

Ion chromatography as highly suitable method for rapid and accurate determination of antibiotic fosfomycin in pharmaceutical wastewater

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ABSTRACT

A rapid and accurate ion chromatography (IC) method (limit of detection as low as 0.06 mg L^{-1}) for fosfomycin concentration determination in pharmaceutical industrial wastewater was developed. This method was compared with the performance of high performance liquid chromatography determination (with a high detection limit of 96.0 mg L^{-1}) and ultraviolet spectrometry after reacting with alizarin (difficult to perform in colored solutions). The accuracy of the IC method was established in the linear range of $1.0\text{--}15.0 \text{ mg L}^{-1}$ and a linear correlation was found with a correlation coefficient of 0.9998. The recoveries of fosfomycin from industrial pharmaceutical wastewater at spiking concentrations of 2.0, 5.0 and 8.0 mg L^{-1} ranged from 81.91 to 94.74%, with a relative standard deviation (RSD) from 1 to 4%. The recoveries of effluent from a sequencing batch reactor treated fosfomycin with activated sludge at spiking concentrations of 5.0, 8.0, 10.0 mg L^{-1} ranging from 98.25 to 99.91%, with a RSD from 1 to 2%. The developed IC procedure provided a rapid, reliable and sensitive method for the determination of fosfomycin concentration in industrial pharmaceutical wastewater and samples containing complex components.

Key words | fosfomycin, high performance liquid chromatography, ion chromatography, limit of detection, pharmaceutical wastewater

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INTRODUCTION

Fosfomycin (1R-2S-epoxypropyl phosphonic acid) is a unique, broad spectrum antibiotic that is chemically unrelated to any other known antibacterial agent. It was discovered in Spain in 1969, isolated from fermentation broth of *Streptomyces fradiae* (Popovic *et al.* 2010). It is a phosphonic acid derivative, and shows almost no binding to proteins. Fosfomycin is a bactericidal antibiotic that interferes with cell wall synthesis in both Gram-positive and Gram-negative bacteria by inhibiting the initial step involving phosphoenol pyruvate synthetase. In clinical medicine, calcium fosfomycin or sodium fosfomycin is applied.

Fosfomycin is quite difficult to analyze in medicine as it is a strongly polar and small molecule. No ultraviolet (UV)

absorbance is detected even at wavelengths lower than 200 nm. In the China pharmacopeia (National Pharmacopoeia Committee 2010), the traditional microorganism detection method is applied which is the typical method for antibiotic detection. Pretreatment for this method is simple, the cost is quite low and a large quantity of samples could be detected. However, the low sensitivity, long operation time and complicated operation and disturbance by other antibiotics hinder the large scale application of the detection method. Other methods, such as ion exchange chromatography to detect pharmaceutical products in the range of $5\text{--}40 \text{ mmol L}^{-1}$, with relative standard deviation (RSD) of 2.58% and recovery of 91.8%–100.5% (Hu *et al.* 1999a),

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spectrophotometry, applied in standard solution with detection range of 1.4–60 mg L⁻¹ and recovery of 99.6%–100.5% (Liu *et al.* 2010), high performance liquid chromatography (HPLC)-evaporative light scattering detector (ELSD), testing standard solution and pharmaceutical products with detection range of 0.9–2.4 mg L⁻¹ and average recovery of 100.4% (Zhong *et al.* 2006), capillary zone electrophoresis with indirect UV detection testing pus samples with detection range of 10–300 mg L⁻¹, limit of detection (LOD) of 4.5 mg L⁻¹, limit of quantitation (LOQ) of 15 mg L⁻¹ (Baillet *et al.* 1993; Levêque *et al.* 1994; Petsch *et al.* 2005), derivatization gas chromatography to determine fosfomycin in biological and bacterial or cellular culture medium with detection limit of 1 mg L⁻¹ (Shafer *et al.* 1970; Dessalles *et al.* 1987), flow injection spectrophotometry, testing pharmaceutical products and urine samples with a detection range of 0.4–40 mg L⁻¹ (Paraskevas *et al.* 2002), liquid chromatographic/tandem mass spectrometric method testing human plasma samples with a detection range of 0.1–12 mg L⁻¹ (Li *et al.* 2007), indirect spectrophotometric method detecting pharmaceutical products with a detection range of 0–5 mg L⁻¹ (Hu *et al.* 1999b), ion chromatography (IC) with indirect spectrophotometric detection applied in plasma samples (Pianetti *et al.* 1993), ion monitoring method to measure blood and urine samples (Longo *et al.* 1981), have been developed. However, these methods were established for single molecule detection, not for a mixture of molecules encountered in real wastewater. For the industrial wastewater sample containing several contaminant molecules or the effluent from the reactor where fosfomycin is treated by activated sludge, there is no information about whether these methods could be applied and about their detection limit and precision.

In this study, alizarin spectrophotometry, HPLC and IC were investigated to find the best method for fosfomycin determination in industrial wastewater.

METHODS

Chemicals

Fosfomycin standard (>99%, purity) was purchased from National Institutes for Food and Drug Control (Beijing, China). A stock standard solution of fosfomycin (concentration 9.6 mg mL⁻¹) was prepared and could be stable for 4 months when kept at -80 °C. Fosfomycin disodium salt was purchased from Northeast Pharmaceutical Factory (Shenyang, China). A stock solution of fosfomycin disodium (concentration 20.0 g L⁻¹) was prepared and kept at 4 °C.

Methanol was from Fisher (North Salt Lake, USA) at chromatographic grade; the other chemicals such as alcohol, alizarin, KH₂PO₄ were analytical grade. Ultrapure water was used with resistance >18.0 MΩcm, prepared by a Millipore Milli-Q integral 5 apparatus (Bedford, MA, USA).

Instruments

A UV 4802 spectrophotometer (UNIC, USA) was used with 1-cm light-path cuvettes. An Agilent 1260 apparatus for HPLC analysis (Agilent Technologies, Palo Alto, CA, USA), was equipped with a Zorbax-SAX column (4.6 mm × 250 mm). DIONEX ICS-2100 for IC analysis (DIONEX ICS-2100, Dionex, USA) was operated with a Dionex IonPac™ AG11-HC anion protection column (50 × 4 mm, Dionex, USA), and a Dionex IonPac™ AS11-HC anion column (250 × 4 mm). Data were analyzed by a Chameleon 6.8 chromatography workstation (Dionex, USA).

Water samples

The original industrial wastewater containing fosfomycin was collected directly from a pharmaceutical factory. The smelly wastewater showed a dark brown color. The industrial wastewater possesses a chemical oxygen demand content of 60.0–80.0 g L⁻¹, total organic phosphorus content of 8.0–10.0 g L⁻¹, PO₄³⁻-P content of 0.8–1.2 g L⁻¹, pH value of 11.0–12.0, which has been described by Qiu *et al.* (2011). The industrial wastewater samples were diluted from original industrial wastewater.

A 2.0 L bench scale sequencing batch reactor (SBR) was operated to cultivate activated sludge with fosfomycin. The fosfomycin sodium (200.0 mg L⁻¹) diluted from stock fosfomycin sodium solution was supplied as the sole carbon and energy source since the concentrations of organic phosphorus changed from several mg/L to almost 10,000 mg/L in the real pharmaceutical wastewater. The nutrients consisted of the following chemicals (mg L⁻¹): (NH₄)₂SO₄ 1,000, KH₂PO₄ 800, K₂HPO₄ 200, MgSO₄·7H₂O 500, FeSO₄ 10, CaCl₂ 50, NiSO₄ 32, Na₂BO₇·H₂O 7.2, (NH₄)₆Mo₇O₂₄·H₂O 14.4, ZnCl₂ 23, CoCl₂·H₂O, 21, CuCl₂·2H₂O 10 and MnCl₂·4H₂O 30 (Zeng *et al.* 2007). The effluent from SBR was collected for fosfomycin analysis by IC.

Pretreatment

Before analysis, the wastewater sample was filtered with 0.45 μm filter paper and then passed through a C18 column (500 mg, 6 ml).

Procedure

UV spectrophotometry

In a 25 ml tube, the fosfomycin standard solution or water samples were reacted with alizarin (1.0×10^{-3} mol L⁻¹) in 99.7% alcohol solution at a molar ratio of 1:1. The formed products caused the color change to be purple. Fifteen minutes later, the absorbance of samples were measured at a wavelength of 545 nm by UV spectrometry (UNIC 4802, UNIC, USA).

High performance liquid chromatography

Concentrations of fosfomycin were determined by HPLC (Agilent 1260, Agilent, USA). The separation of the fosfomycin from the other molecules of the wastewater was obtained by a Zorbax-SAX column (4.6 mm \times 250 mm). The mobile phase was a mixture of 50 mmol/L KH₂PO₄ and methanol in a 92.4/7.6 (v/v) ratio. The applied flow rate was 1 mL min⁻¹. The separated molecules were detected using a UV spectrophotometer at a wavelength of 200 nm. The typical retention time for fosfomycin was 10.022 min (Hu et al. 1999a).

Ion chromatography

Concentrations of fosfomycin were determined by IC (DIONEX ICS-2100, Dionex, USA). The separation of the fosfomycin was obtained using a Dionex IonPacTM AG11-HC anion protection column (50 \times 4 mm, Dionex, USA), and Dionex IonPacTM AS11-HC anion column (250 \times 4 mm, Dionex, USA). The suppressor was ASRS300 with electric conductance. The mobile phase was 30 mmol KCl and the current was 75 mA. The applied flow rate was 1 mL min⁻¹. The column temperature was 30 °C. The injection volume was 25 μ L. The typical retention time for fosfomycin was 3.847 min.

Effect of seven typical anions on the determination of fosfomycin determination

To investigate the ion interfering with fosfomycin, the mixing samples of fosfomycin (8 mg L⁻¹) with seven anions (F⁻ of 2 mg L⁻¹, Cl⁻ of 5 mg L⁻¹, Br⁻ of 10 mg L⁻¹, NO₂⁻ of 8.8 mg L⁻¹, NO₃⁻ of 12 mg L⁻¹, SO₄²⁻ of 9.5 mg L⁻¹, PO₄³⁻ of 20 mg L⁻¹) were injected into the IC. To confirm the interference between chloride and fosfomycin, 10 mg L⁻¹ fosfomycin standard solution with different concentrations of chloride (10 and 50 mg L⁻¹), 1,000 times

dilution with fosfomycin industrial wastewater with different concentrations chloride (10, 100 and 1,000 mg L⁻¹), as well as effluent from SBR treating fosfomycin wastewater (dilution 20 times) with different concentrations of chloride (10, and 50 mg L⁻¹), were injected into IC.

Quantitation, repeatability, accuracy, LOD and LOQ determination

Repeatability, accuracy, LOD and LOQ were determined according to the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines (Green 1996; I-G 1996; Armbruster & Pry 2008). The repeatability of the determination of the peak area of fosfomycin (described by RSD, %) was derived from 10 replicate injections of samples at a concentration level. Accuracy and precision was determined by measuring the recovery of the wastewater samples after addition to blank (Green 1996; I-G 1996; Armbruster & Pry 2008). The method was performed at three different concentrations of fosfomycin industrial wastewater and effluent discharged from SBR fed with fosfomycin wastewater.

RESULTS AND DISCUSSION

Alizarin spectrophotometry

The fosfomycin was analyzed by UV spectrometry at a wavelength of 545 nm after it reacted with alizarin to form a complex indicated by the color change. Standard solutions with concentrations of 2.5, 5.0, 7.5, 10.0, 12.5, 15.0 mg L⁻¹ were made from the stock standard solution of fosfomycin by dilution. The linear relationship between the concentration of fosfomycin and absorbance at 545 nm was observed with a good correlation coefficient of 0.995 and LOD of 1.4 mg L⁻¹. However, the deep color of industrial wastewater caused by its complex properties made it difficult to detect fosfomycin in the industrial wastewater. Thus, the alizarin spectrophotometry was not suitable for the determination of fosfomycin in industrial wastewater.

High performance liquid chromatography

The HPLC chromatograms of fosfomycin standard solution and industrial wastewater for the identification of fosfomycin are shown in Figure 1, where in both water samples, the retention time of fosfomycin was at 10.022 min.

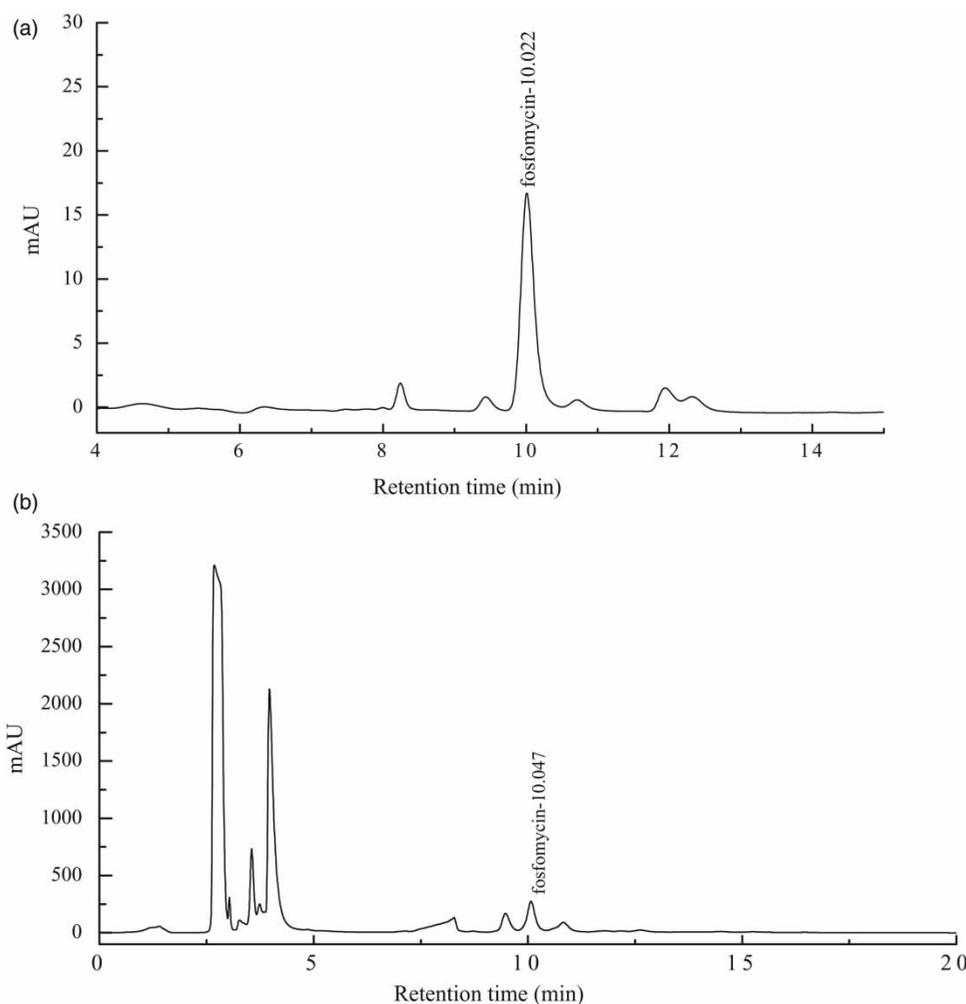


Figure 1 | HPLC chromatograms of fosfomycin in (a) pure aqueous sample (concentration 240 mg L^{-1}) and (b) industrial wastewater sample.

Standard solutions with concentrations of 240.0 , 480.0 , 720.0 , 960.0 , $1,200.0$, $1,440.0 \text{ mg L}^{-1}$ were prepared from the stock standard solution of fosfomycin and analyzed by HPLC. A good linear relationship between the concentration of fosfomycin and peak area was found with a high correlation coefficient (0.9998). However the LOD was high at 96.0 mg L^{-1} . In the effluent of wastewater treatment plant, the concentration of fosfomycin maybe as low as several mg L^{-1} , or lower than 1.0 mg L^{-1} . Therefore, a method with low LOD was needed for the determination of fosfomycin concentration in water sample with low concentration.

Ion chromatography

IC was performed with fosfomycin-contained pure aqueous solution and wastewater samples for the identification of

fosfomycin (Figure 2). In both water samples, the fosfomycin appeared at the retention time of 3.847 min . The detection range was performed for standard solutions of concentrations between 1.0 and 20.0 mg L^{-1} . The according calibration line (expressed as $y = a + bx$, where x is the concentration of fosfomycin in mg L^{-1} , and y is peak area; a is the intercept, both in conductivity) was constructed by external calibration by the aid of seven concentrations of fosfomycin in the linear range between 1.0 and 15.0 mg L^{-1} . The resulting equation is $y = 20.4082x - 0.1224$ with the linear correlation coefficient (R) of 0.9998 (Table 1). Accordingly, the LOD (for a signal to noise ratio (S/N) of 3) is 0.06 mg L^{-1} , and the LOQ (signal to noise ratio 10) is 0.19 mg L^{-1} fosfomycin.

The repeatability of the determination of the peak area of fosfomycin was derived from 10 replicate tests of fosfomycin standard solution at the concentration level of 0.45 ,

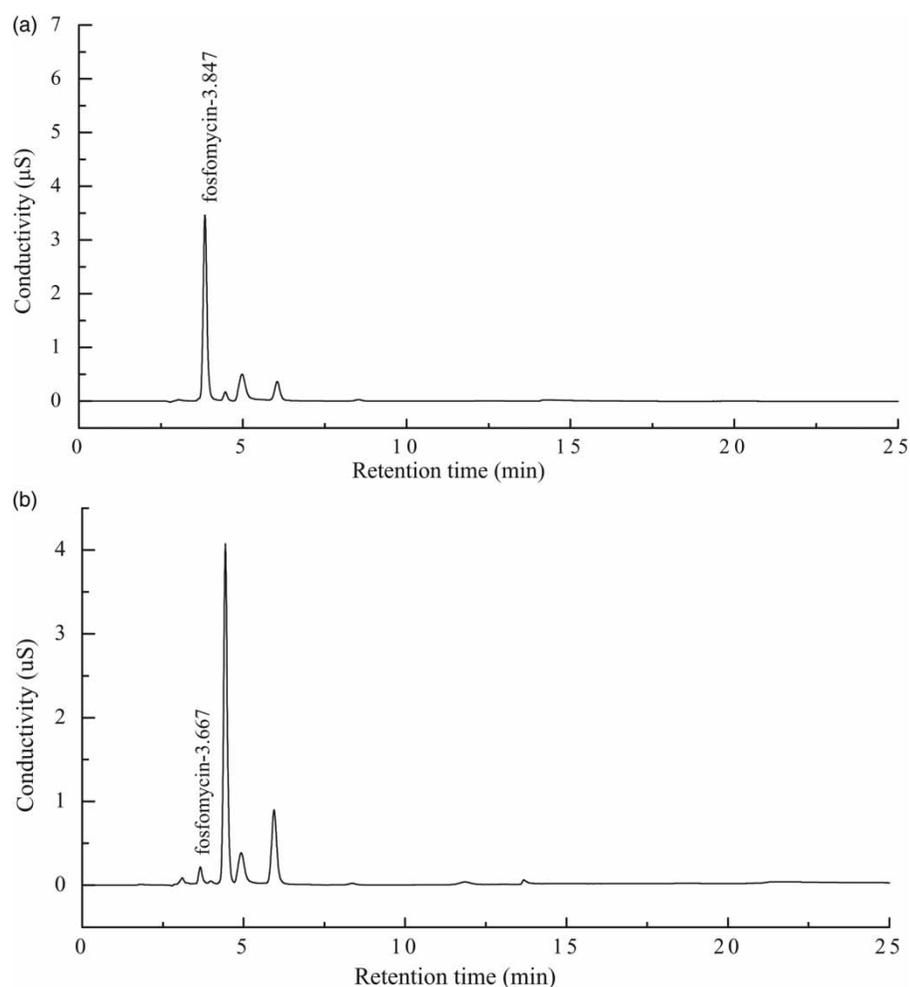


Figure 2 | IC chromatograms of fosfomycin in (a) pure aqueous sample (concentration 15 mg L^{-1}) and (b) industrial wastewater sample (dilution 2500).

Table 1 | Precision and recovery of fosfomycin determination in pharmaceutical wastewater samples by IC

Samples	Fosfomycin concentration			
	Background of industrial wastewater (mg L^{-1})	Standard addition 2.0 mg L^{-1}	Standard addition 5.0 mg L^{-1}	Standard addition 8.0 mg L^{-1}
Sample 1	0.43	1.98	4.99	7.70
Sample 2	0.43	1.96	4.86	7.86
Sample 3	0.45	1.91	4.90	7.65
Average value	0.43	1.95	4.92	7.74
Standard deviation	0.02	0.03	0.06	0.05
RSD	0.04	0.01	0.01	0.01
Recovery (%)		81.91	91.96	94.74

4.70 and 48.00 mg L^{-1} spiked to blank. The samples were filtered before injection into the IC machine. The measurements were performed on three different days and led to the following results: 0.28% ($n = 10$) for

concentration of 0.45 mg L^{-1} ; 0.81% ($n = 10$) for concentration of 4.70 mg L^{-1} ; 0.25% ($n = 10$) for concentration of 48.00 mg L^{-1} . The resulting RSD is acceptable for the present goal.

Table 2 | Precision and recovery of fosfomycin determination in water samples discharged from SBR by IC

Samples	Fosfomycin concentration			
	Effluent from SBR (mg L ⁻¹)	Standard addition 5.0 mg L ⁻¹	Standard addition 8.0 mg L ⁻¹	Standard addition 10.0 mg L ⁻¹
Sample 1	4.00	8.89	11.94	13.75
Sample 2	3.89	8.88	11.92	13.62
Sample 3	3.97	8.88	11.83	13.63
Theoretical standard addition		8.91	11.91	13.91
Average value	3.92	8.86	11.81	13.76
Standard deviation	0.04	0.00	0.04	0.06
RSD	0.02	0.00	0.01	0.01
Recovery (%)		99.69	99.91	98.25

Table 3 | Fosfomycin determination in a real fosfomycin pharmaceutical wastewater

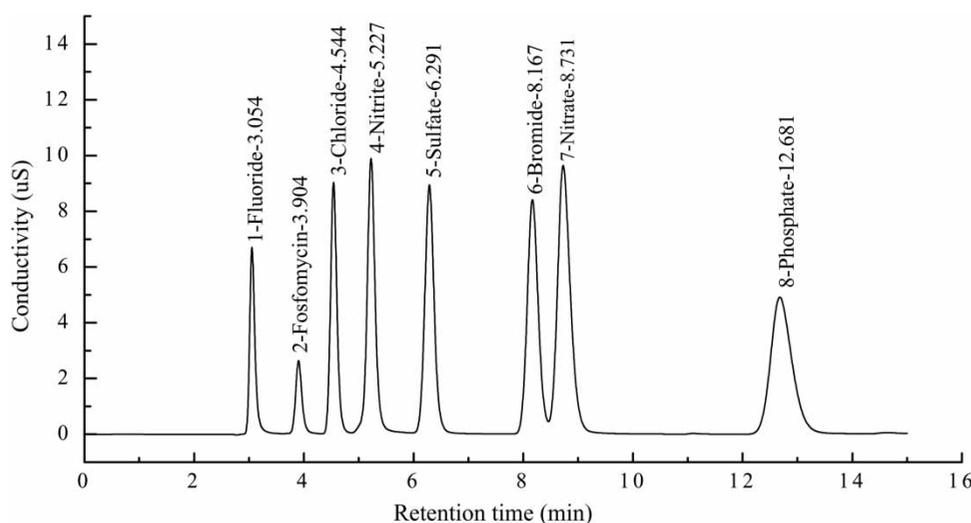
samples	influent	Effluent from hydrolytic acidification	Effluent from contact oxidation	effluent
Fosfomycin concentration (mg/L)	18.11	13.55	2.70	2.10

To evaluate the precision of IC for the determination of fosfomycin concentration in industrial wastewater, the recovery of fosfomycin spiked in different water samples collected from pharmaceutical industrial wastewater, and effluent from SBR fed with fosfomycin wastewater were investigated (Tables 1 and 2). For the industrial pharmaceutical wastewater samples, the standard deviation (SD)

ranged from 0.02 to 0.06 mg L⁻¹ and this corresponded to 81.91 to 94.74% of expected results. For effluent collected from SBR, the SD ranged from 0 to 0.02 mg L⁻¹ and this corresponded to 98.25 to 99.91% of expected results. It was in the range of acceptability (Green 1996; I-G 1996; Armbruster & Pry 2008).

The fosfomycin detection method by IC was applied to detect the real fosfomycin pharmaceutical wastewater samples. The samples were collected from a real fosfomycin pharmaceutical wastewater treatment plant along the flow chart: influent, hydrolytic acidification tank, contact oxidation tank, second sediment tank. The test results are listed in Table 3 and reflected the fosfomycin degradation in the wastewater treatment plant.

To be sure of the specificity of the IC detection method for fosfomycin analysis, the mixing samples of fosfomycin

**Figure 3** | IC chromatograms of fosfomycin and seven anions (1 fluoride, 2 fosfomycin, 3 chloride, 4 nitrite, 5 sulfate, 6 bromide, 7 nitrate, 8 phosphate).

with seven anions were injected into the IC, which is shown in Figure 3. There was good separation of fosfomycin with other anions when analyzed by this method. Comparing the retention time of the seven anions with fosfomycin, the retention time of chloride is closest to the

fosfomycin. To understand whether there was interference between fosfomycin and chloride and in what conditions there was interference, the mixture of fosfomycin and chloride was analyzed by this method (Figure 4). For fosfomycin standard solution, there was no interference with

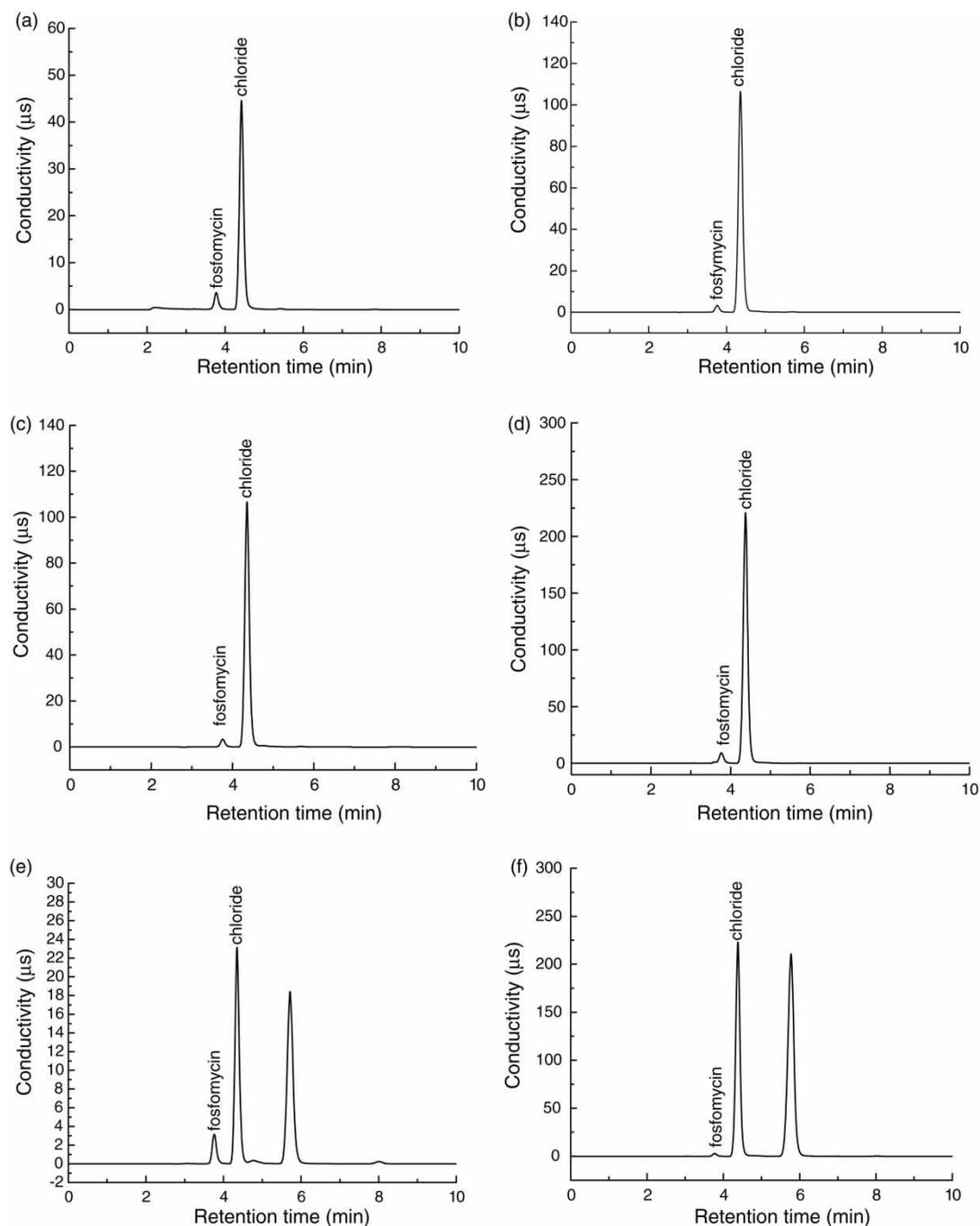


Figure 4 | IC chromatograms of fosfomycin and chloride anion (a) 10 ppm fosfomycin standard + 10 ppm chloride, (b) 10 ppm fosfomycin standard + 50 ppm chloride, (c) fosfomycin industrial wastewater (dilution 1,000) + 10 ppm chloride, (d) fosfomycin industrial wastewater (dilution 1,000) + 1,000 ppm chloride, (e) effluent from SBR (dilution 20) + 10 ppm chloride, (f) effluent from SBR (dilution 20) + 50 ppm chloride.

each other when chloride concentration was five times that of fosfomycin. For the fosfomycin industrial wastewater, there was no interference even when chloride concentration was several hundred times that of fosfomycin. For the effluent from SBR treating fosfomycin wastewater by activated sludge, no interference was observed when chloride was 50.0 mg L^{-1} and fosfomycin was lower than 1.0 mg L^{-1} . Thus, it could be concluded that there was no interference between fosfomycin and chloride.

Based on the detection and analysis carried out, the IC method was found to be highly suitable to determine fosfomycin concentration in industrial wastewaters and samples containing complex components, such as effluent from activated sludge systems.

CONCLUSIONS

Different methods for the determination of fosfomycin concentration have been analyzed for pure water samples, industrial wastewater samples and the effluent from activated sludge systems. Alizarin spectrophotometry based on the reaction of fosfomycin and alizarin to form a colored product easily identified by UV was highly valuable for pure water analysis (with a detection limit of 1.4 mg L^{-1}) but not for industrial wastewater which was often colored and contained sediments. HPLC was found to be a powerful method for pure water and wastewater analysis if the concentration in fosfomycin was higher than 96.0 mg L^{-1} , which was the LOD of the method. IC was the most suitable method to analyze pure water and industrial wastewater with a detection limit as low as 0.06 mg L^{-1} . This method meets the requirements of rapidity, high precision and a low detection limit. IC was highly suitable for the determination of fosfomycin in pharmaceutical wastewaters.

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