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Determination of ofloxacin enantiomers in sewage using two-step solid-phase extraction and liquid chromatography with fluorescence detection

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Abstract

A robust analytical method has been developed and validated for the trace analysis of ofloxacin enantiomers in sewage using two-step solid-phase extraction purification and liquid chromatography with fluorescence detection (LC-FL). Ofloxacin enantiomers were separated on an Aglient TC-C-18 column using MeOH–water containing 4 mmol/L $CuSO_4$ and 5 mmol/L L-isoleucine as mobile phase. The ofloxacin enantiomers were first extracted by a weak cation–exchange resin (WCX) and eluted with acidified MeOH (0.5% formic acid), then further purified by mixed mode of anion–exchange resin (MAX), resulting in ofloxacin recoveries generally above 95%. The limit of quantification was 0.08 μg/L for each enantiomer. No significant matrix effect was found during the analytical procedure and standard solution calibration curves could be used for quantification. Total concentrations of both enantiomers in real sewage samples based on LC-FL method were consistent with those obtained upon liquid chromatography using tandem mass spectrometry (LC–MS/MS) method.

Keywords: Ofloxacin enantiomers; Solid-phase extraction; LC-FL

1. Introduction

In recent years, "emerging" contaminants including pharmaceuticals and personal care products [1] have raised great concern. Among these contaminants, antibiotic drugs have received much attention due to the recognition of the development of antibacterial resistance among organisms, which presents an ever-increasing global public health threat involving all major microbial pathogens and antimicrobial drugs [2–4]. These drugs are continuously being released into the environment mainly as a result of the manufacturing process, the disposal of unused or expired products, and excretion after use. Numerous papers have reported the levels of antibiotic drugs [1,5–7] and drug-resistant strains and genes [8–11] in environment. When such genetic elements are transferred, they create "superbugs" that are resistant to many distinct antibiotics [8,11]. More and more frequently, we are seeing outbreaks of dangerous infections caused by such superbugs [11].

Ofloxacin is a broad-spectrum antibiotic commonly used in human and veterinary medicine to prevent or treat bacterial infections throughout the world [12,13]. It is estimated that the outputs of ofloxacin were about 1200 t in 2002 in China [14] and more than 70% were excreted as original form after dosing [15,16]. Ofloxacin is a chiral fluoroquinolone possessing two optical isomers. Previous researches have confirmed that spatial configuration affects antibacterial activity: (*S*)-ofloxacin is 8–128 times more active in vitro than (*R*)-ofloxacin [17–19]. Consequently, the environmental effect of ofloxacin enantiomers is potentially different. It is therefore of great importance to establish a powerful analytical method for the two ofloxacin enantiomers in sewage so as to understand their environmental fate.

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Analytical methodologies based on ligand-exchange chromatography [20–22], capillary electrophoresis [23,24], flow injection chemiluminescence [25] and enantioselective liquid chromatography [26] have been used for separation of ofloxacin enantiomers in pharmaceuticals and biosamples. Sample-preparation methods include extracting high levels of ofloxacin from pharmaceuticals using organic solvents and centrifugation. However, these procedures are unsuitable for the municipal sewage analysis due to the trace level of analytes and the complicated matrix. Mass spectrometry has proven to be the most useful technique for the analysis of the trace levels of pharmaceuticals including ofloxacin in environment [9]. However, it requires the compatibility between the mobile phases of chromatography and ionization mode. Ligand-exchange chromatography containing involatile salt such as $CuSO_4$ in the mobile phase may cause strong ion competition between target compounds and inorganic ions and suppress the ionization of ofloxacin when using electrospray ionization source. The enantioselective liquid chromatography using nonpolar solvent as mobile phases may be a potential method which can be compatible with atmospheric chemical ionization (APCI). However, the high polarity of ofloxacin determines its low sensitivity when using APCI mass spectrometry. Therefore, liquid chromatographic methods using ligand-exchange or enantioselective exchange are more preferable in ofloxacin enantiomers separation. For enantioselective LC, the wider peak limited its use for complicated matrix. Therefore, ligand-exchange LC with fluorescence (FL) detection was used in this study for its relatively high selectivity and sensitivity.

Existence of ofloxacin in sewage was frequently reported in past few years [27–31]. In these papers, solid-phase extraction (SPE) method based on various adsorbents including the HLB [27,28], C18 [29] and mixed phase cation–exchange phases [30,31] has been successfully used for the analysis of the trace levels of ofloxacin in sewage. More recently, Lee et al. [32] reported a selective SPE method using a weak cation–exchanger (WCX) cartridge to extraction fluoroquinones including ofloxacin, norfloxacin, and ciprofloxacin in municipal wastewater samples. However, the procedure is lack of usage upon FL detector due to both the less specification of detector and the presence of interference in the extract [32]. Therefore, to achieve the quantitation of ofloxacin enantiomers in sewage sample, an improved extraction and purification procedure is needed. In this paper, a comprehensive method based on a two-step SPE procedure and conventional ligand-exchange chromatography was developed for the analysis of ofloxacin enantiomers in sewage.

2. Experimental

2.1. Chemicals and reagents

Racemic mixtures (99%) containing (*S*)- and (*R*)-ofloxacin, pipemidic acid, lomefloxacin, ciprofloxacin, norfloxacin and L-isoleucine (99.5%) were obtained from Sigma–Aldrich (St. Louis, MO, USA). (*S*)-Ofloxacin, i.e. levofloxacin, was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All solvents used in sample preparation and chromatographic separation were HPLC grade. Methanol (MeOH) was supplied by Fisher Scientific (Fair Lawn, NJ, USA). Formic acid (HCOOH, 99%) was from Acros Organics (Morris Plains, NJ, USA). Ultra-pure-water (UPW) was made using a Milli-Q Ultra-pure System (Millipore, Bedford, MA, USA). Acetic acid (99%), ethylenediamine tetraacetic acid disodium salt ($Na_2EDTA{\cdot}H_2O$), anhydrous copper sulphate and ammonia (25–28% NH_3) were all from Beijing Chemical Co. (Beijing, China).

Stock standard solutions of racemic and (*S*)-ofloxacin were prepared by dissolving 10 mg powder, accurately weighed, in 10 mL methanol, obtaining a final concentration of 1000 μg/mL. These solutions were stored at −20 °C. Working solutions used for LC analysis and sample fortification were obtained by diluting the stock solutions with LC mobile phase and the initial mobile phase of mass detection, respectively.

2.2. Sampling and preparation

In April 2007 and September 2007, samples from two local sewage treatment plants (STPs), G and Q, were collected over a 24-h period for each sampling campaign. Both of the STPs include an anoxic–anaerobic process unit and a continuous aerobic activated sludge treatment, with the total hydraulic residence time of about 8 h. No rain event was registered either during the previous 10 days or on the sampling days. Composite raw sewage and final effluent samples were collected in 4-L brown glass bottles which were rinsed with water samples three times before final sampling was performed. The samples were stored at 4 °C in the dark for at most 2 days.

Analytes were extracted using 150-mg (6-mL) Oasis WCX cartridges (Waters, Milford, MA, USA) with a mixed-mode weak cation–exchange and reversed-phase sorbent resin, i.e. a copolymer of poly(divinylbenzene)-*co*-*N*-vinylpyrrolidone containing carboxylic acid groups. The cartridges were preconditioned with 6 mL of MeOH, 6 mL UPW and 6 mL 10 mmol/L Na_2EDTA solution (pH 3). After filtering through the GF/A glass fiber membrane (1.6 μm, Waterman, UN), sample aliquots (250 mL), to which 0.2 g Na_2EDTA was added, were adjusted to pH 3, and extracted through the WCX cartridges at a flow rate of ∼5 mL/min using a 24-position vacuum manifold (Waters, Milford, MA, USA). After extraction, the cartridge was rinsed with 2 mL × 6 mL of UPW and 2 mL of UPW–MeOH (90:10, v/v) solution containing 0.5% formic acid, and vacuum-dried for 2 min. Ofloxacin was subsequently eluted with 10 mL of UPW–MeOH (50:50, v/v) solution containing 0.2% formic acid. The SPE eluent was evaporated under a gentle stream of nitrogen at 40 °C to remove the MeOH. The residual solutions were adjusted to pH 9 and reconstituted to ∼50 mL for further purification using a 60-mg (3-mL) Oasis MAX SPE cartridge (Waters, Milford, MA, USA), mixed-mode anion–exchange and reversed-phase sorbent, i.e. a copolymer of poly(divinylbenzene)-*co*-*N*-vinylpyrrolidone containing quaternary ammonium salt groups. MAX cartridge was preconditioned with 3 mL of MeOH and 3 mL basified UPW (pH 9). Sample

aliquots mentioned above (~50 mL) were passed through the MAX cartridges at a flow rate of ~5 mL/min. After being rinsed with 2 mL UPW containing 5% ammonia and 2 mL MeOH and vacuum-dried for ~5 min, ofloxacin enantiomers were subsequently eluted with 4 mL of UPW–MeOH (20:80, v/v) solution containing 0.3% formic acid in a glass test tube. The SPE eluent was evaporated under a gentle stream of nitrogen at 40 °C to near dryness and reconstituted to 1.0 mL using LC mobile phase. To remove particulates, the extract was centrifuged at 10,000 rpm for 10 min, transferred to an autosampler vial, and stored at −4 °C until analysis. No filtration was done before injection because of the significant loss of analytes.

2.3. LC-FL method

Waters Alliance 2695 HPLC system coupled with a 2475 FL detector (Waters, Milford, MA, USA) was used for the analysis of target compounds. Data acquisition, data processing, and instrument control were performed using Microsoft Windows 2000 based Empower 3.2 software. Separation of ofloxacin enantiomers was performed on a TC-C-18 analytical column (250 mm × 4.6 mm, 5 μm; Agilent Technologies, CA, USA) with isocratic mobile phase at a flow rate of 1 mL/min for 35 min. The mobile phase was a mixture of MeOH–UPW containing 4 mmol/L $CuSO_4$ and 5 mmol/L L-isoleucine (12:88, v/v). The injection volume was 20 μL. The fluorescence detector was operated at $\lambda_{ex} = 303$ nm and $\lambda_{em} = 505$ nm. The column temperature was 40 °C.

Retention factor (k), selectivity (α), and resolution (R_s) were calculated using the following equations: $k = (t - t_0)/t_0$, where t and t_0 are the retention times of analyte and unretained solutes, respectively; $\alpha = k_R/k_S$, where k_S and k_R are the retention factors of (*S*)- and (*R*)-enantiomer, respectively; $R_s = 2(t_R - t_S)/(w_R + w_S)$, where t_S and t_R are retention times of the (*S*)- and (*R*)-enantiomers, respectively, and w_S and w_R are the baseline peak widths of the two enantiomers.

2.4. Validation of the quantitative method

The calibration curve was obtained by injecting, in triplicate, standard racemic ofloxacin solutions at eight concentration levels between 0.02 and 3.0 μg/mL (0.01–1.5 μg/mL for each enantiomer). The linearity acceptance criterion was met if the correlation coefficients (r^2) were higher than 0.99. The matrix-fortified calibration curve was obtained by spiking a series of concentrations of standard solution into the samples.

The accuracy and the precision were calculated by analyzing samples spiked at two concentration levels (1 and 2 μg/L) in replicate of six. The acceptance criteria for accuracy were that recoveries ranged between 70% and 110% and for precision that relative standard deviation (RSD) was lower than 20%. Because of the ubiquitous existence of ofloxacin in sewage, the limits of quantification (LOQ) and limit of detection (LOD) was calculated by determining signal-to-noise ratio of the lowest measured concentrations and extrapolating to S/N values of 10 and 3, respectively.

2.5. Confirmation study

In this paper, liquid chromatography–tandem mass spectrometry (LC–MS/MS) was applied to confirm the sum concentrations of two enantiomers obtained upon LC-FL and develop the sample-preparation protocol. Chromatographic separation was carried out on a Waters Acquity UPLC system (Waters, Milford, MA, USA) using an Acquity UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 μm particle size). The column oven temperature was 40 °C, the flow rate was 0.3 mL/min, and the injection volume was 10 μL. The mobile phase consisted of MeOH (A) and UPW containing 0.1% (v/v) formic acid (B). The initial composition was 80% A and 20% B. A gradient elution was performed where phase A was increased linearly to 10% in the first 4 min, then kept for 0.1 min and finally returned to the initial composition and equilibrated for 3 min before the next injection.

Mass spectrometric data acquisition was performed on a Micromass-Quattro Premier XE mass spectrometer (Waters, Manchester, UK) in positive ionization mode using multiple reaction monitoring (MRM). Two transitions (precursor > product), 362.6 > 261.3 and 362.6 > 318.4 were used for confirmation and quantification, respectively. Acquisition parameters were set following our recent article [33]. Calibration curves were generated using linear regression analysis within the concentration range of 0.2–100 μg/L and gave good fits ($r^2 > 0.99$). It was used for LC–MS/MS analysis because no significant effect was found for the clean-up procedure.

In view of the influence of the reconstituted solution (mobile phase of LC-FL) upon LC–MS/MS analysis, time-segment acquisition using a valve switch at the start of each run to divert $CuSO_4$ into waste before ofloxacin eluted from UPLC column.

3. Results and discussion

3.1. LC-FL methodology

In previous reports [20–22], ligand-exchange reversed-phase mobile phases consisting of MeOH or acetonitrile and water with amino acid and $CuSO_4$ as modifiers were often used to separate ofloxacin enantiomers. Therefore, three factors involved with the separation efficiency were considered: (i) the proper column, (ii) the composition of the mobile phase and the reconstituted extract, and (iii) column temperature.

First, a MeOH–UPW containing 10 mmol/L L-isoleucine and 5 mmol/L $CuSO_4$ (88:12, v/v) was used as the mobile phase. Enantioselective separation was performed on three conventional C18 analytical columns, which were TC-C-18 (250 mm × 4.6 mm, 5 μm, Agilent Technologies), TC-C-18 (100 mm × 4.6 mm, 5 μm, Agilent Technologies) and Xterra C-18 (250 mm × 4.6 mm, 5 μm, Waters). As a result, baseline separation of enantiomers was observed only on TC-C-18 with a length of 250 mm. Poor R_s upon the shorter TC-C-18 and Xterra C-18 might be attributed to the lower column efficiency and the somewhat difference of stationary phase, respectively. C18 columns with different brands have different selectivities for HPLC separation. It is reported that Xterra C-18 was devel-

Table 1
Effect of amino acid (5 mmol/L) on chromatographic selectivity and resolution of oflaxacin enantiomers

Amino acid	k_S	k_R	α	R_s
L-Lysine	4.79	4.79	1.00	0
L-Valine	4.98	5.54	1.12	1.13
L-Phenylalanine	8.52	9.11	1.07	0.76
L-Isoleucine	4.56	5.33	1.18	1.89

Table 2
Effect of isoleucine concentration in mobile phase (MeOH, 12%; $CuSO_4$, 5 mmol/L) on chromatographic selectivity and resolution of oflaxacin enantiomers

Concentration of isoleucine (mmol/L)	k_S	k_R	α	R_s
2	6.90	8.33	1.21	1.45
4	6.28	7.53	1.20	1.51
5	5.98	7.24	1.21	2.22
6	9.24	11.46	1.24	1.91
8	9.79	12.25	1.25	1.85
10	9.93	12.42	1.25	1.90

oped by hybrid particle technology, which might change the selectivity of ofloxacin enantiomers.

Then different amino acids including L-lysine, L-valine, L-phenylalanine, and L-isoleucine were used as additives at a concentration of 5 mmol/L for enantiomers separation, and the R_s and α were compared (Table 1). The results suggested that the mobile phase system using L-isoleucine as additive achieved the highest R_s (1.89), while no separation was found when using L-lysine ($R_s = 0$) or L-phenylalanine ($R_s = 0.76$) as additives, which was different from previous reports [34]. This difference might be due to the different selectivity of column. Therefore, in this study, L-isoleucine was used as additive.

Based on the selection of column and amino acid, the concentration of amino acid, $CuSO_4$, and the ratio between MeOH and aqueous phase were also optimized. The selectivity and resolution are summarized in Tables 2, 3 and 5. An assay of 12% of MeOH, 5 mmol/L L-isoleucine and 4 mmol/L $CuSO_4$ was found as optimal. Cu^{2+} and L-isoleucine in the mobile phase both components of the ligand complex of ofloxacin, thus, both affect the retention and R_s. The high concentrations of Cu^{2+} or L-isoleucine might cause the peaks to widen and the low level of Cu^{2+} or L-isoleucine might lead to incomplete complex,

Table 3
Effect of concentration of $CuSO_4$ in mobile phase (MeOH, 12%; isoleucine, 5 mmol/L) on chromatographic selectivity and resolution of oflaxacin enantiomers

Concentration of $CuSO_4$ (mmol/L)	k_S	k_R	α	R_s
2	7.34	8.91	1.21	1.49
4	7.56	9.21	1.22	1.61
6	5.68	6.84	1.20	1.32
8	6.06	7.37	1.22	1.44
10	5.03	6.04	1.20	1.28

Table 4
Effect of column temperature on chromatographic selectivity and resolution of oflaxacin enantiomers

Column temperature (°C)	k_S	k_R	α	R_s
25	8.44	10.32	1.22	1.04
30	8.03	9.82	1.22	1.23
35	6.78	8.23	1.21	1.89
40	5.75	6.92	1.20	2.38
45	4.9	5.83	1.19	2.31

Table 5
Effect of mobile phase composition (aqueous phase (5 mmol/L isoleucine and 4 mmol/L $CuSO_4$) and MeOH) on chromatographic selectivity and resolution of oflaxacin enantiomers

$V_{aqueous\ phase}$:V_{MeOH}	t_S	t_R	k_S	k_R	α	R_s
95:5	20.93	23.49	6.89	7.86	1.14	2.51
88:12	19.47	23.40	6.35	7.84	1.23	2.57
80:20	15.85	19.00	5.34	6.60	1.24	1.52
70:30	9.43	10.72	2.89	3.43	1.19	1.07
60:40	6.00	6.55	4.32	4.81	1.11	0.92

which resulted in the decreasing of R_s. The results of reconstituted extract optimization (Table 6) showed that R_s between (*R*)- and (*S*)-enantiomer almost remained constant when any ratio of MeOH–UPW containing 5 mmol/L L-isoleucine and 4 mmol/L $CuSO_4$ was used. Thus, the LC mobile phase was used as the reconstituted extract for consistency.

As for the effect of column temperature on the separation, five temperatures (25, 30, 35, 40 and 45 °C) were investigated (Table 4). The results present a similar tendency as that obtained in previous papers [20,35]: the retention factors (k_R and k_S) decreased significantly with increasing column temperature. However, the R_s increased from 1.03 to 2.38 when the temperature changed from 25 to 40 °C. Generally speaking, resistance to mass transfer is reduced at elevated temperature, which could help to increase the separation efficiency and thus improve resolution. Selectivity also changes at increased temperature.

3.2. Sample preparation

Ofloxacin (Fig. 1) contains a carboxyl and a tertiary nitrogen atom group (pK_a1 ~5.9, pK_a2 ~7.6); therefore, SPE cartridges (Oasis MCX and Oasis WCX) containing cation–exchange resins were first selected and compared. It is well known that the pH values are critical for sample preparation when ion exchange

Table 6
Effect of reconstituted solution on chromatographic selectivity and resolution of oflaxacin enantiomers

$V_{aqueous\ phase}$:V_{MeOH}	t_S	t_R	k_S	k_R	α	R_s
100:0	19.61	23.59	6.39	7.89	1.23	2.47
90:10	19.47	23.40	6.35	7.84	1.23	2.57
80:20	19.37	23.30	6.33	7.81	1.23	1.92
70:30	19.33	23.27	4.88	6.07	1.24	1.94
60:40	19.20	23.09	4.82	6.01	1.24	1.68

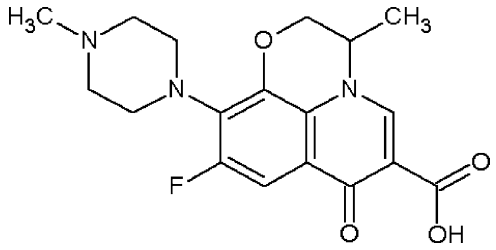

Fig. 1. Chemical structure of ofloxacin.

SPE cartridges are being used. Therefore, screening experiment was performed by loading aliquots of ofloxacin spiked tap water samples (40 ng/L) which was acidified to different pH (2–6) upon Oasis MCX and Oasis WCX, respectively. After sample loading, ofloxacin was directly eluted using MeOH containing 0.5% formic acid and 5% ammonia for WCX and MCX cartridges, respectively, according to the protocol provided by Waters. Fig. 2 depicts the extraction efficiency of samples at different pH values. Similar as reported by Lee et al. [32], recoveries of ofloxacin using Oasis WCX was higher than that using Oasis MCX at the same pH value. In addition, pH 3 was the optimal value for the two cation–exchange cartridges. For the WCX cartridge, the response decreased significantly when the pH was adjusted to 2. At this pH ofloxacin could only interact with stationary phase by reverse-phase mechanism, because the carboxylic acid moiety of the WCX stationary phase (pK_a 5.1) was fully protonated. Lower recovery may be due to the increased overall polarity of protonated ofloxacin at pH 2, which reduced the hydrophobic reverse-phase retention.

It was reported that quinolones have a tendency to form chelate complexes with metal ions through the ring carbonyl and one of the carboxylic oxygen atoms [36,37]. Therefore, ofloxacin could be present mainly in the form of complexes in sewage due to the existence of many metal ions. In order to reduce the chelate complexes, 0.2 g Na_2EDTA was added to sewage influent samples (taken from STP G, April 2007) before SPE and then treated as described in Section 2. These operations were carried out in triplicate. Control assays were conducted in parallel without Na_2EDTA addition. The results indicated the response area of ofloxacin with Na_2EDTA addition was 50% higher than that of the control.

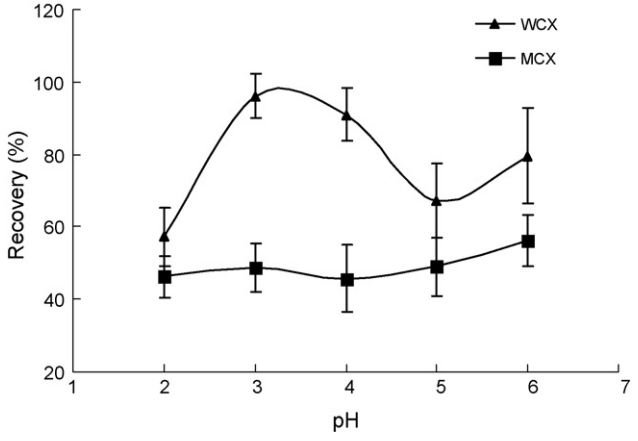

Fig. 2. Recoveries of ofloxacin in WCX and MCX cartridges at different pHs of loading solution (error bars indicate the standard deviations, $n = 3$).

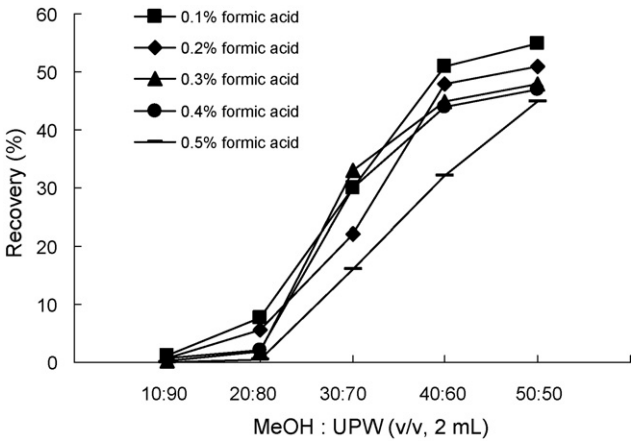

Fig. 3. Washing percentage of ofloxacin in WCX cartridge using different washing solutions.

Based on the above results, the washing solvent for the WCX cartridge was further optimized. Two milliliter of washing solvents containing different concentrations of formic acid and MeOH aqueous were investigated. The results (Fig. 3) suggested that no ofloxacin was eluted when 2 mL of MeOH–UPW (10:90) containing 0.1–0.5% formic acid was used. Considering its relatively high efficiency in impurity removal, 2 mL of MeOH–UPW (10:90, v/v) containing 0.5% formic acid was used as rinse solution. As presented in Fig. 3, whatever percentage of formic acid was present, approximately 50% ofloxacin was eluted using MeOH–UPW (50:50, v/v). Fig. 4 presents the recoveries of ofloxacin using 10 mL MeOH–UPW containing different percentages of formic acid. The results indicated that nearly 100% recovery was achieved using this volume of eluent containing 0.2% or higher formic acid. In this study, 10 mL MeOH–UPW (50:50, v/v) containing 0.2% formic acid was chosen as eluting solution.

For complicated sewage samples, extensive sample preparation is necessary to improve the sensitivity of the method, especially using conventional liquid chromatography coupled with fluorescence or UV detectors. Therefore, a mixed strong

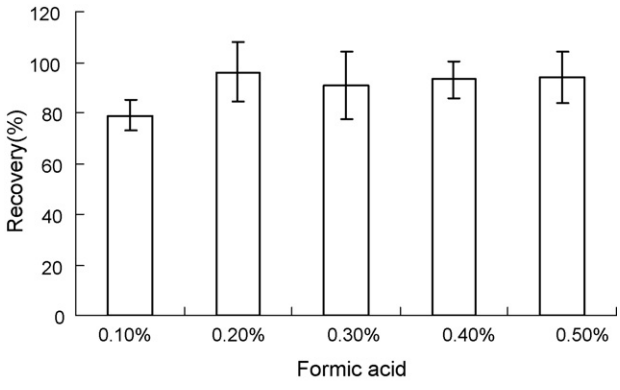

Fig. 4. Recoveries of ofloxacin in WCX cartridge using different concentrations of formic acid in MeOH–UPW (50/50, v/v, 10 mL) as eluting solution (error bars indicate the standard deviations, $n = 3$).

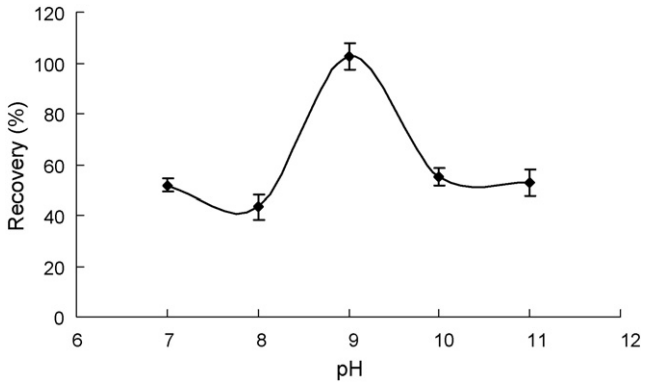

Fig. 5. Recoveries of ofloxacin in MAX cartridge for ofloxacin at different pHs of loading solution (error bars indicate the standard deviations, $n = 3$).

anion ion exchange was used to further purify the samples based on the carboxylic group of ofloxacin. Before sample loading, the pH of each sample was optimized, ranging from 7 to 11. The target analyte was eluted with 4 mL MeOH containing 0.3% formic acid. The recoveries of ofloxacin at different pHs are shown in Fig. 5. The results suggested that the highest recovery of ofloxacin was achieved at pH 9. At this pH the carboxylic acid group (pK_a ~6.0) in the ofloxacin molecule is in its unprotonated form and the tertiary amino group is in its protonated form (pK_a ~7.7). Therefore, the unprotonated carboxylic acid could interact with quaternary amine ions through the electrovalent bond and the protonated tertiary amino group could interact with resin through hydrogen bonding, resulting in higher extraction efficiencies. However, the reason why extract recoveries of ofloxacin decreased significantly from pH 9 to pH 10 is not clear. Based on these results, the washing solvent and eluting solvent were optimized. First, different volumes of MeOH ranging from 0.5 to 2 mL were used to remove impurities. The results indicated that no target analyte was eluted, even when 2 mL of MeOH was used. The elution efficiencies were investigated using 1 mL of MeOH–water solution with different ratios containing different percentages of formic acid. As shown in Fig. 6, more than 50% of analytes were eluted by 1 mL acidified MeOH–water (20:80, v/v); specifically, when MeOH–UPW (20:80, v/v) containing

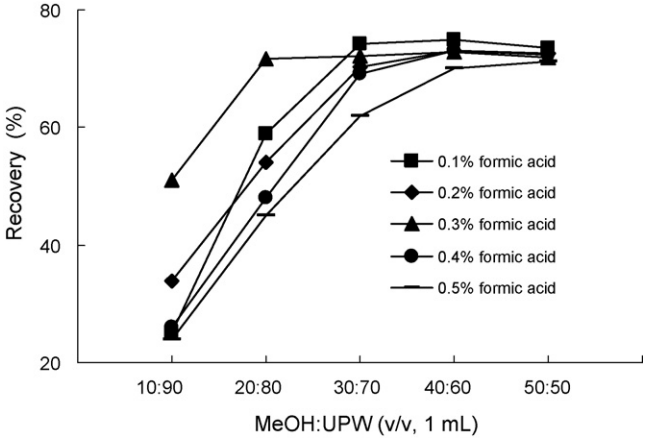

Fig. 6. Washing percentage of ofloxacin in MAX cartridge using different washing solutions.

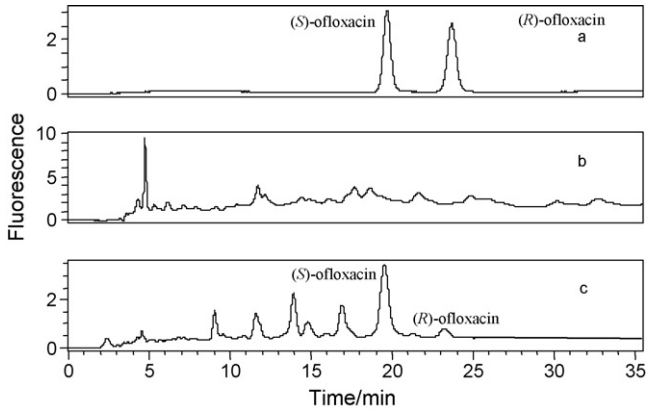

Fig. 7. Chromatograms of oflaxacin enantiomers in (a) standard sample, (b) the sewage influent using WCX SPE procedure, and (c) the sewage influent using WCX–MAX SPE procedure.

0.3% formic acid was used, 70% ofloxacin was eluted. Therefore, MeOH–water (20:80, v/v) containing 0.3% formic acid was used as eluting solvent and the eluting volume was further optimized as 4 mL.

Chromatograms of oflaxacin enantiomers in standard sample and the sewage influent after different SPE procedure were shown in Fig. 7. It can be seen from the figure that well-shaped peaks of oflaxacin enantiomers could be observed from *chromatogram c* (two-step SPE processed sewage sample), while no obvious peaks of the analytes was found from the *chromatogram b* (WCX-processed sample), which might be due to the significant signal suppression. These results indicated that the preparation procedure described above is selective and effective.

Pipemidic acid, lomefloxacin, ofloxacin, ciprofloxacin and norfloxacin are often used in China and are found in sewage and river water in Beijing. To rule out the interference of other quinolones, 0.1 μg of these chemicals were spiked into 250 mL ultra-pure water and prepared according to Section 2 in triplicate. Low recoveries (<20.0%) were achieved for most of the chemicals except for ofloxacin (95.6 ± 5.9%) and lomefloxacin (39.8 ± 4.6%), suggesting that the sample-preparation procedure had good specificity. As shown in Fig. 8, chromatographic separation for the mixture standards of pipemidic acid, lomefloxacin, ciprofloxacin, norfloxacin and oflaxacin enantiomers with acceptable R_s (1.41 for (*S*)-oflaxacin and norfloxacin, 0.68 for norfloxacin and (*R*)-oflaxacin) indicated that other quinolones could not interfere with the analysis of ofloxacin enantiomers.

3.3. Method validation of LC-FL

The linearity of the instrumental response was evaluated by directly injecting standard solutions of the targeted analytes at eight concentration levels (for each enantiomer): 0.010, 0.045, 0.135, 0.225, 0.450, 0.650, 1.000 and 1.500 mg/L. In this range, the analytes are expected to be detected in the final extracts with a sample enrichment factor of 250. The Pearson correlation coefficients (r^2) were typically 0.999. The instrumental detection

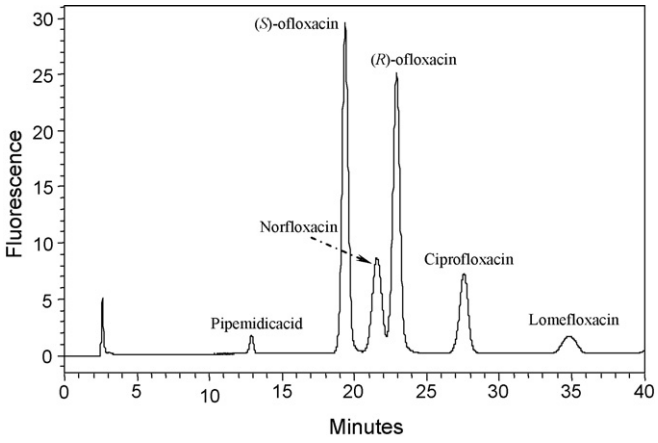

Fig. 8. Liquid chromatogram of mixture standards of pipemidic acid, lomefloxacin, ciprofloxacin, norfloxacin and oflaxacin enantiomers.

limits (IDL) were 10 pg for (*R*)- and (*S*)-ofloxacin calculated as three times the standard deviation of the response from 10 consecutive blank injections.

For method validation, matrix-fortified calibration curves was made based on different samples, including two tap water, two influent and effluent sewage samples, respectively, spiking at concentrations of 0.1, 0.5, 1, 2, 4, and 6 μg/L, with each batch conducted in replicates. The calibration curves were constructed by plotting the background-substracted response factor of ofloxacin enantiomers versus the spiking level. The correlation coefficients (r^2) for all the calibration curves throughout the whole procedure were typically greater than 0.98, indicating good linearity in the tested concentration range. Statistical comparison between the slopes of the standard calibration curves (six calibration curves, ranging from 0.01 to 1.5 μg/mL for each enantiomer) and the six matrix-fortified calibration curves (described above, considering the enrichment factor of 250) was processed by *t*-test using Excel software. This calculation drawn a *P* value of 0.35, which demonstrated that there was no significant difference occurred between all of the calibration curves and the matrix effect on fluorescence detection could be negligible. Therefore, the calibration curves of standard solution could be used for quantitation. For each enantiomer, the LOD and LOQ calculated as described in Section 2.4 were 0.03 μg/L and 0.08 μg/L, respectively.

The recovery of this procedure was evaluated by spiking 0.5 and 1 μg of racemic standard analytes to 250 mL influent samples at two levels in replicates of six, corresponding to 1 and 2 μg/L of enantiomers. As a result, the recoveries of (*R*) and (*S*)-ofloxacin were 101.1 ± 4.7% and 103.6 ± 5.3%, respectively, for the spiked level of 1 μg/L. They were 99.0 ± 10.1% and 97.4 ± 9.6%, respectively, for the spiked level of 2 μg/L.

3.4. Analysis of environmental samples

In order to confirm the utility of the LC-FL methodology for analysis of environmental samples, sewage samples taken from two STPs were determined. It can be seen from Table 7 that (*R*)- and (*S*)-ofloxacin were both found in the influent and effluent. Total concentrations of both enantiomers, with a range of 488–1067 ng/L, were consistent with that reported previously in STPs sewage [28,32]. Much higher concentrations of (*S*)-ofloxacin (393–910 ng/L) than (*R*)-ofloxacin (83–175 ng/L) occurred in all of the samples, which is accordance with their usage in medical treatment. LC–MS/MS analysis used for further confirmation presents a result similar as the sum concentrations of both enantiomers achieved by LC-FL.

Table 7
Concentrations of ofloxacin in sewage influent and final effluent

Sample	(*S*)-Ofloxacin (ng/L)[a]	(*R*)-Ofloxacin (ng/L)[a]	Sum of two enantiomers (ng/L)[b]	Ofloxacin (ng/L)[c]
April 2007				
G influent	892	175	1067	1092
G effluent	910	97	1007	986
Q influent	461	83	544	533
Q effluent	393	92	485	493
September 2007				
G influent	560	122	682	703
G effluent	597	101	698	672
Q influent	551	110	661	653
Q effluent	423	85	508	515

[a] Determined by LC-FL.
[b] Sum of (*S*)- and (*R*)-ofloxacin enantiomers.
[c] Determined by LC–MS/MS.

4. Conclusions

A sensitive and specific method was developed for trace analysis of ofloxacin enantiomers in sewage using two-step solid-phase extraction and ligand-exchange chromatography; the new method generated quantitation limits of 0.08 μg/L. This is the first time that the analysis of ofloxacin enantiomers in sewage has been reported. The developed method is expected to be applicable to investigate the environmental fate of ofloxacin enantiomers during different sewage treatment processes and in the environment.

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