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A Simple and Rapid Method to Measure Residue of Cefexime – a Cephalosporin Antibiotic in the Wastewater of Pharmaceutical Production Plant

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A simple and rapid HPLC method was developed to analyse cefixime, and potentially other cephalosporin antibiotic in wastewater of pharmaceutical production industry. The collected wastewater samples were treated by liquid – liquid extraction using chloroform to eliminate contaminants before instrumental analysis. Separation of the analyte was achieved using a Phenomenex Luna C18 column (250×4.6 mm; 5 μm) and a mixture of methanol and phosphate buffer pH 3.2 as mobile phase in a gradient program. The drug was detected by UV detector at wavelength of 285 nm. The analytical method was first validated using cefixime– spiked samples. Absorbance response to cefixime concentrations is linear from 23 to 1100 ppb with a correlation coefficient > 0.999 and the limit of detection of 6.68 ppb. Repeatability and intermediate precision were tested with RSD of less than 3.0%. The recovery values were found in the range of 97.4–106.0%. The method was then applied to determine concentrations of cefixime in real wastewater samples. The concentrations of cefixime residue measured in the actual samples were in the range of 19.24 to 43.33 ppb which proved the applicability of this analytical method in monitoring the level of contaminated cefixime from the effluent of the pharmaceutical manufacturing plant into environment. The method also showed a potential to apply for other cephalosporin antibiotics.

Key words: cefixime, environmental monitoring, HPLC, water quality

INTRODUCTION

Antibiotics are extensively used in human and veterinary medicine, as well as in aquaculture, for the purpose of preventing (prophylaxis) or treating microbial infections (Kümmerer, 2009a). Although antibiotics are important for the protection of human and animal health from bacterial infections the presence of antibiotic residues in the general aquatic environment may cause an unexpected problem – bacterial resistance to antibiotics, which has been found by several studies in the last decades (Kümmerer, 2009b). This phenomenon together with an imprudent use of antibiotics is probably the main cause of bacterial resistance found in the environment.

Contribution to the mass of antibiotics in the environment is considered to be from the excretion and disposal of drugs for human and animal use, plant agriculture as well as aquaculture and recently from pharmaceutical production plants. Discharge of antibiotics from production plants have received little attention previously but recently it has been found that in some Asian countries concentrations of up to several mg L^{-1} could be found in effluents for single compounds (Larsson *et al*., 2007; Li *et al*., 2008). In developed countries a manufacturing plant can also make a significant contribution to total antibiotic concentration in the influent of a sewage treatment plant, as has been shown only recently for the treatment plant in Oslo (Langford and Thomas, 2009).

With the rapid development of the economy, the consumption and production of antibiotics in Vietnam at a very high rate resulted to, an alarming level of the release of those antibiotics to the environment. However, there are only a few studies investigating the occurrence of antibiotics and antibiotic–resistant bacteria in the environment from different sources (hospital effluents, aquaculture…) (Hoa *et al*., 2011; Duong *et al*., 2008; Hoang *et al*., 2012). No study has yet focus to the source of discharge from pharmaceutical production plants.

Cephalosporins are among the safest and the most effective broad spectrum antibiotics (Péhourcq, 1998). In Vietnam they are probably most prescribed and most produced antibiotics. Measurement of their residues in the aquatic environment is necessary to ensure the quality of the environment, especially in the surrounding of the pharmaceutical production plants. A simple and rapid analytical method is in a demand for analysing those cephalosporins in water. Analytical methods in the literature are either developed for clinical/biological samples (Nemutlu *et al*., 2009; Péhourcq, 1998) and or using high cost instruments such as LC–MS (Meng *et al*., 2005) that are not suitable for the environmental water samples. In this study, we aimed to develop a simple and rapid analytical method for the analysis of cefixime, in effluent of a pharmaceutical plant as a tool for regular environmental monitoring program at local level (factory quality control or local environment authority).

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MATERIALS AND METHODS

Chemicals and reagents

Cefixime (98.88% purity, national standard) was obtained from the National Institute for Drug Quality Control (Hanoi, Vietnam). Working standards of cefuroxime sodium, cefadroxil, cefdinir, cefoperazon (other cephalosporin antibiotics) were also obtained from the National Institute for Drug Quality Control (Hanoi, Vietnam). Methanol and acetonitrile (HPLC–grade), dichloromethane, chloroform, diethyl ether, cyclohexane, sodium dihydrophosphate, acid hydrochloric, acid phosphoric were purchased from Merck (Darmstadt, Germany). Deionized water was used throughout the study.

Preparation of standard solutions

Standard working solution of cefixime was prepared by dissolving appropriate amount of cefixime in deionized water to give concentration of about 50 μg/ml. The working solutions of cefuroxime sodium, cefadroxil, cefdinir, cefoperazon also at concentration of 50 μg/ml were obtained directly from the standard stock of the National Institute for Drug Quality Control (Hanoi, Vietnam) and were used for comparison purpose.

A calibration curve was prepared by diluting the working standard of cefixime to obtain a series of solutions with concentrations of 25, 50, 100, 200, 500, 800, 1000, 1200 ng/ml water.

Spike samples were prepared by spiked working solution of cefixime (or of other cephalosporins) to matrix blank samples at appropriate concentrations for validation purposes.

Sample preparation

Wastewater samples were collected in an effluent system of a pharmaceutical plant located in Hanoi, Vietnam. A series of tests were conducted to find the optimal treatment for the water samples. In general, samples were filtered through a 0.45 μm filter paper and then were used directly or were acidified by HCl 1N to pH around 3. Samples were then poured into a test tube of 15 mL before an organic solvent or a mixture of them was added in different ratio for cleaning up purpose. The mixture was well shaken in 10 min by a Vortex device and then was centrifuged in 5 min at the speed of 3000 rpm. The aqueous phase was taken and filtered again through a 0.45 μm filter paper before analysis. Organic solvents to be tested include acetonitrile, chloroform, dichloromethane, diethyl ether and cyclohexane.

Chromatographic conditions

Chromatography was performed using a Merck–Hitachi LiChrom HPLC system equipped with a pump, on–line vacuum degasser, auto–sampler, column oven, and UV–Vis detector (Merck–Hitachi, Darmstadt, Germany). The chromatographic data was analyzed using Multi–system Manager connected to the D7000 Interface to control the HPLC system.

Cefixime and other cephalosporins were separated by a Phenomenex Luna C18 column (250×4.6 mm; 5 μm). The oven was kept at 32°C. After a first screening run to determine the maximum absorbance of cefixime, the UV detector was set at 285 nm wavelength. Injection volume was set at 200 μL to achieve the highest detection limit while still maintain a good separation/peak shape. An initial test of the mobile phase indicated that a suitable mobile would consist of methanol and a phosphate buffer at pH 3.2 (solution of sodium dihydrophosphate 40 mM, with pH adjusted by condensed phosphoric acid), using a gradient program as presented in Table 1.

Data analysis

Qualitative: Identification of cefixime in the samples (spiked or real) by comparing the retention time and UV spectrum of peaks in chromatographs of tested samples with those of the standards. All data obtained from precision and accuracy tests were analysed according to ICH guideline for method validation (ICH, 2005).

Quantitative: Using linear regression to construct a calibration curve and a corresponding regression equation. Concentrations of cefixime in the tested samples were calculated from this equation.

RESULTS AND DISCUSSIONS

Optimisation of analytical conditions

Optimisation of sample preparation

Collected wastewater samples were acidified at pH 3 to facilitate the condition of HPLC analytical assay. However a value of $P_{o/w}$ –0.64 of cefixime at pH around 3 hinders the drug from partioning from the aqueous phase into the organic phase. Therefore, samples were treated to remove interference of lipophilic compounds using organic solvents while cefixime was remained in the aqueous phase. A number of solvents including solvent mixtures with different ratios were tested to find an optimal condition, namely simple, less interference, and acceptable recovery.

Samples were first treated with acetonitrile to precipitate proteins in the samples as usually recommended for biological samples (Péhourcq, 1998) and were then treated with single or a mixture of chloroform, dichloromethane and diethyl ether. Table 2 presents organic solvents with different ratios of combination used for elimination of lipophilic contaminants. The combination of acetonitrile and dichloromethane was shown to delivere the best recovery among those mixtures but the use of acetonitrile is not suitable since the maximum recovery was about 80% (data not shown). It is probably due to the dilution effect of acetonitrile (which is miscible in water) to the sample. Therefore, acetonitrile should be avoided for sample preparation and but immiscible solvents such as chloroform, diethyl ether and cyclohexane were recommended.

It is clear that acidification of the sample, which led to stronger ionic force of cefixime and other cephalosporin antibiotics with an amino group (Péhourcq, 1998), showed an advantage over non–acidification in

term of keeping the target analyte in the aqueous phase for a higher recovery.

Those results were evaluated together with the chromatograms shown in Fig. 1 to decide which treatment would be used for actual samples. Less interference in the chromatograph (Fig. 1) and a high recovery (Table 1) of acidified samples treated with chloroform (sample/solvent ratio of 5:3 v/v) demonstrated suitability of chloroform as a cleaning solvent. Moreover, good recoveries for 4 other cephalosporin antibiotics were also obtained when treated with chloroform.

Data on the robustness of the sample treatment presented in Table 3 indicate that the selected treatment protocol is suitable to apply for actual wastewater samples and has potential to extend for treating other cephalosporin antibiotics in the sample with recovery value over 93%.

Optimisation of instrumental conditions

It is usual for a pharmaceutical company to produce different cephalosporin antibiotics at the same period of time. Therefore, it is important for the method to separate and quantify cefixime in the presence of other cephalosporins in the sample background. We have screened a range of different mobile phase parameters (compositions and flow rates) and injection volumes to find the optimal chromatographic conditions as stated in previous sentence.

A simple isocratic mobile phase was tested against a gradient program in this study. Although simple the isocratic condition did not provide satisfactory outcomes: cefixime was not completely separated from other cephalosporins and the peak stretched as well as asymmetric (Fig. 2a). Meanwhile, the gradient program successfully separated cefixime from other peaks and the cefixime peak was symmetric (Fig. 2b). The detailed gradient program was provided in Table 1.

For a simple method, increasing injection volume is an approach to improve the limit of detection. However, care should be taken to ensure the quality of the target peak (symmetric) and the overall chromatogram (level of noise). After testing different injection volumes in the range of 20–200 μl, it was found that the volume of 200 μl is suitable for this method.

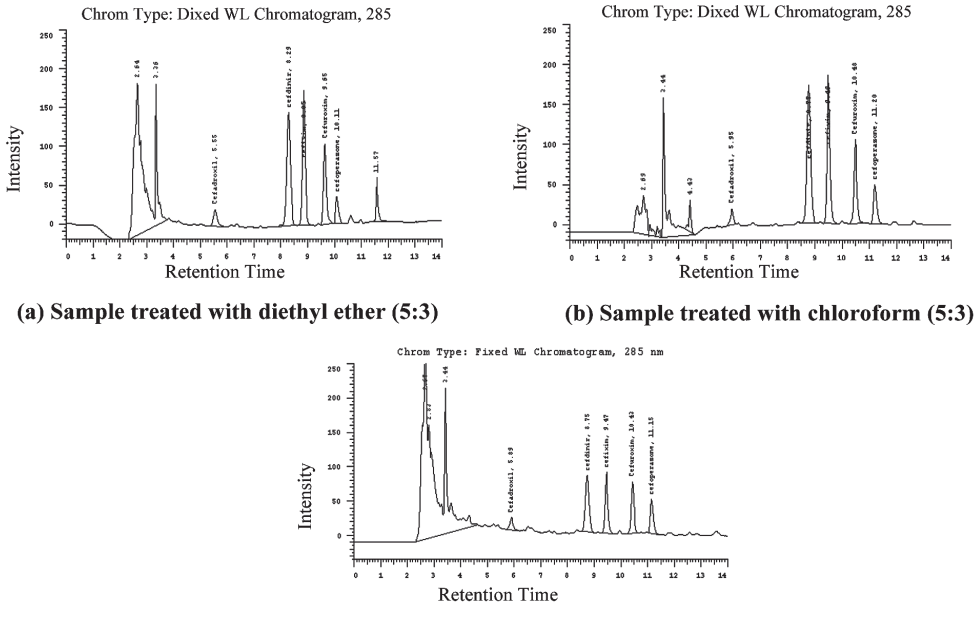
Although it is reviewed that cefixime can be detected in different UV wavelength (Péhourcq, 1998), a spectrum screen from 200–350 nm for the sample

Table 1. Gradient program of the mobile phase

Time (min)	MeOH (%)	Phosphate buffer solution (%)	Flow rate (ml/min)
0	18	82	1.0
6	45	55	1.0
13	55	45	0.85
14	18	82	0.85

Table 2. Different organic solvent with different ratio of samples and solvents used for sample treatment

Solvent	Sample:solvent volume ratio	Non–acidified sample	Acidified sample (pH 3)
		Recovery (%)	Recovery (%)
Diethyl ether	4 : 4	70.7	80.5
	5 : 3	78.3	83.5
Chloroform	4 : 4	41.1	95.4
	5 : 3	40.9	96.8
Cyclohexane	4 : 4	18.1	48.6
	5 : 3	16.5	47.1



(a) Sample treated with diethyl ether (5:3)

(b) Sample treated with chloroform (5:3)

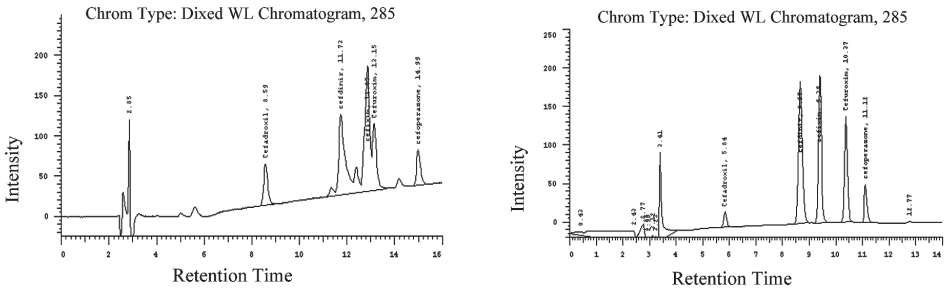
(c) Sample treated with cyclohexane (5:3)

Fig. 1. Chromatograms of acidified extracts using different solvents.

Table 3. Robustness of sample treatment for different cephalosporin antibiotics

	Recovery (%)*				
	Cefixime	Cefdinir	Cefadroxil	Cefuroxime	Cefoperazone
Ave	96.0	100.2	101.5	96.9	93.8
Sdev	0.55	0.59	1.35	1.52	0.30
RSD	0.57	0.59	1.33	1.57	0.32

** 6 samples were used for each test and the average value was used.*



a. Isocratic mobile phase **b. Gradient mobile phase**

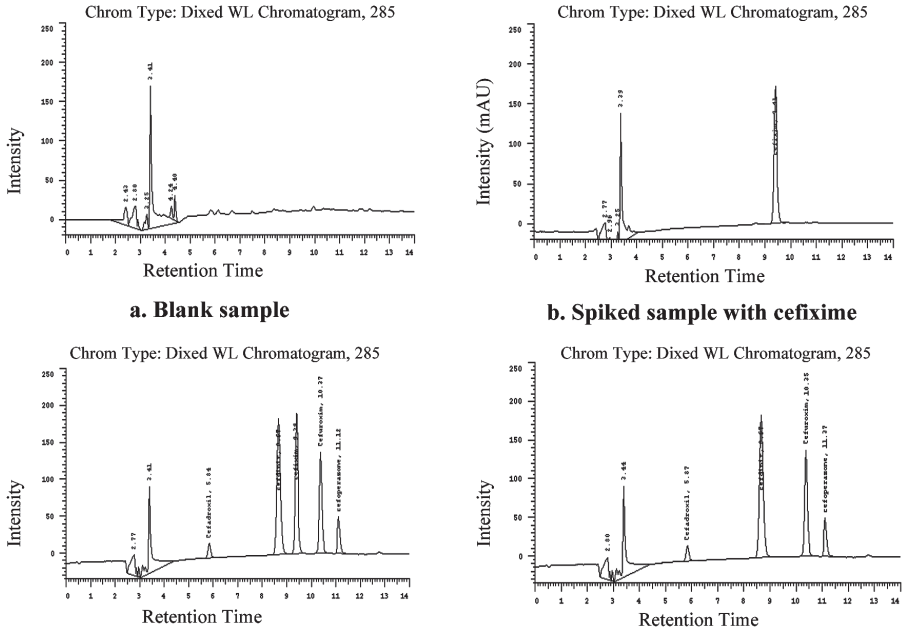
Fig. 2. Comparison of different mobile phase programs.

extracts of this study indicated the maximum absorbance at 285 nm. It is in agreement with another study by Khan *et al.* (2011) in which the response at 285 nm was concluded to give a maximum absorbance of cefixime while minimised extraneous peaks. Thus, a wavelength of 285 nm was selected for cefixim determination.

Method validation

After the optimisation of all parameters, the method was validated according to the ICH guideline for method validation (ICH, 2005). The matrix in use was the wastewater from a pharmaceutical production factory in Vietnam.

The selectivity of the method was evaluated by comparing a retention time of cefixime peak and aspectra of peaks between standard and test samples. The linearity of the calibration curve was assessed based on the regression between cefixime concentrations and the



a. Blank sample **b. Spiked sample with cefixime**

c. Sample containing 5 cephalosporins **d. Sample containing 4 cephalosporins (without cefixime)**

Fig. 3. Chromatograms of samples during optimisation of instrumental conditions.

Table 4. Results of the method validation

Parameter	AOAC recommended threshold	Results
Selectivity	Retention time (t_R) of peak on chromatogram of test sample coincide with that of standard sample.	t_R standard ~ 9.43 min t_R test sample ~ 9.52 min
	No peak on chromatogram of blank sample at the same retention time with the target analyte	There was no peak in the chromatogram of blank sample at the retention time of cefixime
	UV–VIS spectrum of cefixime in standard and test samples gave an $SI > 0.99$	$SI = 0.9993$
System suitability	RSD of peak area $\leq 2.0\%$ ($n = 6$)	1.41%
	RSD of $t_R \leq 1.0\%$ ($n = 6$)	0.46%
	Resolution of cefixime peak and a close peak (cefnidir) > 1.5	R_s =1.6
Linearity	Linear range	22.98 – 1103.14 ng/ml
	$r \geq 0.998$	$r = 0.99998$
	Slope	0.001803
	Intercept	0.003693
Accuracy (recovery)	@ low level ≈ 70 ng/ml: 60 – 115%	100.2 – 106.0%. *Ave* = 103.0%
	@ medium level ≈ 500 ng/ml: 80 – 110%	97.4 – 104.5%. *Ave* = 103.0%
	@ high level ≈ 1000 ng/ml: 80 – 110%	97.7 – 104.7%. *Ave* = 101.5%
Precision	@ low level ≈ 70 ng/ml: $RSD \leq 21.0\%$ ($n = 6$)	1.50%
	@ medium level ≈ 500ng/ml: $RSD \leq 15.0\%$ ($n = 6$)	1.04%
	@ high level ≈ 1000 ng/ml: $RSD \leq 15.0\%$ ($n = 6$)	1.64%
Reproducibility	@ low level ≈ 70 ng/ml: $RSD \leq 21.0\%$ ($n = 12$)	1.35%
	@ medium level ≈ 500 ng/ml: $RSD \leq 15.0\%$ ($n = 12$)	2.61%
	@ high level ≈ 1000 ng/ml: $RSD \leq 15.0\%$ ($n = 12$)	2.14%
Limit of detection	Concentration at which cefixime response is higher than 3 times background signal	6.68 ng/ml
Limit of quantification	Concentration at which cefixime response is higher than 10 times background signal	22.0 ng/ml

areas of cefixime peak in the chromatogram. The limit of detection of the method was calculated from the slope of the calibration curve and the standard deviation of the response.

The precision and the accuracy of the method were assessed at three concentration levels (low, medium and high level). The results of the validation tests together with the recommended thresholds of AOAC (AOAC, 2011) were presented in Tables 4 and Fig. 3.

The results showed that the newly developed method has good selectivity with high precision and accuracy (according to AOAC standards). The linearity ranges in 3 orders of magnitude which is suitable to measure cefixime in wastewater because of the large variation of cefixime concentration in this media.

Additionally, the stability of test samples was also evaluated after storing samples at ambient temperature for 4 and 24 hours and at –20˚C for 5 and 10 days. The results of the stability test were presented in Table 5. The stability test indicated that cefixime could be preserved in the samples in a short period of time at ambient temperature (1.5% difference) but would start to degrade after 24 hours (over 5% difference) while the freezer condition of –20˚C can keep the samples well preserved for at least 10 days.

Table 5. Results of the stability test

Storage condition	Storage time	% difference*
Ambient temperature	After 4 hours	–1.5
	After 24 hours	–5.8
– 20°C	After 5 days	–2.8
	After 10 days	–3.8

** 6 samples were used for each test and the average value was used.*

Applications to real samples

Actual wastewater samples were taken from the sewer of a pharmaceutical production company during the period where production of cefixime happened. Sampling was carried out in 3 consecutive days in August 2013 and samples were transferred directly to

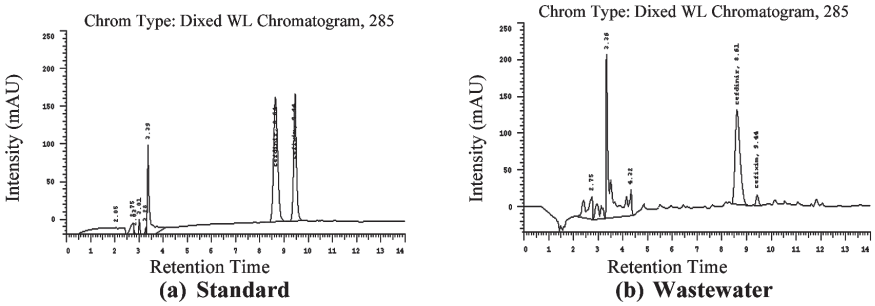


Fig. 4. Chromatograms of standard and actual wastewater samples taken from the sewer.

Table 6. Concentrations of cefixime in actual samples

Day	Replicate	Cefixime concentration (ng/ml)	Average value (ave ± st dev)
I	1	29.96	30.9 ± 2.8
	2	28.71	
	3	34.11	
II	1	42.12	43.3 ± 1.1
	2	43.70	
	3	44.18	
III	1	18.86	19.2 ± 0.6*
	2	19.96	
	3	18.89	

** concentration at the level close to the limit of quantification but the results of triplicate samples confirmed the presence of cefixime.*

the laboratory for analysis.

Samples were treated and analysed using the method described above. Results were presented in Table 6 and Fig. 4. The chromatogram (with spiked cefdinir as reference compound) showed a clearly separated peak of cefixime in the samples. The concentrations of cefixime measured in those 3 samples were above the detection limit and may pose some risk to the environment. Therefore, the newly developed method is applicable to measure the residue of cefixime in wastewater of the pharmaceutical industry.

DISCUSSION

Preventing the occurrence of antibiotic resistant bacteria is an important task in the cause of protecting human and animal health. Monitoring the residue of antibiotics in the environment, especially the aquatic environment would help authorities to tighten their regulation on waste water treatment. To carry out this task in developing countries where analytical cost is an obstacle for many monitoring programs, a simple (low cost) and rapid analytical method is needed. The newly developed method has satisfied those criteria because:

1. Sample treatment by chloroform is simple, rapid and cheap but the recovery and robustness are high. Additionally, this protocol can also be applied to treat samples containing 4 other cephalosporins (cefadroxile, cefdinir, cefuroxime and cefoperazone) with recovery of more than 90%.
2. The separation is carried by a HPLC–UV system which is widely equipped in even small laboratory.
3. The limit of detection of this method is considered relatively high but is capable of detecting cefixime in actual wastewater samples. However, further work is needed to lower the limit of detection to meet the common standard of 1 ng/ml.

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