Fosfomycin determination in serum by capillary zone electrophoresis with indirect ultraviolet detection

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ABSTRACT

Capillary zone electrophoresis with indirect ultraviolet detection was used for the determination of fosfomycin in serum. Running buffer consisted of a mixture of 200 m*M* sodium borate with 10 m*M* phenylphosphonic acid used as ultraviolet absorbing background electrolyte. Relationships between the pH of the buffer and the efficiency of the separation (migration times and selectivities) or the sensitivity of detection were investigated. The method was then validated over a 10–100 $\mu g\ ml^{-1}$ concentration range to be applied to further therapeutic drug monitoring. The choice of ethylphosphonic acid as internal standard is discussed. The specificity and the linearity of the technique are demonstrated. The inter-day precision was satisfactory with a relative standard deviation of less than 2%. Accuracy was calculated with a standard error near 0.5 and 18% for 100 and 10 $\mu g\ ml^{-1}$, respectively.

INTRODUCTION

Fosfomycin, the sodium salt of *cis*-(3-methyloxiranyl)phosphonic acid (Fig. 1), was the first phosphonic acid to be extensively used as a bactericidal antibiotic in severe infections [1–3]. It was mainly administered parenterally, together with other antibiotics. Therapeutic drug monitoring is necessary to determine a dosage regimen able to keep the fosfomycin plasma levels above the minimal inhibitory concentration (between 30 and 60 $\mu g\ ml^{-1}$), depending on the microorganisms involved [4].

One important area is concerned with fosfomycin determination in blood samples. The polar structure of this molecule and the absence of chromophores or fluorophores cause numerous problems in drug extraction and detection. The first pharmacokinetic studies of fosfomycin measured its plasma levels microbiologically [5]. The main advantage of this method is that it does not require any drug extraction, which is not an easy task because of the solubility of fosfomycin in water. However, microbiological analysis is not consistent with polyantibiotic therapy.

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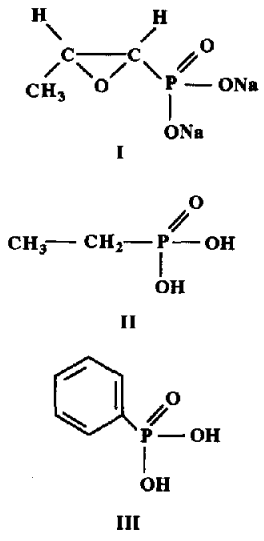

Fig. 1. Structures of (I) fosfomycin, (II) ethylphosphonic acid and (III) phenylphosphonic acid.

Gas chromatographic techniques after trimethylsilyl derivatization have been described [6,7] with or without a selective-ion monitoring (SIM) procedure [8,9]. This detection method (SIM) showed a very high sensitivity (0.25 μg ml^{-1}). A flash methylation method has also been presented [10] using thermoionic detection with a limit of detection of 1.0 μg ml^{-1}. However, all these techniques were carried out using derivatized samples and were therefore time-consuming. Recently we proposed a quicker method using anion-exchange liquid chromatography with direct conductometric detection for the separation of fosfomycin and other alkylphosphonic acids [11].

Capillary zone electrophoresis (CZE) appears to be an alternative method with a very important potential for ionized species separation. In a previous work [12], we reported the separation of a series of homologous alkylphosphonic acids using CZE with indirect UV detection. This method allowed good sensitivities, and a high efficiency of separation was attained. In the present study, we firstly applied this technique to fosfomycin analysis and we secondly adapted the experimental parameters to the determination of fosfomycin in serum. Validation was then carried out using ethylphosphonic acid (Fig. 1) as internal standard. The technique appears sensitive and precise enough to allow a reliable therapeutic monitoring of fosfomycin.

EXPERIMENTAL

Reagents

All chemicals were of analytical reagent grade unless stated otherwise. Deionized water was prepared with a Milli-Q system (Millipore, St. Quentin-en-Yvelines, France). Fosfomycin sodium salt (FOF) was obtained from Clin Midy (Paris, France) and ethylphosphonic acid (EPA) was purchased from Aldrich-Chimie (Strasbourg, France). Human serum pool was provided by the Hôpital Robert Debré (Paris, France).

Equipments

The CZE system (Model P/ACE 2000) with a UV detector (set at 254 nm) was purchased from Beckman (Gagny, France). Temperature was set at 33°C. A fused capillary with 50 cm effective length (57 cm total length) and 75 μm I.D. was used for separation. A positive high voltage of 30 kV was applied to the capillary.

Pretreatment of the capillary column

All new capillary columns were flushed with 1.0 *M* sodium hydroxide solution for 30 min. Before each injection, the column was washed with 0.1 *M* sodium hydroxide, deionized water and equilibrated finally with the background electrolyte.

Preparation of background electrolyte

To prepare background electrolyte 10 m*M* phenylphosphonic acid was added to a 200 m*M* sodium borate buffer. By adding sodium hydroxide the pH was maintained in the range 6.0–8.5 for pH studies and at 8.2 for human serum analysis. The running buffer was then filtered through a 0.22-μm Millex membrane (Millipore) prior to use.

Spiked serum sample preparation

A stock solution of fosfomycin (100 μg ml^{-1})

was prepared in methanol. The required serum concentrations (10, 20, 40, 80 and 100 $\mu g\ ml^{-1}$) were obtained by adding 20, 40, 80, 160 and 200 μl of the fosfomycin stock solution, respectively, to 200-μl aliquots of human serum pool obtained from five different healthy volunteers. Ethylphosphonic acid (200 μl of a 100 $\mu g\ ml^{-1}$ methanolic solution) was then added as an internal standard. Spiked serum samples were vortex-mixed for 15 s. Deproteinization was carried out by adding appropriate volumes of methanol to adjust each sample to a final volume of 2.0 ml. After vigorous vortex-mixing and a centrifugation at 6000 *g* for 20 min at 0°C, the supernatant was transferred into screw-capped vials, dried under nitrogen and reconstituted with 200 μl of deionized water. These reconstituted samples were filtered through a 0.11-μm filter (Millipore) and then hydrodynamically injected for 2 s into the capillary electrophoresis system.

RESULTS AND DISCUSSION

The potential of CZE, with indirect photometric detection, for the analysis of alkylphosphonic acids was previously investigated by Pianetti *et al.* [12]. To achieve the highest sensitivity, phenylphosphonic acid (Fig. 1) was chosen as the absorbing background electrolyte because its mobility is close to that of the alkylphosphonic acids studied [13,14]. The experimental conditions (200 m*M* sodium borate at pH 6.0 with 10 m*M* phenylphosphonic acid) were selected after an optimization of the electrophoretic parameters, such as pH and concentration of both electrolyte and UV-absorbing ion. The applied voltage was set at 30 kV and the temperature at 33°C.

We applied these conditions to the analysis of deproteinized blank serum and 100 $\mu g\ ml^{-1}$ fosfomycin-spiked serum samples to evaluate the extent of disturbing matrix components. Ethylphosphonic acid was used as internal standard. Of the alkylphosphonic acids tested, it appeared to be the most suitable for several reasons. First, its mobility was closer to that of fosfomycin than butyl- and propylphoshonic acids. Second, it produced a more symmetrical peak than methylphosphonic acid. Unfortunately, using the conditions previously described, an overlap was observed between fosfomycin and the endogenous compounds whatever the serum analysed (Fig. 2).

CZE has been sparingly employed for the quantitation of drugs in serum, and analyses were usually extracted from serum samples with organic solvents before their introduction into the analytical system [15,16]. However, the extraction of fosfomycin led to very poor yields whatever organic solvent was used because of its significant hydrophilicity. We decided to carry out a simple methanolic precipitation to eliminate interfering proteins. Consequently, some ions were still present in the injected samples and a good separation was possible through a modification of the electrophoretic conditions. In order to resolve the mixture completely, we studied the influence of the buffer pH from 6.0 to 8.5. The

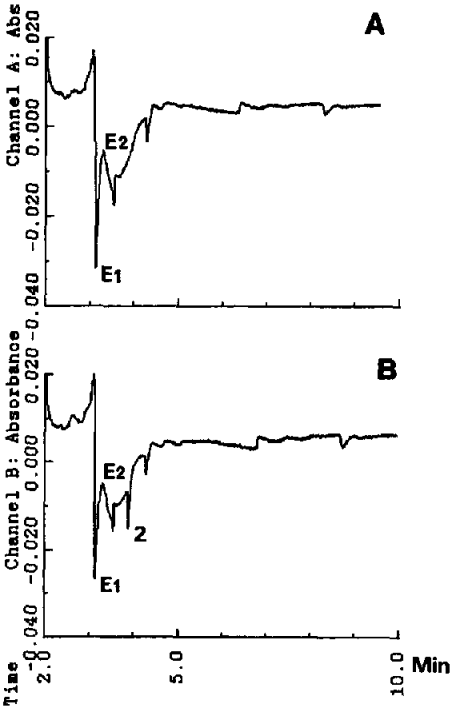

Fig. 2. Electropherograms of (A) a blank serum and (B) a 60 $\mu g\ ml^{-1}$ spiked fosfomycin serum sample obtained at the initial conditions (pH 6.0). Note overlapping between fosfomycin and endogenous compounds in the serum (E_1 and E_2).

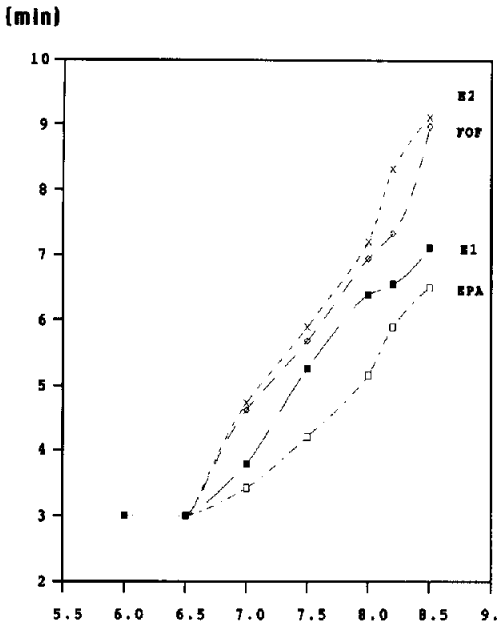

Fig. 3. Migration times of compounds in the analysed plasma *versus* pH values. E_1 and E_2, endogenous compounds; FOF, fosfomycin; EPA, ethylphosphonic acid. For electrophoretic conditions, see Experimental section.

other conditions were unchanged. With increasing pH, the migration times of fosfomycin and ethylphosphonic acid increased (Fig. 3). For borate–sodium hydroxide buffers, an increase in pH resulted in an increase in both the ionization of the analytes and the ionic strength of the buffer. The ionization of the analytes increased according to their pK_a (pK_{a2} fosfomycin = 6.20, pK_{a2} ethylphosphonic acid = 8.50): the ratio of diionized to monoionized forms varies in the pH range investigated from 0.6 to 200 and from 0.006 to 2.0 for fosfomycin and ethylphosphonic acid, respectively. Because of the difference in their ionization constants, the slope of rising in the migration velocities is higher for fosfomycin than for ethylphosphonic acid. In spite of the increase in the ionization of silanol goups in the inner capillary wall, the increase in pH induced a slight decrease in the electro-osmotic flow [17]. This could be attributed to an increase in the buffer ionic strength due to the combined effects of an increase in the ionization of both phenylphosphonic acid and sodium borate and the addition of sodium hydroxide for adjustment of buffer pH. These two phenomena led finally to a global increase in the effective mobilities of both the analytes of interest and the endogenous compounds. As a result, the differences in the degree of ionization of the species were favourable for achieving separation of the compounds. Fig. 4 illustrates the variation in the selectivity factor (α) with pH. These data indicate that the optimum pH value is 8.2. The corresponding electropherograms presented in Fig. 5 show that the spiked serum sample was resolved within a short analysis time, less than 10 min.

Another major consideration in the optimization stage is the sensitivity of detection. Since the concentration of the UV-absorbing background electrolyte has been optimized previously, we explored only the influence of buffer pH on the detection. With increasing pH, both phenylphosphonic acid (pK_{a2} = 6.70) and fosfomycin (pK_{a2} = 6.20) were gradually transformed to deionized species. The transfer ratio, defined as the number of chromophore molecules displaced by one analyte molecule, was slightly decreased from 1.20 (pH 6.5) to 1.00 (pH 8.5). Unfortunately, owing

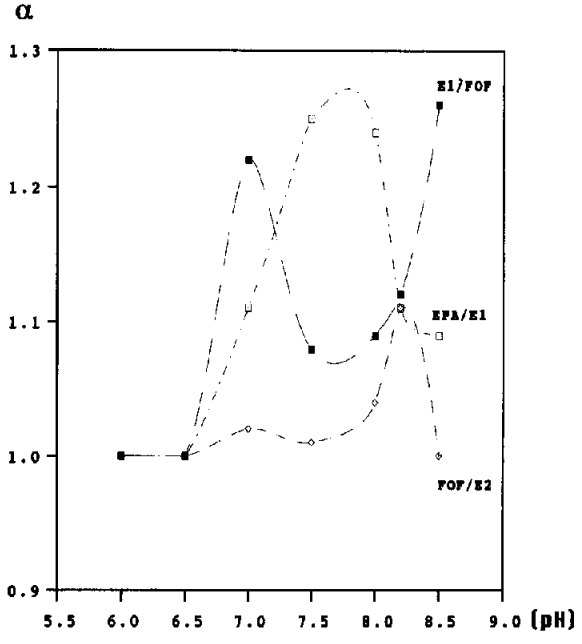

Fig. 4. Relationship between selectivity and buffer pH.

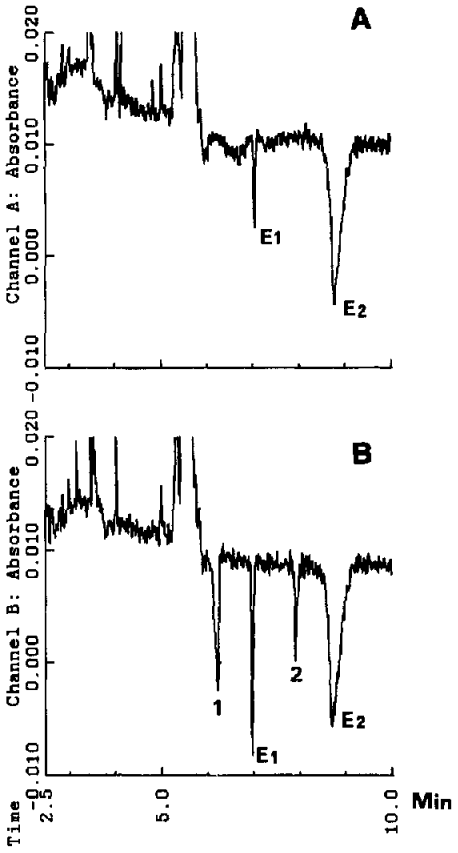

Fig. 5. Electrophoric profiles of (A) a blank serum sample and (B) a spiked fosfomycin serum sample (60 μg ml^{-1}). Peaks: 1 = ethylphosphonic acid as internal standard; 2 = fosfomycin; E_1 and E_2 = endogenous compounds. Experimental conditions: 200 m*M* sodium borate buffer (pH 8.2)–10 m*M* phenylphosphonic acid; fused silica, 50 cm × 75 μm I.D.; applied voltage, 30 kV; temperature, 33 °C.

to a dramatic increase in the noise from pH 7.5 to 8.5, the signal-to-noise ratio is decreased by four-fold. Moreover, we observed a peak broadening at these higher pH values. This appears to be a consequence of the higher peak dispersion that occurs at higher migration times and of the increase in joule heating resulting from the increase in current. The limit of detection, expressed as a signal-to-noise ratio of 2, was 6 μg ml^{-1} for fosfomycin. The viscosity of the injected sample was still a limiting factor. Therefore, this detection limit could not be improved even by increasing the injection volume (nearly 5 nl s^{-1} for 2 s). Based on these limits, consistent with the residual serum levels, a pH of 8.2 was found to be the best compromise between sensitivity of detection and efficiency of separation: when the pH was increased from 7.0 to 8.2, the sensitivity was reduced by at least by 10%, while the resolution for the less resolved adjacent peaks (E_2/fosfomycin) was improved five-fold.

Validation of the method was then carried out to perform further pharmacokinetic studies. The migration times remained stable within a three-day analysis period. The value was 7.83 ± 0.05 min (0.62% R.S.D., n = 15) for sodium fosfomycin and 5.94 ± 0.03 min (1.02% R.S.D., n = 15) for ethylphosphonic acid. The reproducibility of the method was studied by four repetitive injections of spiked serum samples over three days. The overall intra-assay precision of fosfomycin/ethylphosphonic acid peak-height ratio was satisfactory (1.66%). The results summarized in Table I indicate that the internal standard improved

TABLE I

INTER-ASSAY REPRODUCIBILITY OF MIGRATION TIMES, PEAK HEIGHTS OF FOSFOMYCIN AND FOSFOMYCIN/ETHYLPHOSPHONIC ACID PEAK-HEIGHT RATIOS IN SERUM SAMPLES

Values in brackets indicate the R.S.D.

Concentration (μg ml^{-1})	Migration time (min)	Peak height of fosfomycin	Fosfomycin/ethylphosphonic acid peak-height ratio
100.0	7.904 ± 0.142 (1.80%)	10.71 ± 0.17 (1.59%)	1.346 ± 0.002 (0.15%)
80.0	7.878 ± 0.065 (0.83%)	8.69 ± 0.09 (1.07%)	1.093 ± 0.002 (0.15%)
40.0	7.964 ± 0.048 (0.61%)	4.31 ± 0.13 (2.98%)	0.542 ± 0.006 (1.11%)
20.0	7.989 ± 0.083 (1.04%)	2.18 ± 0.04 (1.65%)	0.274 ± 0.003 (1.09%)
10.0	7.801 ± 0.046 (0.62%)	1.36 ± 0.04 (2.86%)	0.171 ± 0.003 (1.75%)

the inter-assay precision, compensating for the slight variation in injection volumes and the peak broadening observed at higher concentrations due to overloading of the capillary. We also observed a similar reproducibility of the peak height whatever sample concentration was studied. The calibration curve showed a good linearity over the range 10–100 $\mu g\ ml^{-1}$ ($y = 0.0135 + 0.0134x$) with a correlation coefficient of 0.999. The absolute recovery of fosfomycin from plasma was determined by the addition of known concentrations (10 and 100 $\mu g\ ml^{-1}$) of drug and internal standard to drug-free plasma. The accuracy of the method was evaluated by calculating the recovery, which was up to 99.5% for 100 $\mu g\ ml^{-1}$ concentration [intra-assay R.S.D. ($n = 4$) = 1.1%; inter-assay ml^{-1} R.S.D. ($n = 3$) = 2.7%] and 82.0% for 10 $\mu g\ ml^{-1}$ concentration [intra-assay R.S.D. ($n = 4$) = 2.4%; inter-assay R.S.D. ($n = 3$) = 3.3%].

CONCLUSION

Fosfomycin was successfully determined in serum by CZE. Although the detection limit of the method is higher than that previously reported for gas chromatography, the described technique using indirect UV detection is sensitive enough to allow adequate therapeutic drug monitoring. Moreover, this technique offers a good efficiency and a very short analysis time considering that no derivatization is required. Similar electropherograms were obtained whatever human serum was used. These results indicate that this technique is able to separate fosfomycin from endogenous compounds and is therefore reliable for the monitoring of fosfomycin after its administration to human patients.

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