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Journal of Chromatography B, 655 (1994) 320–324

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short Communication

Determination of fosfomycin in biological fluids by capillary electrophoresis☆

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(First received September 23rd, 1993; revised manuscript received January 19th, 1994)

Abstract

A capillary electrophoresis method was developed for the determination of the antibiotic fosfomycin in serum, cerebrospinal fluid and aqueous humor. The technique uses indirect UV detection and the working buffer includes an organic cation to improve fosfomycin mobility. The electrophoretic time of migration is less than 7 min in both fluids. The limit of quantification is 2.5 and 1 μg/ml in serum and aqueous fluids, respectively (signal-to-noise ratio = 3). The method was validated in serum and water over the concentration range 2.5–200 μg/ml. The calibration graph for serum was linear with a correlation coefficient $r = 0.999$. At a fosfomycin concentration of 2.5 μg/ml in serum, the intra- and inter-day precisions (coefficients of variation) were 5 and 5.2%, respectively. The mean recovery in serum was 94.5% (S.D. = 2.4%).

1. Introduction

Fosfomycin (*cis*-1,2-epoxyphosphonic acid) is a broad-spectrum antibacterial agent particularly active against *Staphylococcus aureus* and *Streptococcus* spp., but also Gram-negative bacteria such as *Pseudomonas aeruginosa*. Its bactericidal activity results from its ability to inhibit bacterial cell wall synthesis. Fosfomycin has a low molecular mass (138) and its chemical structure, which includes a phosphonic acid group, is unrelated to that of any other antibacterial agent (Fig. 1). This particular structure has led to difficulties in its analysis, and it is non-UV-absorbing. Fosfomycin has been determined in biological fluids by microbiological methods [1,2] or by gas chromatography coupled with mass spectrometry [3]. The gas chromatographic procedure is not suitable for routine analysis and bioassay suffer from errors inherent both in the difficulties encountered when testing fosfomycin *in vitro* and in bioassays in general. It is noteworthy that the *in vitro* activity of this drug is affected by many

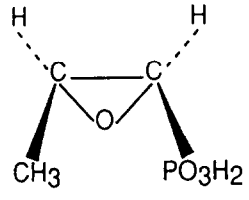

Fig. 1. Structure of fosfomycin.

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☆ This work was presented in part at the 45th National Meeting of the American Association for Clinical Chemistry, New York, July 1993, and was published as an abstract in *Clinical Chemistry*, 39 (1993) 1232.

0378-4347/94/$07.00
SSDI 0378-4347(94)00095-M

factors, such as constituents of the culture medium (glucose, phosphate) [4]. Therefore, a rapid and sensitive assay is needed for both pharmacokinetic studies and therapeutic drug monitoring. This prompted us to develop a high-performance capillary electrophoretic (CE) technique with indirect UV detection allowing the determination of fosfomycin in serum, cerebrospinal fluid and aqueous humor.

2. Experimental

2.1. Reagents

Fosfomycin (sodium salt) as pure powder was kindly provided by Sanofi-Winthrop Labs. (Paris, France). Dichloromethane, acetonitrile, both of chromatographic grade, phosphate buffer (Titrisol, pH 7) and lithium hydroxide monohydrate were provided by Merck (Darmstadt, Germany). Sodium 4-hydroxybenzoate (4-hydroxybenzoic acid sodium salt) and cetrimide (hexadecyltrimethylammonium bromide) were obtained from Fluka (Buchs, Switzerland).

2.2. Capillary electrophoresis system

A Waters Quanta 4000 system (Millipore, Milford, MA, USA) was used and data analysis was performed using Waters Baseline 810 software (Millipore).

Separations were carried out under a 15-kV negative voltage in a fused-silica capillary (Beckman, Palo Alto, CA, USA) of 75 μm I.D., total length 57.5 cm, distance between injection and detection 50 cm. Samples were loaded by hydrostatic injection (10-cm height for 40 s) and analysed by indirect UV detection at 254 nm.

The buffer used for electrophoresis was a phosphate (1.5 m*M*)–sodium 4-hydroxybenzoate (15.4 m*M*) solution containing 1.3 m*M* cetrimide, 25 m*M* lithium hydroxide and 2.5% (v/v) methanol (pH 11.4). All stock solutions were filtered through a 0.22-μm filter (Millipore). The final solution was degassed before use. Between runs, the capillary was washed with distilled water (0.5 min) followed by reconditioning with the running buffer (2 min).

2.3. Sample preparation

Serum (500 μl) was deproteinized with 500 μl of acetonitrile. After mixing on a vortex-mixer, the tubes were gently shaken by rotation (20 rpm, 10 min). After centrifugation (1000 *g*), the supernatant was mixed with 3.5 ml of dichloromethane to remove the excess of acetonitrile (10 min). A 50-μl volume of the supernatant resulting from a 10-min centrifugation (1000 *g*) were then ultrafiltered (molecular mass cut-off 5000) for 10 min (1000 *g*) using Ultrafree-MC filter units (Millipore). The resulting ultrafiltrate was then available for analysis. Cerebrospinal fluid and aqueous humor samples were only ultrafiltered before analysis.

2.4. Validation procedure

Intra- and inter-day precisions were studied by analysing ten samples (serum and aqueous solution) at concentrations of 2.5, 10 and 100 μg/ml. To establish the linearity, three calibration graphs were constructed over the concentration range 2.5–200 μg/ml. The recovery was investigated by analysing ten samples at concentrations of 2.5, 10 and 100 μg/ml.

3. Results and discussion

As fosfomycin is not a UV-absorbing drug, we used indirect UV detection with a buffer containing a UV-absorbing salt (sodium 4-hydroxybenzoate). Under these conditions, fosfomycin detection is facilitated by a decrease in absorbance. In addition, fosfomycin is negatively charged at pH 11.4 and its migration from the cathode towards the anode (detection side) is due to its electrophoretic mobility lowered by the electroosmotic flow. This mobility can be enhanced by the introduction in the working buffer of an organic cation (cetrimide), which reverses the natural electroosmotic flow direction of fused-silica capillaries. Hence, the natural

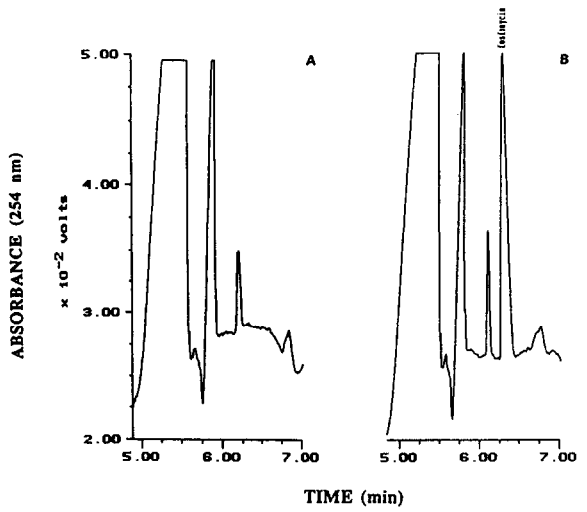

Fig. 2. Electropherograms of (A) human blank serum and (B) serum obtained from the same patient treated with a 4-g intravenous injection of fosfomycin. Buffer, 1.5 m*M* phosphate buffer–15.4 m*M* sodium 4-hydroxybenzoate containing 1.3 m*M* cetrimide–25 m*M* lithium hydroxide and 2.5% (v/v) methanol (pH 11.4); capillary, 50 cm × 75 μm I.D. fused silica; separation potential, 15 kV; hydrostatic injection, 10-cm height for 40 s; detection, 254 nm.

flow is reversed to an anodic flow, which enhances the mobility of the fosfomycin anion towards the detector. Improved resolution occurs with 50- rather than 75-μm I.D. capillaries, but unfortunately to the detriment of the limit of quantification, so we decided to work with 75-μm I.D. capillaries. Between runs, a water wash was preferred to an alkali wash as the latter was found to interfere with cetrimide and to unbalance the capillary. We combined deproteination with ultrafiltration to purify the extract and obtain cleaner electropherograms and to enhance the reproducibility.

Fig. 2 presents electropherograms obtained from analysis of (A) a blank serum and (B) a serum obtained from a patient treated with fosfomycin administered intravenously at the standard dosage (4 g). The migration time is 6.3 min. For cerebrospinal fluid and aqueous humor samples, the electropherograms are shown in Figs. 3 and 4. The migration times are 6.4 and 6.8 min, respectively.

The assay was validated over the concentration range 2.5–200 μg/ml. For serum, the intra-assay precision was characterized by coefficients of variation (C.V.'s) of 5% (2.5 μg/ml), 1.8% (10 μg/ml) and 1.6% (100 μg/ml) (Table 1). The values for the inter-assay precision respectively were 5.2, 4.0 and 4.4%. The mean recovery was 94.5% (S.D. = 2.4%) and the limit of quantification was 2.5 μg/ml (signal-to-noise ratio = 3). Accuracy was characterized by values

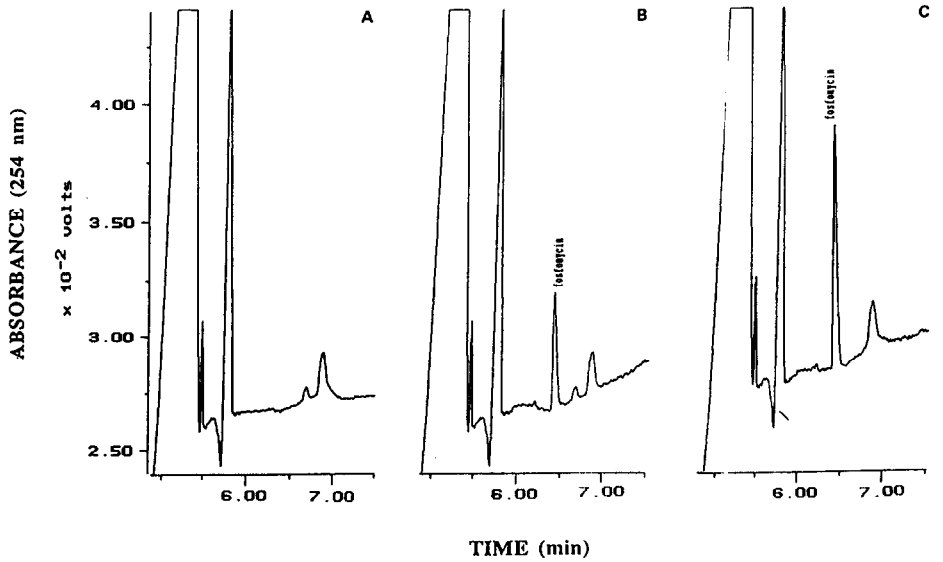

Fig. 3. Electropherograms of cerebrospinal fluid. (A) Blank; (B) spiked with 25 μg/ml; (C) spiked with 50 μg/ml of fosfomycin.

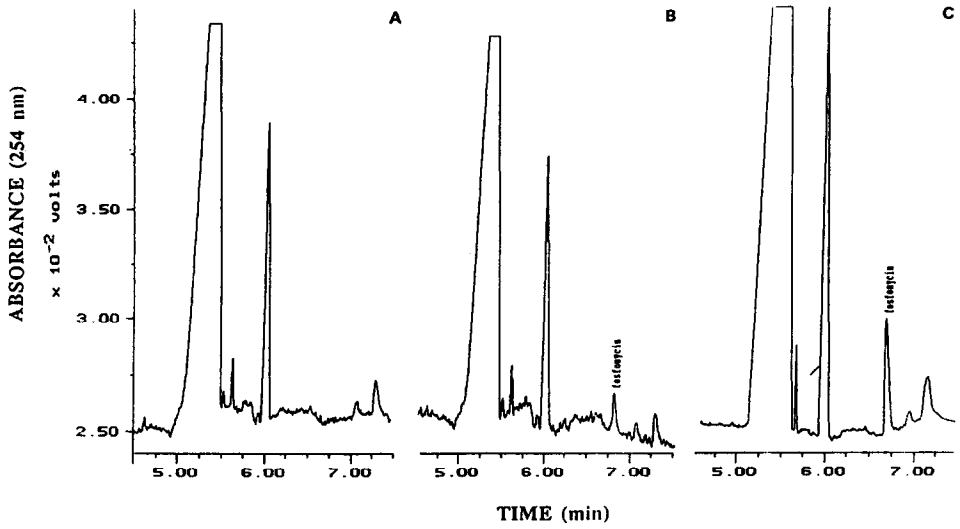

Fig. 4. Electropherograms of aqueous humor. (A) Blank; (B) spiked with 5 μg/ml; (C) spiked with 20 μg/ml of fosfomycin.

within 10% of the expected values except for the limit of quantification (12%). The assay was linear between the limit of quantification of 2.5 μg/ml and 200 μg/ml with a correlation coefficient $r = 0.999$.

For aqueous samples (cerebrospinal fluid and aqueous humor), the intra-day C.V.'s were within 3.5% and the inter-day C.V.s were 4.5%. The recovery was 100.5% (S.D. = 1.4%) and the limit of quantification was 1 μg/ml (signal-to-noise ratio = 3). This assay proved to be very specific as no interferences occurred when studies were carried out with numerous drugs that may be co-administered with fosfomycin, particularly any available antibiotics at high concentration and non-UV-absorbing components such as cisplatin and aminoglycosides.

Table 1
Precision of the fosfomycin assay

Concentration (μg/ml)	Sample ($n = 10$)	Intra-assay C.V. (%)	Inter-assay C.V. (%)
2.5	Serum	5.0	5.2
	Water	3.5	4.5
10	Serum	1.8	4.0
	Water	3.1	4.1
100	Serum	1.6	4.4
	Water	2.8	3.7

Parallel to our work, Baillet *et al.* [5] also develop a CE technique with indirect UV detection for the determination of fosfomycin in serum. The main differences between the two assays consist in the applied voltage (higher in Baillet *et al.*'s method), the composition of the working buffer and the preparation of the samples (use of an internal standard in Baillet *et al.*'s method). The migration time for fosfomycin is shorter in our method (6.3 *versus* 7.9 min). This could be due to the use of cetrimide, which enhances the mobility of fosfomycin. We do not need an internal standard as the extraction recovery is good and the within- and between-day reproducibilities and linearity have been demonstrated. Further, the limit of detection of our method is lower (2.5 *versus* 6 μg/ml in serum) and the validation study was performed over a wider range of concentrations (2.5–200 μg/ml). This last point is important as the peak value obtained after the intravenous administration of the standard dosage (4 g) is *ca.* 200 μg/ml [6].

In conclusion, we have developed a method that allows the rapid determination of fosfomycin in biological fluids. This capillary electrophoresis technique has proved to be much more reliable than bioassays for both therapeutic drug monitoring and pharmacokinetic studies.

4. References

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