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Determination of fluorinated quinolone antibacterials by ion chromatography with fluorescence detection*

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Abstract: For preparing fluorinated quinolone antibiotic medicine locally used in stomatology, simultaneous determination of norfloxacin, ciprofloxacin, and enoxacin was carried out by multiphase ion chromatography with fluorescence detection. Quinolone antibiotics were separated by Dionex OmniPac PAX-500 column with an eluent of 15 mmol/L H_2SO_4 and 35% methanol (v/v) at a flow-rate of 1.0 ml/min and detected with fluorescence with excitation and emission wave lengths of 347 nm and 420 nm respectively. The detection limits (*S*/*N*=3) of norfloxacin, ciprofloxacin and enoxacin were 50, 105 and 80 ng/ml respectively. The relative standard deviations of retention time, peak area and peak height were less than 1.1% and good linear relationship resulted. The developed method was applied to pharmaceutical formulations and biological fluids.

Key words: Fluorinated quinolone, Ion chromatography, Fluorescence detection
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INTRODUCTION

Most antimicrobial agents introduced for clinical use in recent years are nalidixic acid derivatives. Their common skeleton is termed 4-oxo-1,4-dihydroguionoline. These medicines (Fig.1) are, however, better known under their generic name, 4-quinolones. These compounds act directly on bacterial DNA by inhibiting topoisomerase which leads to cell death, so they are bactericidal (Appelbaum and Hunter, 2000; Bryskier and Chantot, 1995; Asahina *et al*., 1992; Morissey *et al*., 1996). These agents are widely used in clinical applications and in the treatment and prevention of oral ulcers and periodontitis with obvious effects (Yang *et al*., 1997; Duan *et al*., 2002). In order to prepare a new compound medicine, it is necessary to analyse fluorinated quinolone antibacterials.

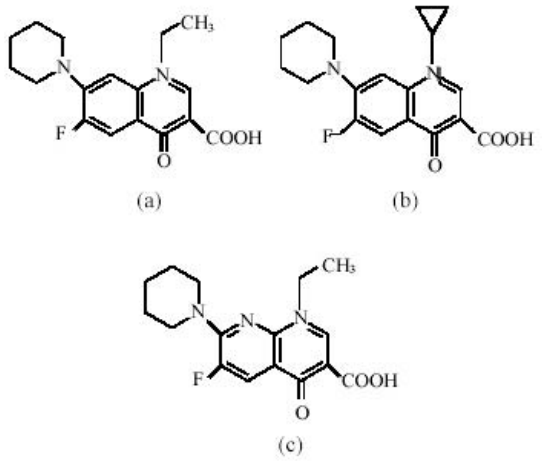

Fig.1 Structural formulas of the norfloxacin (a), ciprofloxacin (b) and enoxacin (c)

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The methods available for fluorinated quinolone antibacterials assays are thin-layer chromatography (TLC)-fluorescence (Simonovska *et al*., 1999; Novakovic *et al*., 2001; Riesbeck and Forsgren, 1994), capillary electrophoresis-UV or fluorescence (Faria *et al*., 2006; Awadallah *et al*., 2003; Schmitt-Kopplin *et al*., 1999), high-performance liquid chromatography (HPLC)-UV or fluorescence (Grellet *et al*., 2002; Carlucci, 1998; Gasparrini *et al*., 2001; Lee and Hong, 2000; Brodfuehrer *et al*., 1998). The majority of these methods were developed to analyse fluorinated quinolone antibacterials in biological samples such as serum or urine, residues in fish and meat or medicine tablets.

As most of the fluorinated quinolone antibacterials are quinolinecarboxylic acids, they can be separated by anion exchange chromatography and detected by fluorescence detection. In this paper, a novel analysis system to determine three fluorinated quinolone antibacterials simultaneously was developed by using ion chromatography with multi-phase ion chromatography and fluorescence detection.

EXPERIMENT

Chemicals and reagents

Methanol was HPLC-grade, and H_2SO_4 was analytical-reagent grade (purchased from Shanghai Chemical Reagents, Shanghai, China). Norfloxcin, ciprofloxacin and enoxacin were biochemical reagents (purchased from Shanghai Chemical Reagents, Shanghai, China).

Apparatus

The chromatographic system consisted of a Dionex 4000I ion chromatograph pump (Sunnyvale, CA, USA) equipped with Rheodyne-7125 injection valve (Rheodyne, Cotati, CA, USA) and Shimadzu RF-535 fluorescence detector (Shimadzu, Kyoto, Japan). Dionex OmniPac PAX-500 (250 mm×4.6 mm) column. Data plotting and analysis were done with Yingpu chromatographic data station (Yingpu, Hangzhou, Zhejiang, China) software installed on a PII computer.

Chromatographic conditions

The mobile phase consisted of a mixture of 35% methanol (v/v) and 15 mmol/L H_2SO_4. The eluent was carefully degassed and filtered prior to use at a flow-rate of 1 ml/min. The injection volume was 50 μl. Detection was performed at excitation wavelength of 347 nm and emission wavelength of 420 nm.

Standard solutions

A stock solution of fluorinated quinolone was prepared to 1 mg/ml with methanol. This solution was stored at 4 °C for no longer than two months.

Standard work solutions were prepared every day, and diluted with distilled water.

Procedure

Samples were dissolved in distilled water, adjusted into the concentration range of 1 to 20 μg/ml and filtered with 0.45 μm membrane filter and then injected directly.

RESULTS AND DISCUSSION

Optimization of the wavelength for fluorescence detection

The optimal wavelength for simultaneous detection of three fluorinated quinolone antibacterials was observed. As shown in Fig.2, it was found that the excitation wavelength of 347 nm and emission

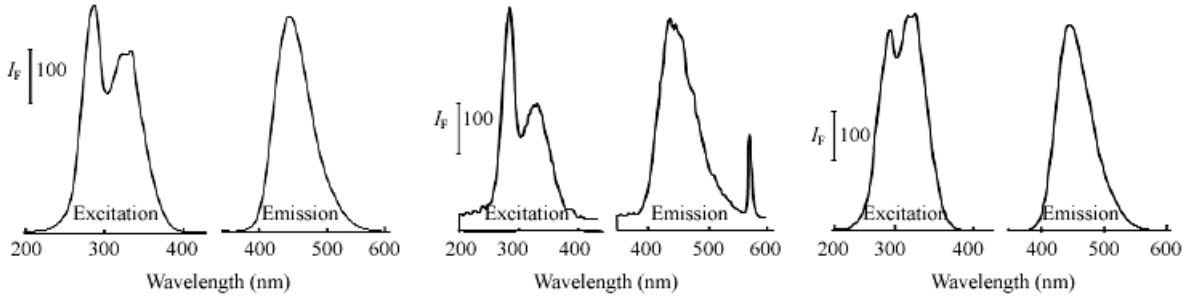

Fig.2 Excitation and emission in fluorescence of enoxacin (a), ciprofloxacin (b) and norfloxacin (c)

wavelength of 420 nm were the most suitable for detection of the three fluorinated quinolone antibacterials simultaneously.

Optimization of the separation

The separation of the fluorinated quinolone antibacterials was achieved using a multi-phase stationary phase which combines anion exchange and reversed-phase mechanisms with mixtures of sulfuric acid and methanol as eluent. The role of the sulfuric acid was to protonate the analytes and to also provide SO_4^{2-} as the eluent competing ion. The methanol was added to reduce hydrophobic absorption of the analytes and to decrease the retention times. With the elution of 0.05 mol/L H_2SO_4, the relationship of retention time and resolution with different concentrations of methanol is shown in Table 1 and Fig.3.

Table 1 Relationships between concentration of methanol and resolutions of the quinolone antibacterials

Concentration of CH_3OH (%)	Resolution	
	R_1*	R_2*
60	0.140	0.090
50	0.521	0.210
40	0.579	0.563
35	0.919	0.820
30	0.902	0.815

*R_1: The resolution between of norfloxacin and ciprofloxacin; R_2: The resolution between of ciprofloxacin and enoxacin

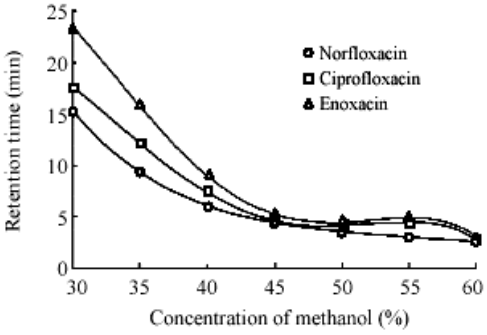

Fig.3 Changes of retention time of fluorinated quinolone antibacterials against the different concentration of methanol in elution with 0.05 mol/L of H_2SO_4

Judging from the experimental results, the retention time of the analyte was suitable and resolution was also good when the concentration of methanol was 35% (v/v), so eluent containing 35% (v/v) methanol was chosen in the experiments.

With the concentration of methanol set at 35% (v/v), the relationship of retention time and resolution with the different concentration of H_2SO_4 is shown in Table 2 and Fig.4.

Table 2 Relationships between concentration of sulfuric acids and resolutions of the quinolone antibacterials

Concentration of H_2SO_4 (mol/L)	Resolution	
	R_1*	R_2*
0.005	0.919	0.820
0.010	1.089	0.980
0.015	1.498	0.980
0.020	1.171	0.994
0.025	1.050	1.020

*R_1: The resolution between of norfloxacin and ciprofloxacin; R_2: The resolution between of ciprofloxacin and enoxacin

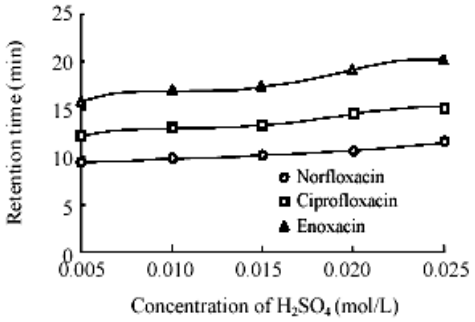

Fig.4 Changes of retention time of fluorinated quinolone antibacterials against the different concentration of H_2SO_4 in elution with 35% (v/v) methanol

The concentration of H_2SO_4 set at 0.015 mol/L was considered to be suitable for detection of the three fluorinated quinolones. In the above-mentioned experimental conditions, the resolution of each fluorinated quinolone was more than 1, and the retention time of the analyte was as expected appropriate.

So eluent containing 35% (v/v) methanol and 0.015 mol/L H_2SO_4 was chosen in the experiments for the simultaneous determination of the three fluorinated quinolone antibacterials.

Calibration, limit of detection

A calibration curve was described by the equation $A(H)=mx+b$, where $A(H)$ represents the response value of the analyte in the sample (peak area or peak height). The x in the equation represents the concentration of μg/ml of each fluorinated quinolone injected into the column. The mean correlation coefficient, intercept and slope values of all the three quinolones tested are indicated in Table 3. The chroma-

tographic method was demonstrated to be linear from 1 to 100 μg/ml of each fluorinated quinolone injected (r≥0.9995).

Table 3 Correlation values of fluorinated quinolones

Fuorinated quinolones	Calibration graph	Correlation coefficient
Norfloxacin	A=1.256×10^6x+4.113×10^5	0.9998
	H=1.655×10^4x+8.490×10^3	0.9993
Ciprofloxacin	A=8.528×10^6x+5.905×10^5	0.9996
	H=9.718×10^3x+4.527×10^3	0.9995
Enoxacin	A=1.177×10^6x+2.122×10^5	0.9998
	H=1.098×10^4x+3.369×10^3	0.9995

The lower detection limit for each fluorinated quinolone was determined by analysing calibration standards from 20 to 200 ng/ml of each fluorinated quinolone injected. The detection limits (S/N=3) of norfloxacin, ciprofloxacin and enoxacin were 50, 105 and 80 ng/ml, respectively.

Fig.5 shows a typical chromatogram of the standard stock solution containing norfloxacin, ciprofloxacin and enoxacin.

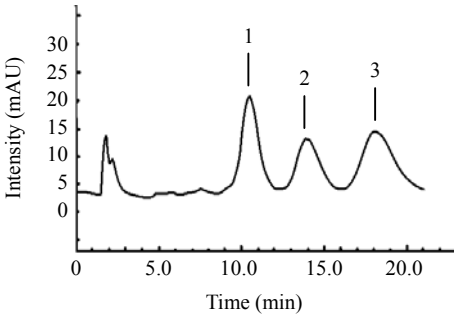

Fig.5 Typical chromatogram of the standard solution (5 μg/ml) in the optimal conditions

1. Norfloxacin; 2. Ciprofloxacin; 3. Enoxacin

Repeatability

Precision studies for 10 replicate injections of 5 μg/ml standard stock solution of the three fluorinated quinolones gave the percentage relative standard deviations of the three analytes in the range of 0.85%~1.0% for retention time, 0.73%~1.2% for peak area, and 0.63%~0.96% for peak height.

Application

The method described above was successfully applied for direct analysis of the mixed diluent solutions of norfloxacin and ciprofloxacin in pharmaceutical formulations. The recoveries of norfloxacin and ciprofloxacin were calculated by comparison of peak heights of the injected standards. The recoveries were in the range 100.1% to 103.5% when standard 0.42 μg/ml norfloxacin and 8.6 μg/ml ciprofloxacin were spiked into the sample for nine injections. Results are shown in Table 4. Fig.6 shows the chromatogram from the analysis of diluent solutions of pharmaceutical formulations.

Table 4 Percentage recoveries of two quinolones in pharmaceutical formulations

Diluent solutions in pharmaceutical formulations	Concentration (μg/ml)			Recovery (%)
	Analysed	Added	Total	
Norfloxacin in tablets	0.422	0.420	0.856	104
Ciprofloxacin in tablets	8.513	8.600	17.100	100

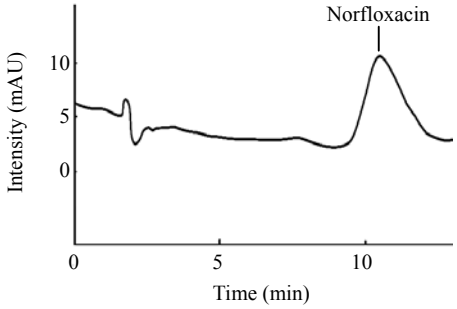

Fig.6 Chromatogram of dilute pharmaceutical formulation containing norfloxacin

The developed method was used for qualitative measurement of the fluorinated quinolones from human urine. As the half-life of ciprofloxacin was 3 to 4 h, measurements of the diluted human urine (1:100) of patient given oral dose of 0.25 g for 4 h were performed. Fig.7 shows the chromatogram from diluted human urine.

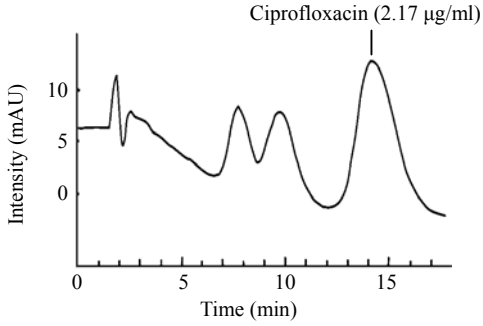

Fig.7 Chromatogram of dilute human urine (1:100) of patient with oral 0.25 g ciprofloxacin

CONCLUSION

A rapid and convenient procedure for separating and detecting the fluorinated quinolone antibacterials simultaneously has been developed and validated by ion chromatography-fluorescence detection. The method was stable and highly sensitive, so it may be applicable for monitoring fluorinated quinolone antibacterials in human body and biological tissues.

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