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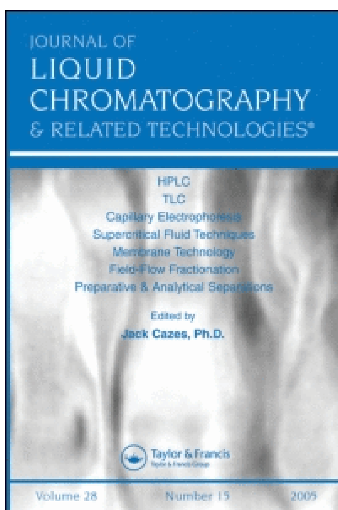

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
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Determination of Fosfomycin Trometamol and its Related Substances in the Bulk Drug by Ion-Pair HPLC with Evaporative Light Scattering Detection

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To cite this Article Liu, Hao , Wang, Hongwu and Sunderland, V. Bruce(2006) 'Determination of Fosfomycin Trometamol and its Related Substances in the Bulk Drug by Ion-Pair HPLC with Evaporative Light Scattering Detection', Journal of Liquid Chromatography & Related Technologies, 29: 1, 15 — 24
To link to this Article: DOI: 10.1080/10826070500358209
URL: http://dx.doi.org/10.1080/10826070500358209

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Journal of Liquid Chromatography & Related Technologies®, 29: 15–24, 2006
Copyright © Taylor & Francis, Inc.
ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070500358209

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Abstract: Ion-pair high performance liquid chromatography (HPLC) with an evaporative light scattering detector was used to develop a method for the determination of fosfomycin trometamol and its related substances in the bulk drug. Utilizing a Luna 5 μm C_{18} column at 35°C, evaporation temperature of 45°C, and nebulizing gas pressure of 3.5 bar, the optimized mobile phase was a 15 mM octylamine solution (adjusted to pH 5.2 with glacial acetic acid) with acetonitrile (92 : 8), and the flow rate was maintained at 1.5 mL/min. Validation of the method was performed, and specificity, reproducibility, precision, and accuracy were confirmed. The linear range for fosfomycin trometamol and fosfomycin trometamol impurity A was 48.7–292 μg/mL and 10.5–84 μg/mL, respectively. The limit of quantitation was 16.3 μg/mL for fosfomycin trometamol and 10.7 μg/mL for fosfomycin trometamol impurity A. Due to its simplicity and accuracy, this method is particularly suitable for routine quality control of fosfomycin trometamol, and can probably be applied to the

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determination of fosfomycin and related substances in fosfomycin sodium and fosfomycin calcium.

Keywords: Fosfomycin, HPLC, ELSD detection, Rapid analysis

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INTRODUCTION

Fosfomycin is an antibiotic that inhibits the synthesis of bacterial cell walls by preventing peptidoglycan synthesis. Due to its unique structure, which differs from other antibiotics, and its activity on the synthesis of bacterial cell walls, which are unaffected by other agents, hardly any cross resistances to other antibiotics are observed.[1] Fosfomycin as its trometamol salt (Fig. 1), despite many years of usage, continues to be characterized by an extremely low incidence of *E. coli* resistant strains (about 1%) worldwide, while antibiotic resistance to commonly used agents, such as trimethoprim and ampicillin, often now exceeds 30–50%.[2] Although nowadays, microbial resistance limits available resources and some drugs can no longer be recommended as reliable agents, fosfomycin trometamol, because of its properties, remains a drug of choice for the eradication of uncomplicated urinary tract infections.[2]

Since fosfomycin has no UV-Vis absorption or fluorescence properties only a few analytical methods are available for its determination. Current methods include microbiological analysis,[3] thermal analysis,[4] indirect photometric analysis,[5] flow injection analysis,[6] gas chromatography after derivation,[7] non-suppressed ion chromatography with indirect UV detection,[8] high performance liquid chromatography (HPLC) using indirect UV detection,[9] ion-exchange HPLC coupled with refractive index (RI) detection,[10] and capillary zone electrophoresis using indirect UV and contactless conductivity detection.[1] Among these methods, only ion-exchange HPLC, i.e., the British Pharmacopoeia (BP) method[10] can provide separation of fosfomycin and its related substances. Utilizing the BP method, an 80 mM potassium dihydrogen phosphate solution (pH 4.5, not buffer but "salt" solution) is used as mobile phase to operate an NH_2 column in an anion-exchange mode. Since the RI detector has a poor sensitivity, a large amount of sample (0.6 mg, i.e., 5 µL of 0.12 g/mL) is injected, which causes the analytical column to be heavily overloaded. As a result, the peak shape of

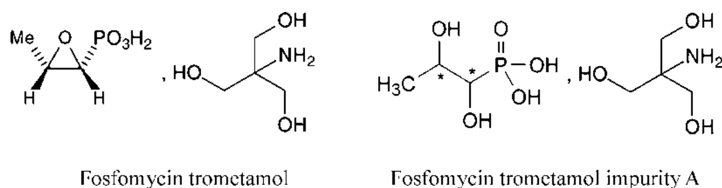

Figure 1. Structures of fosfomycin trometamol and fosfomycin trometamol impurity A.

fosfomycin is broadened and fronting, the resolution between fosfomycin and its related substances might, therefore, be reduced. Hence, the resolution between the peaks corresponding to fosfomycin and its impurity A (Fig. 1) is only required at not less than 1.5. Moreover, due to the poor buffer capacity of the mobile phase and much lower stability of the NH_2 column, the BP method is not robust.

The principal aim of this study was to develop a rapid and accurate HPLC method capable of resolving fosfomycin and its related substances without the use of a NH_2 column and RI detector. In this paper, an ion-pair HPLC method coupled with evaporative light scattering detection (ELSD) for the determination of fosfomycin trometamol and its related substances in the bulk drug is presented and validated.

EXPERIMENTAL

Reagents

Fosfomycin trometamol was obtained from Zambon Group Inpharzam S.p.A. (Milan, Italy). A batch of fosfomycin trometamol rich in impurities was kindly provided by Northeast Pharmaceutical Factory (Shenyang, P.R. China). Fosfomycin trometamol chemical reference standard (CRS) and fosfomycin trometamol impurity A CRS were purchased from the European Pharmacopoeia. Fosfomycin sodium and fosfomycin calcium were kindly provided by Shanghai Asia Pioneer Pharmaceuticals Co. Ltd (Shanghai, P.R. China). Glacial acetic acid and trometamol were from SCR (Shanghai, P.R. China). Octylamine was purchased from Aldrich (St. Louis, MO, USA). Acetonitrile was HPLC grade and obtained from Merck (Darmstadt, Germany). All other chemicals were at least of analytical grade. All solutions were prepared with distilled water for HPLC.

Solution Preparation

A 0.97 mg/mL fosfomycin trometamol CRS stock solution was prepared in mobile phase and stored protected from light in a refrigerator. Working standard solutions in the range 48.7–292 μg/mL were freshly prepared in mobile phase. Sample solutions used for assay were typically prepared by weighing a 20 mg sample into a 100 mL volumetric flask and diluted to volume with mobile phase.

For fosfomycin trometamol impurity A calibration, a 0.42 mg/mL fosfomycin trometamol impurity A CRS stock solution was prepared in mobile phase and stored protected from light in the refrigerator. Working standard solutions in the range 10.5–84 μg/mL were freshly prepared in mobile phase. Sample solutions used for impurity detection were typically prepared

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by weighing a 50 mg sample into a 10 mL volumetric flask and diluted to volume with mobile phase.

Liquid Chromatography

Isocratic elution was performed with an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany) consisting of a quaternary pump, an autosampler, a mobile phase de-gaser, and a heated column thermostat. A Sedex 75 low temperature evaporative light scattering detector (Sedere, Alforville, France) was used for detection.

Chromatographic Conditions

The mobile phase contained 15 mM octylamine solution (adjusted to pH 5.2 with glacial acetic acid)-acetonitrile (92 : 8) and the flow rate was maintained at 1.5 mL/min. Chromatographic separations were performed at 35°C on a 250 × 4.6 mm i.d. column packed with 5 μm Luna 5 μ C_{18}[2] (Phenomenex, Torrance, CA, USA) and the injection volume was 50 μL. The detector was operated at 45°C, 3.5 bar nebulizing gas (dried and filtered air) and a gain of eight throughout the experiments.

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RESULTS AND DISCUSSION

Method Development and Optimization

Fosfomycin and its four known related substances[10] all possess negative charge in aqueous solution. Due to their relatively hydrophilic properties, they are eluted near the dead volume in a typical reversed phase HPLC system. For this reason, an ion-pair reagent such as octylamine could be added to the mobile phase to form, with the ionic analytes (e.g., fosfomycin and its related substances), ion-pairs, which according to their hydrophobicity, will be retained on the reversed stationary phase.

Evaporative light scattering detection (ELSD) is described as a quasi-universal detection mode suitable for non-absorbing analytes. The mobile phase is nebulized with gas and evaporated in a drift tube. The remaining particles are detected by light-scattering. The response does not depend on the solute optical properties, any compound less volatile than the mobile phase could be detected. The detector response shows a double logarithmic relationship between this signal and the analyte concentration. Its has an ability to perform quantitation of a substance without a standard, since it shows nearly equal response factors for molecules with about equal molecular mass and similar structural formula, and is well-established.[11] Hence, with the

logarithmic calibration curve of the fosfomycin trometamol impurity A, hence, the content of unknown impurities of fosfomycin trometamol can be measured.

Since ELSD demands the evaporation of the mobile phase prior to the light-scattering step, mobile phases of high volatility are required. Octylamine and acetic acid, which are used to adjust the pH of mobile phases and to provide buffer capacity for the mobile phase, are applicable.

Due to the lack of other known related substances of fosfomycin trometamol except impurity A, a batch of fosfomycin trometamol rich in impurities was used for the system suitability test.

The experimental results indicated that at pH 5.2 ± 0.2 and 6–10% acetonitrile content in the mobile phase, fosfomycin and its related substances could be satisfactorily separated. Addition of acetonitrile to the mobile phase resulted in decreased retention of all analytes. Maintaining the pH buffer at 5.2, as the concentration of octylamine was increased from 5 mM to 20 mM, caused the retention times of all analytes to be substantially increased. Further increased concentrations of octylamine increased the baseline noise significantly.

During the development of this method, it was found that as the concentration of octylamine was decreased from 15 mM to 5 mM, the fosfomycin peak tended to be broadened or deformed. When samples were dissolved in water and the injection volume was very large (more than 50 μL), the peak shapes of fosfomycin were severely deformed and even spilt. Substituting mobile phase for water as solvent or reducing the injection volume, resulted in the peak shapes being significantly improved. This phenomenon is the result of an interaction of the fosfomycin zone with the hydrophobic octylammonium (ionized form of octylamine) system zone at an early stage of their migration along the column.

It is well known that system peaks (zones) will develop at all times when a sample deviating from the mobile phase is injected. Large deviations should result in large system peaks and might cause disturbances of analyte peak shapes due to interfering system peaks. Because of their hydrophobic alkyl groups, organic ionic components (i.e., pairing-ions) added to the mobile phase in ion-pair HPLC systems often have retention volumes similar to the analytes. The performance of analyte peaks might be influenced by system peaks derived from such mobile phase components, and the analyte peak shapes are more easily deformed by counter-ion system peaks than by co-ion system peaks.[12] In systems where components have no UV-Vis absorption or fluorescence properties, the presence of system peaks is often indicated only by the distorted analyte peak shapes. Therefore, the use of an RI detector is often recommended in order to reveal the existence of system peaks during the development of ion-pair HPLC methods. In this study, when samples were dissolved in water, a positive system peak derived from octylammonium was detected by an Agilent RI detector, while a negative octylammonium system peak appeared if samples were dissolved in mobile

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phase lacking octylamine. Samples dissolved in mobile phase only resulted in a small positive octylammonium system peak.

The guidelines, provided by Fornstedt and Westerlund,[12] for the design of general ion-pair HPLC systems, which do not suffer from peak distortion effects, suggest that when an organic ion (i.e., pairing-ion) is used as a counter-ion, a positive system peak should preferably be regulated to elute before the analytes, the injection volume should be as small as possible and the sample when possible should be dissolved in the mobile phase. Furthermore, if peak deformation occurs, the concentration of the organic ion should be changed in order to improve the separation between the analytes and the system peak. The retention of the analytes will increase at an increasing counter-ion concentration, while the retention of the system peak decreases at the same time.

In this study, the ion-pair HPLC system was optimized according to the above guidelines. The optimum mobile phase contained 15 mM octylamine solution (adjusted to pH 5.2 with glacial acetic acid), acetonitrile (92 : 8), the samples were dissolved in the mobile phase, and the injection volume was fixed at 50 μL. A particle of $CH_3(CH_2)_7NH_3^+$-fosfomycin will be formed during desolvation and subsequently detected by light scattering. In the case of trometamol, trometamol-CH_3COO^- is formed under these conditions and subsequently detected. A very small positive octylammonium system peak with a retention time of around 7.5 min was detected by RI detection in this optimized system, and had minimal effect on the analyte peaks. The separation of fosfomycin and its related substances are well illustrated in Figs 2 and 3, which show baseline separations with good peak symmetries. Trometamol was eluted near the dead volume due to its relatively high hydrophilicity and possessing a positive charge in the mobile phase. Figure 4 shows a chromatogram for a sample solution used in the assay. The theoretical plate number and the tailing factor of the fosfomycin peak were around 23,000 and 1.02, respectively (Fig. 4).

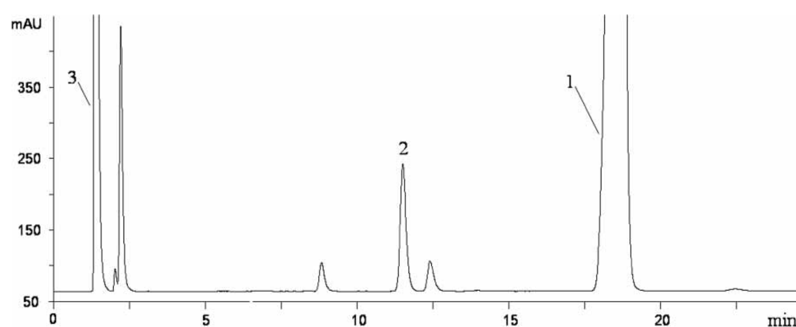

Figure 2. Chromatogram obtained from system suitability test (2.1 mg/mL). Peak: 1. fosfomycin; 2. fosfomycin impurity A; 3. trometamol. See operating conditions in the experimental section.

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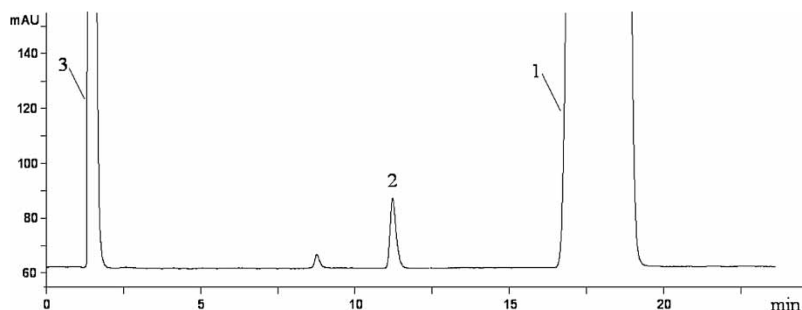

Figure 3. Chromatogram obtained from fosfomycin trometamol sample solution (5.2 mg/mL). Peak: 1. fosfomycin; 2. fosfomycin impurity A; 3. trometamol. See operating conditions in the experimental section.

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The chromatograms illustrated in Figs 5 and 6 indicate that this method can probably be applied to the determination of fosfomycin and related substances in fosfomycin sodium and fosfomycin calcium. According to the BP, fosfomycin sodium and fosfomycin calcium, as well as their impurity A in bulk drug, are determined by a titrimetric method which lacks accuracy, and other related substances can not be controlled.[13]

Method Validation

Linearity

A series of working standard solutions (48.7–292 μg/mL of fosfomycin trometamol) was analyzed in duplicate and the peak areas (A) were used to construct a logarithmic calibration curve: $\log A = b \log C_{\mu g/mL} + \log \alpha$. The regression equation and the correlation coefficient (r^2) were $\log A = 1.4977 \log C_{\mu g/mL} + 2.2442$, and 0.9996 (n = 6), respectively.

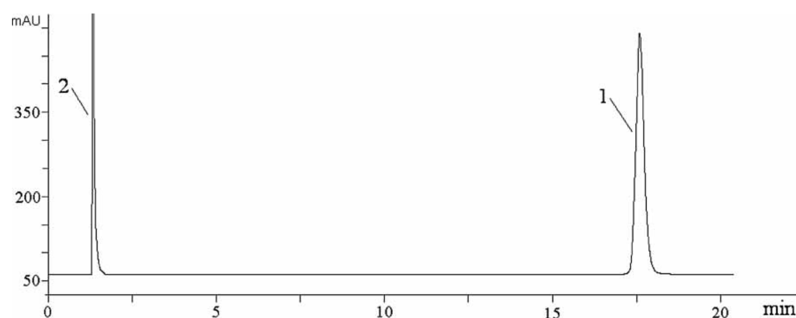

Figure 4. Chromatogram obtained from fosfomycin trometamol sample solution (0.20 mg/mL). Peak: 1. fosfomycin; 2. trometamol. See operating conditions in the experimental section.

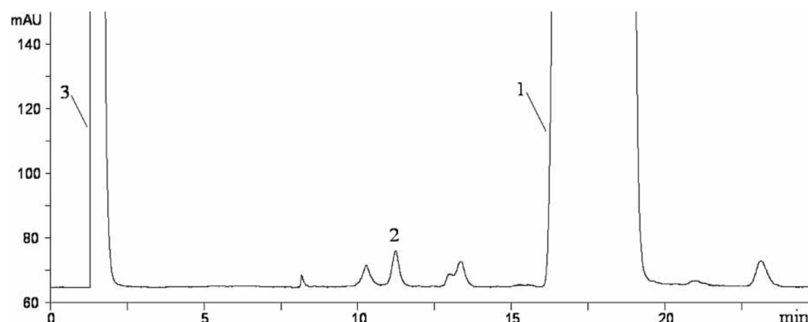

Figure 5. Chromatogram obtained from fosfomycin sodium sample solution (6.4 mg/mL). Peak: 1. fosfomycin; 2. fosfomycin impurity A; 3. sodium. See operating conditions in the experimental section.

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For fosfomycin trometamol impurity A, a series of working standard solutions (10.5–84 μg/mL fosfomycin trometamol impurity A) was analyzed in duplicate and the peak areas were used to construct a logarithmic calibration curve. The regression equation was $\log A = 1.4848 \log C_{\mu g/mL} + 2.3625$ ($r^2 = 0.9991$, n = 6).

Accuracy

The results of a batch sample determined with this method were in good agreement with those obtained with the BP method (Table 1).

Precision

Within-day precision of the assay was determined by analysis of replicate (n = 5) samples of three different concentrations (0.16, 0.20, and 0.24 mg/mL)

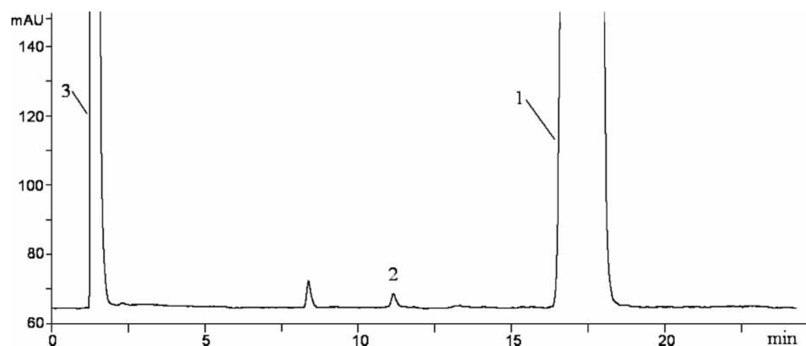

Figure 6. Chromatogram obtained from fosfomycin calcium sample solution (2.1 mg/mL). Due to its relatively lower solubility in the mobile phase, the concentration of sample was decreased, and 100 μL was used as compromising injection volume. Peak: 1. fosfomycin; 2. fosfomycin impurity A; 3. calcium. See operating conditions in the experimental section.

Table 1. Comparison between this method and the BP method for the analysis of bulk drug samples (n = 6)

	Fosfomycin trometamol (%)	Impurity A (%)	Total related substances (%)
This method	98.8	0.51	0.60[a]
BP method	99.0	0.45	0.53[b]

[a]The content of unknown impurity was measured with the logarithmic standard calibration curve of impurity A.

[b]The content of unknown impurity was measured with the external standard calibration curve of impurity A.

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on the same day. Within-day relative standard deviation (RSD) values for the fosfomycin trometamol assay ranged from 0.8–1.2%.

The RSD of the $\log A$ values of 6 consecutive injections of fosfomycin trometamol impurity A reference standard solution (42 μg/mL) was 0.8%.

Limits of Detection and Quantitation

The limits of detection of fosfomycin trometamol and its impurity A were estimated at 4.9 and 3.2 μg/mL (signal to noise of 3), respectively. The limits of quantitation of fosfomycin trometamol and its impurity A were estimated at 16.3 and 10.7 μg/mL (signal to noise of 10), respectively.

Repeatability

Six individual sample weighings of the same batch were performed and analyzed. The RSD of results was 0.5%.

For fosfomycin trometamol impurity A, six individual sample weighings of the same batch were taken and analyzed. The RSD of results was 2.6%.

Sample Solution Stability

Three sample solutions (stored at ambient temperature: 23°C) were separately injected at 0, 1, 2, and 4 h. The results remained almost unchanged and no significant degradation was observed within the given period, indicating that the sample solutions were stable for at least 4 h.

CONCLUSIONS

This study has demonstrated the application of ion-pair HPLC-ELSD to the determination of fosfomycin trometamol and its related substances in bulk drug. Good method performance was obtained for selectivity, linearity, accuracy, precision, repeatability, and detection limits. It is satisfactory for the described application and can be considered for quality control of fosfomycin

trometamol. This method can probably be applied to the determination of fosfomycin and related substances in fosfomycin sodium and fosfomycin calcium.

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Received May 28, 2005
Accepted July 2, 2005
Manuscript 6670

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