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Determination of fluoroquinolones in human urine by liquid chromatography coupled to pneumatically assisted electrospray ionization mass spectrometry☆

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Abstract

The fluoroquinolones are synthetic antimicrobial agents widely used in human and veterinary medicine. The aim of this work was to develop a method of characterization and determination of three widely used fluoroquinolones (norfloxacin, ciprofloxacin and ofloxacin) in human urine by liquid chromatography (LC) coupled to pneumatically assisted electrospray ionization (ESI) mass spectrometry (MS). For this purpose, the operational parameters of the electrospray interface were optimized in order to obtain the best signal stability and the highest sensitivity of the fluoroquinolones. The three fluoroquinolones studied and enrofloxacin, used as internal standard, were extracted from human urine samples by solid-phase extraction (SPE) and the previously established LC-UV method was successfully coupled with the MS system. The mass spectra obtained provide adequate information for identification purposes. Quality parameters were determined and satisfactory results were obtained. Likewise, the method detection limit was about 10 ng ml^{-1} for the three fluoroquinolones studied employing selected-ion monitoring mode.

Keywords: Fluoroquinolones

1. Introduction

The fluoroquinolones have emerged as one of the most important class of antibiotics and comprise a relatively large and constantly expanding group of substances. This group of synthetic drugs has shown excellent activity against both pathogenic gram-negative and gram-positive bacteria, as well as anaerobes. These compounds are widely used in human medicine for the treatment of pulmonary, urinary and digestive infections with good localized action on infected sites. The bactericidal action of fluoroquinolones results from interference with enzyme DNA gyrase that is needed for the synthesis of bacterial DNA [1–3]. Their common skeleton is 4-oxo-1,4-dihydroquinoline, where the pharmacophoric unit consists of a pyridone ring with a carboxyl group, a piperazinyl group and a fluorine atom placed at positions 3, 7 and 6, respectively.

Usually, liquid chromatography (LC) methods with UV and fluorescence detection have been published for the determination of fluoroquinolones in biological samples [4–12] but, to date, only some works use LC coupled with mass spectrometry for the confirmation and determination of fluoroquinolones in biological samples [12–13]. For confirmatory methods, which need a high degree of certainty in the identify, mass spectral analysis is the preferred technique for the confirmation of fluoroquinolone owing to its specificity and sensitivity [14]. The proportion of the organic modifier in the aqueous-organic mobile phase was previously optimized for separation and determination of fluoroquinolones by liquid chromatography with UV detection, using the linear solvation energy relationships (LSER) method, which is based on the single-solvent parameter E_T^N [15–18]. Also, the pH of acetonitrile–water mixtures and their correlation with the retention factor, k, were used to optimize mobile phase pH for the required separations [19–21]. Moreover, liquid chromatography coupled with electrospray ionization–mass

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spectrometry (LC–ESI–MS) combines high specificity, sensitivity and allows rapid and multiresidue determinations in complex matrices, together with structural information [13,22–23].

This paper describes a simple method for the identification, characterization and determination of three fluoroquinolones (norfloxacin, ofloxacin and ciprofloxacin) in human urine by LC–ESI–MS, using enrofloxacin as internal standard. The optimization of the different mass spectrometric operating parameters was performed in order to obtain the best signal and the highest sensitivity for the fluoroquinolone studied. Solid-phase extraction (SPE) was used to extract the antibiotics from human urine and the previously established LC-UV separation method was successfully coupled with the MS system. Finally, quality parameters were determined and satisfactory results were obtained.

2. Experimental

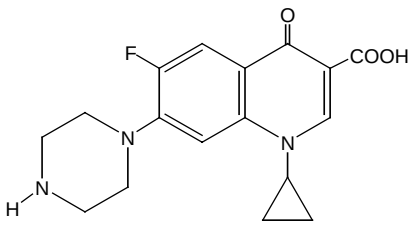
2.1. Chemicals and reagents

All reagents were of analytical grade. Water with a conductivity lower than 0.05 μS cm^{-1} and acetonitrile (Merck) were LC grade. Ammonium acetate, formic acid, potassium bromide, phosphoric acid and potassium hydrogen phthalate (dried at 110 °C before use) were analytical grade and obtained from Merck. The eluents were filtered through a 0.22 μm nylon filter membrane (MSI) and degassed by bubbling helium. The mobile phases used as eluents were acetonitrile–water, 20 mM ammonium acetate, adjusted the mobile phase pH with formic acid. The fluoroquinolones, Fig. 1, were obtained from different pharmaceutical firms: norfloxacin, NOR, (Liade, Boral Química); ofloxacin, OFL, (Hoescht); ciprofloxacin, CIP, (Lasa); and enrofloxacin, ENR, (Cenavisa). Hundred milligrams per liter individual stock solutions of each fluoroquinolone were prepared in acetonitrile–water mixtures (20:80 v/v), and suitable dilutions were made to obtain a mixture of the four fluoroquinolones. The samples were filtered through a 0.45 μm nylon filter membrane (MSI) before injection. All standards were protected from light and kept at 4 °C until they were used. MPC cartridges (3 ml) were manufactured by 3 M under the trademark Empore, and were purchased from Scharlau.

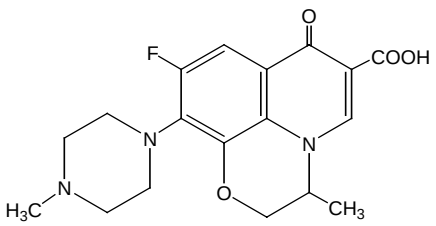
2.2. Apparatus

LC-UV measurements were performed using an ISCO model 2350 pump with an injection valve equipped with a 20 μl sample loop and a variable-wavelength V^4 absorbance detector (ISCO) operating at 280 nm. The chromatographic system was controlled by ChemResearch Chromatographic Data Management System Controller software (ISCO). A Kromasil C_8 column, 4.6 mm i.d. × 250 mm length (Teknokroma), was used at ambient temperature.

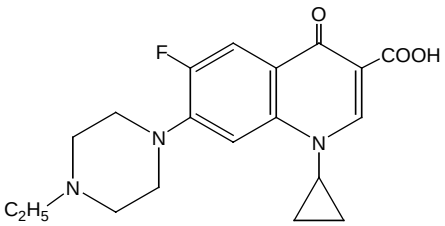
LC–ESI–MS measurements were performed using a Waters pump (Alliance), model 2690 automatic injector, coupled to a VG Platform II single quadrupole mass spectrometer (Micromass) equipped with a nebulizer-assisted



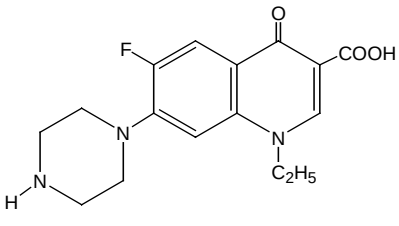
CIPROFLOXACIN
$C_{17}H_{18}FN_3O_3$
MW: 331.4



OFLOXACIN
$C_{18}H_{20}FN_3O_4$
MW: 361.4



ENROFLOXACIN (IS)
$C_{19}H_{22}FN_3O_3$
MW: 359.4



NORFLOXACIN
$C_{16}H_{18}FN_3O_3$
MW: 319.3

Fig. 1. Structures of the fluoroquinolones.

electrospray source. A Kromasil C_8 column, 4.6 mm i.d. × 250 mm length (Teknokroma), at room temperature, with an injection valve of 100 μl sample loop and with a post-column split 1/10 was used. Instrument control and data analysis were performed using MassLynx application software from Micromass.

The emf values used to evaluate the pH of the mobile phase were measured with a CRISON 2002 potentiometer (±0.1 mV) using an Orion 8102 ROSS combination pH electrode. The electrode was stabilized in the appropriate acetonitrile–water mixtures prior to the emf measurements, and the measurements were performed by triplicate to ensure stability and reproducibility of the potentiometric system. The potentiometric system was calibrated using a standard reference solution of potassium hydrogenphthalate 0.05 mol kg^{-1} whose reference pH values in the acetonitrile–water mixtures studied were previously assigned [24,25].

2.3. Experimental procedure

2.3.1. Optimization of the LC mobile phase

The concomitant effects of acetonitrile concentration and pH were previously studied for all solutes on the Kromasil column and are described in detail elsewhere [18]. Good chromatographic separation of the fluoroquinolones was achieved by the use of a mobile phase composition of 20:80 (v/v) acetonitrile–ammonium-acetate buffer, adjusted to pH 2.5 with formic acid.

2.3.2. Optimization of the operational parameters of the mass spectrometer

For the optimization of the ESI source and the analyzer parameters of the mass spectrometer, direct injection of a 10 mg l^{-1} solution of norfloxacin and ciprofloxacin was performed, working at a flow rate of 100 μl min^{-1} and using the data acquisition full scan mode (*m*/*z* 150–500). Source and analyzer parameters of ESI–MS system were optimized in order to obtain the best signal and the highest sensitivity for identification/quantification purposes of the fluoroquinolones. The positive ion ESI mode was selected for the fluoroquinolones because of improved sensitivity due to the presence of an amino group, which is easily protonated under the acidic mobile phase conditions (pH 2.5). The optimized parameters were cone voltage, source temperature, capillary voltage and counter electrode voltage. For the improvement of the system performance and the prevention of source contamination, higher temperatures were used, resulting in higher fragmentation of the molecules. In order to counteract this effect and to avoid an excessive fragmentation, cone voltage was adjusted. The fragment ions obtained can be monitored in the SIM mode to increase sensitivity in the detection. The best results are obtained when the operating conditions were: electrospray probe (capillary) voltage, 4.5 kV; counter electrode (HV lens) voltage, 0.5 kV; sample cone voltage, 60 V; source temperature, 150 °C. The spectrometer commonly works with a drying nitrogen gas flow of 300–400 l h^{-1} and a nebulizing nitrogen gas flow of 10–20 l h^{-1}.

2.3.3. Treatment of urine samples

Human urine samples were collected from healthy male and female volunteers. The samples were centrifuged for 10 min at 398 rad/s and filtered through a cellulose acetate filter (0.45 μm pore size, Millipore HA WP 04700). The filtrates were collected in glass containers, which had been carefully cleaned with hydrochloric acid and washed with deionized water and stored at 4 °C to prevent bacterial growth and proteolysis. Spiked urine samples were obtained by addition of different aliquots of 5 mg ml^{-1} fluoroquinolones solution to 2 ml of human urine, yielding a final concentration from 0.01 to 1.00 μg ml^{-1}. Moreover, 8 ml of water and 0.1 ml of H_3PO_4 were added, and adjusted at pH 3 with NaOH.

2.3.4. Solid-phase extraction procedure

Extraction cartridges (3M-Empore MPC) were placed on a vacuum elution manifold (Supelco) and rinsed with 2 ml of methanol followed by 2 ml of purified water. Care was taken that the cartridges did not run dry. Ten milliliters of the spiked urine samples was loaded onto the cartridges and drain by applying vacuum. The cartridges were then washed with 2 ml of water followed by vacuum suction for 1 min 3 ml of acetonitrile–TFA 2% (25:75 v/v) and 0.5 ml of acetonitrile were used to elute the adsorbed analytes, which were transferred into a vial and were evaporated at 50 °C under a stream of nitrogen. The residue was regenerated with 0.25 ml of mobile phase.

2.3.5. Quality parameters

The quality parameters were obtained using solutions prepared in drug-free human urine that was spiked before the extraction. The repeatability ($n = 5$) and the long-term reproducibility ($n = 15$) were determined by injecting the spiked solution at individual final concentrations of fluoroquinolones of 1 μg ml^{-1} in the optimized experimental conditions, five times in 1 day and for three different days respectively. The repeatability and long-term reproducibility of retention times and areas were calculated as the percentage of relative standard deviation (% RSD). Calibration curves were constructed using ratios of the observed analyte peak area to internal standard versus concentration of analyte. The linear range was established by injecting spiked solutions with individual quinolone concentrations ranging from 0.01 to 1.00 μg ml^{-1}. Linear regression analysis of the data gave slope, intercept and correlation coefficients, which were used to determine the concentration of each analyte in the quality control samples. The limits of detection (LODs) were calculated by using a signal to noise ratio of three (the ratio between the peak intensity and the noise intensity was used). Also, the limits of quantification (LOQs) were calculated by using a signal-to-noise ratio of ten.

Recoveries were obtained using solutions prepared in drug-free human urine and spiked with the internal standard before the extraction and with the three fluoroquinolones, NOR, CIP and OFL, at different concentrations, after the extraction. All samples were analyzed, and the ratio of NOR, CIP and OFL to internal standard for the set of samples was then compared with the ratio obtained for calibration curves.

3. Results and discussion

Many procedures have been described in the literature for the determination of quinolones using liquid chromatography [12] and most of them use C_{18}, C_8 or polymeric columns as stationary phase and acetonitrile as organic modifier in the mobile phase. The best results were obtained with a C_8 column [18]. Acetonitrile–water mixtures are suitable for chromatography of quinolones but good peak symmetry is only obtained if an organic competing base or an ammonium salt is added [12,16,26]. Moreover, the widely used phosphate buffers [12] are not compatible with mass spectrometric detection. For those reasons we chose in our studies a C_8 stationary phase and a mobile phase consisting of acetonitrile and a 0.020 mol l^{-1} ammonium acetate solution, while the pH was adjusted with formic acid.

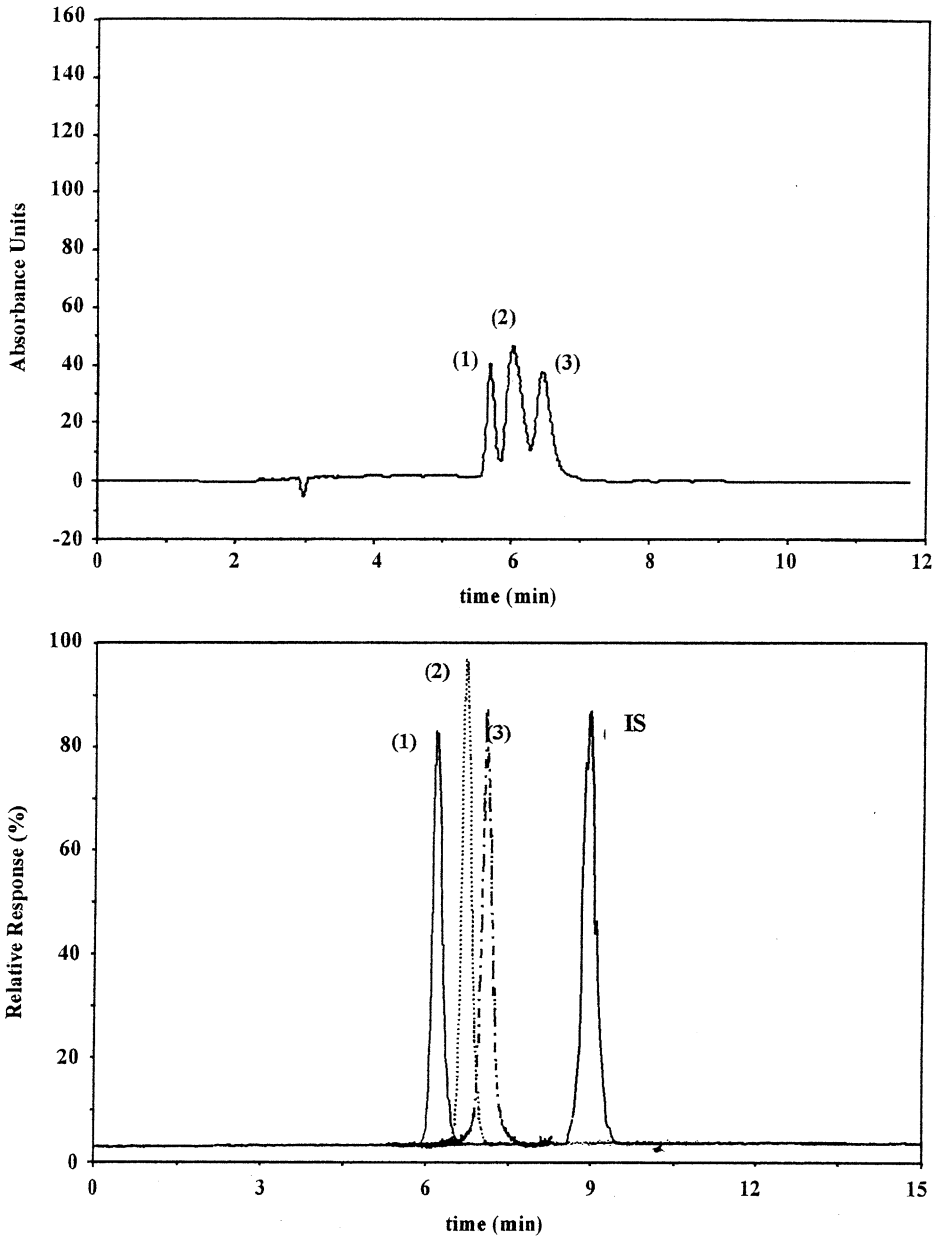


Fig. 2. LC-UV (A) and LC–ESI–MS (B) chromatograms of a solution containing 1 mg l^{-1} of norfloxacin (1); ofloxacin (2), ciprofloxacin (3). The (B) also contains enrofloxacin (IS).

Table 1
Fragments chosen for the SIM mode and retention times

Substances	Fragments (*m*/*z*)	t_r (min)
Norfloxacin	320, 302, 276, 233	6.20
Ofloxacin	362, 344, 318, 261	6.73
Ciprofloxacin	332, 314, 288, 245	7.11
Enrofloxacin	360, 342, 316, 245	8.96

From the relationships previously established [18] between quinolone retention and the solvent pH and composition, optimal mobile phase conditions of acetonitrile–0.02 M ammonium acetate 20:80 v/v, adjusted to pH = 2.5with formic acid were obtained in order to separate the three fluoroquinolones considered by LC-UV. The chromatogram obtained with the optimized mobile phase is show in Fig. 2A.

Total ion chromatogram (TIC) of the fluoroquinolones studied, obtained using this chromatographic system and the ESI optimized conditions, is shown in Fig. 2B. Similar separation of the fluoroquinolones with both detection systems was obtained. The order of elution was: norfloxacin, ofloxacin, ciprofloxacin, and enrofloxacin. The chromatographic retention times are given in Table 1.

Fig. 3 shows the full scan spectra associated with each fluoroquinolone obtained by injecting individually in the optimal conditions. ESI mass spectra of most of substances analyzed show a protonated molecular ion $[M + H]^+$. Two major fragmentation pathways were observed in the full scan spectra for fluoroquinolones (norfloxacin, ofloxacin, enrofloxacin, and ciprofloxacin): (a) Loss of H_2O from the carboxyl function at C-3, followed by loss of R_1 (as C_2H_4 or C_3H_4) from the ethyl or cyclopropyl groups at N-1. Only norfloxacin exhibits this last step (loss of C_2H_4). (b) Initial loss of CO_2 from the carboxyl group, followed by loss of C_2H_5N (norfloxacin and ciprofloxacin), C_3H_7N (ofloxacin) or C_4H_9N (enrofloxacin). These fragmentation pathways are shown for norfloxacin in Fig. 4 [13]. Table 2 summarizes the most relevant *m*/*z* values observed with the relative intensities of the peaks between parentheses and structural assignments of the fragments. The SIM mode was employed to achieve suitable sensitivity, and four ions were monitored to each fluoroquinolone (Table 1).

Solid-phase extraction was used to isolate the analytes from the urine samples. During development of the solid-phase extraction method, a series of different extraction cartridges were investigated [27]. These include silica-bonded phases such as Bond-Elut C_{18} and C_8 cartridges (Varian) and MPC-SD extraction disk cartridges (C_8 and benzenesulfonic groups, from 3M Empore). Several poly(styrene-divinylbenzene) copolymer sorbents had also been used, some of them including cation exchange groups: Oasis HLB (polymeric, from Waters) Oasis MCX (polymeric cationic, from Waters) and Isolute-ENV+ (polymeric, from IST). 3M Empore cartridges did not show any loss of analytes when loading spiked urine samples [27] and they provided the highest recoveries for all drugs analyzed in this study. The recovery values for fluoroquinolones studied with these cartridges were obtained following the methodology previously described [11] and are shown in the Table 3.

Injections of a standard mixture, an urine blank (full scan mode) and an urine spiked with the fluoroquinolones studied (SIM mode) in the LC–ESI–MS system at the optimal conditions give the chromatograms shown in Fig. 5.

Relative standard deviations (% RSD) based on peak area and retention time in the range from 4.9 to 7.5% were obtained when repeatability was calculated. Also, relative in the range from 9.5 to 13.0%, respectively, were obtained when testing the day-to-day reproducibility. Repeatability and reproducibility were obtained working in data acquisition mode of full scan. It has been reported by other authors that the reproducibility often exceeds 20%. Calculation of % RSD values using an internal standard give better results.

Table 2
Results obtained by LC–ESI–MS: mass-to-charge ratios (*m*/*z*) with relative intensities in parenthesis and structural assignments of the fragments

Substances	*m*/*z* (relative abundance)					
	$[M + H]^+$	$[M - H_2O + H]^+$ (A)	$[M - CO_2 + H]^+$ (B)	(B)–Pip	(A)–R_1	Other ions
Norfloxacin	320 (50)	302 (90)	276 (100)	233 (80)	274 (10)	300 (5); 256 (10); 219 (15); 205 (5)
Ofloxacin	362 (30)	344 (15)	318 (100)	261 (30)		
Ciprofloxacin	332 (45)	314 (55)	288 (100)	245 (30)		268 (10); 231 (5); 205 (5)
Enrofloxacin	360 (55)	342 (45)	316 (100)	245 (20)		

Table 3
Calibration curves, linear range, detection and quantification limits, and recovery of the solid-phase extraction

Parameter	Intercept	Slope ($l\,\mu g^{-1}$)	LR ($\mu g\,l^{-1}$)	LOD ($\mu g\,l^{-1}$)	LOQ ($\mu g\,l^{-1}$)	Recovery (%)
Norfloxacin	0.05 ± 0.01	1.56 ± 0.01	44–1000	13	44	46.0
Ofloxacin	0.05 ± 0.06	8.24 ± 0.13	71–1000	21	71	65.1
Ciprofloxacin	−0.01 ± 0.02	4.21 ± 0.04	58–1000	17	58	61.9

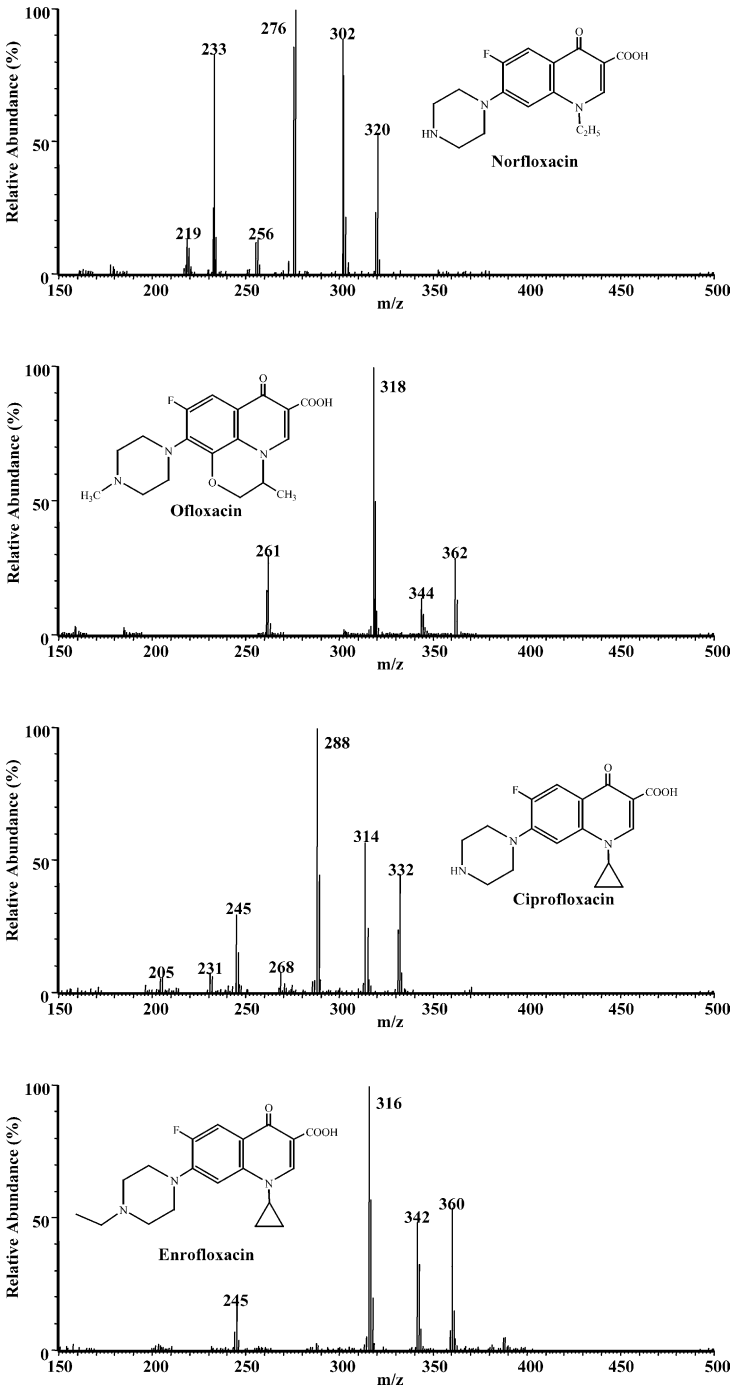


Fig. 3. Full scan spectra of the fluoroquinolones.

% RSD values based on peak area ratios were in the range from 1.6 to 3.4% for repeatability and 4.7 to 5.3% for reproducibility.

The applicability of LC–ESI–MS method for the determination of fluoroquinolones was verified using an internal standard for quantification. Enrofloxacin was used as internal standard for determination of norfloxacin, ofloxacin and ciprofloxacin. The quantification was performed by internal calibration in urine samples, plotting peak area ratios (area quinolone/area IS) versus quinolone concentration. Calibration graphs were obtained (seven points) for solutions between 0.01 and 1.00 $\mu g\,ml^{-1}$, under the same conditions previously described. The mode SIM was employed to achieve suitable sensitivity. Four ions were monitored for each fluoroquinolone (Table 1). The system was linear in all the cases with correlation factors of $r^2 > 0.99$. Detection and quantification limits were calculated as the concentration corresponding to signal of 3–10 times the standard deviation of the baseline, respectively. The detection limits obtained were 13 $\mu g\,l^{-1}$ for NFL, 21 $\mu g\,l^{-1}$ for

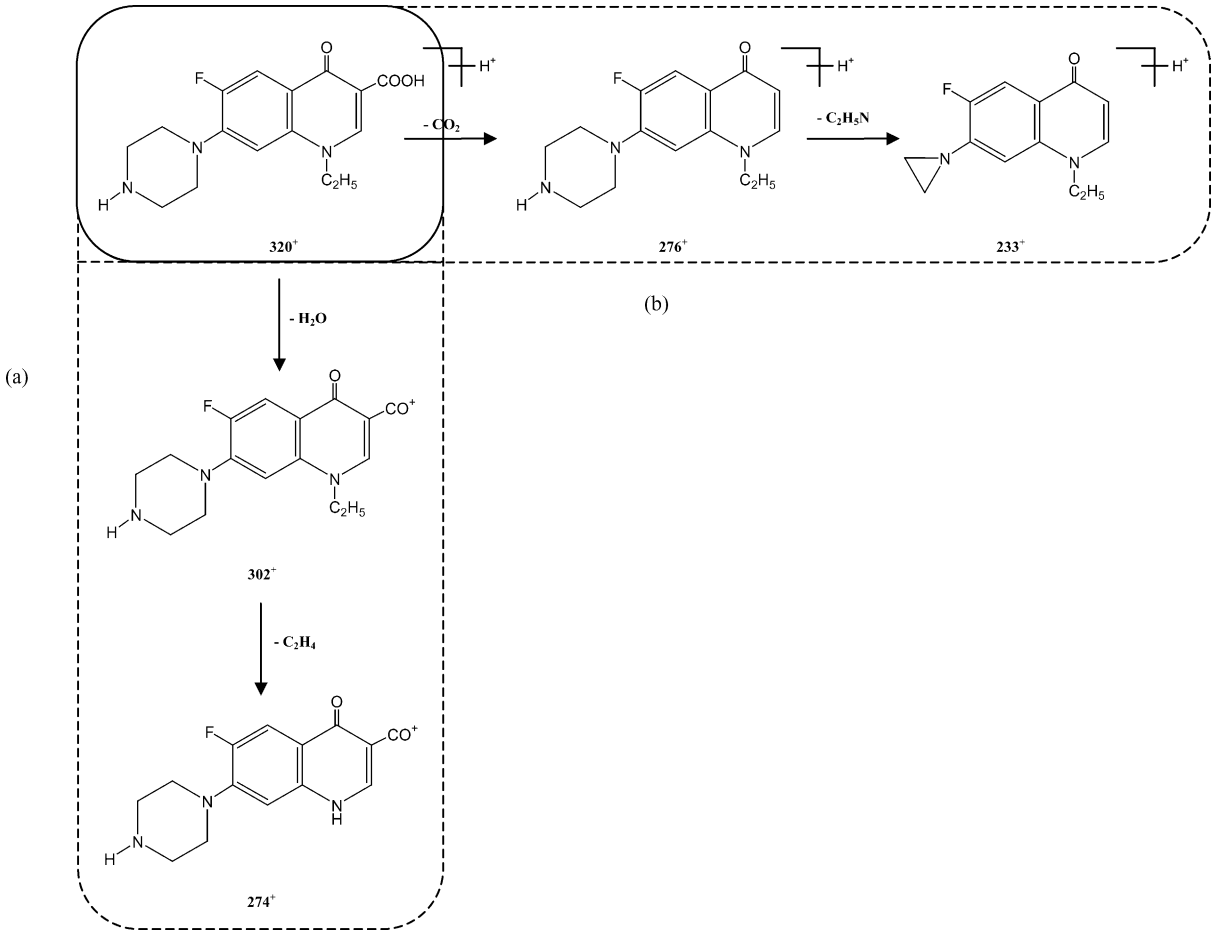


Fig. 4. Proposed fragmentation pathways of norfloxacin.

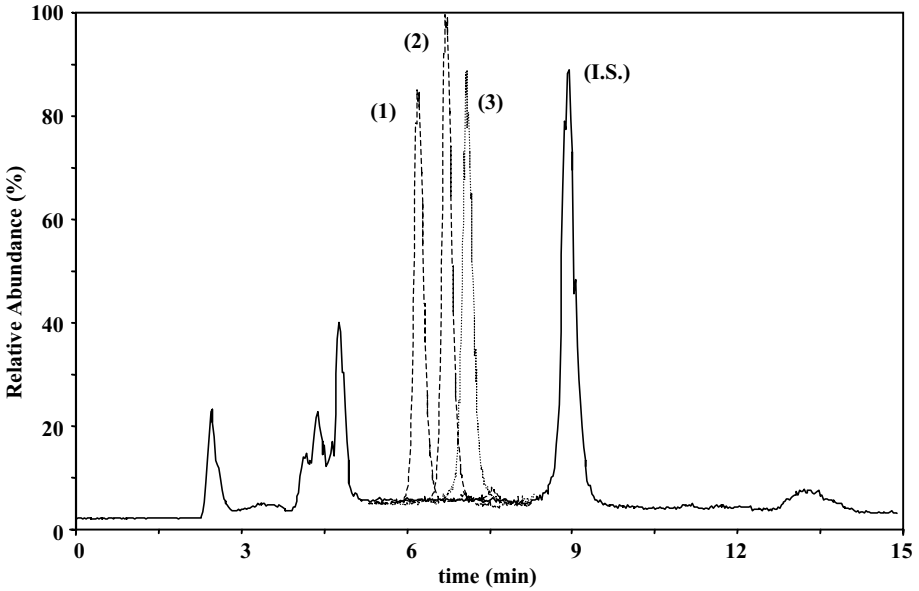


Fig. 5. ESI–MS chromatograms of spiked samples using a mobile phase of (20:80) (v/v) acetonitrile–water, that contains 20 mM ammonium-acetate buffer at pH 2.5, adjusted with formic acid. Solid line corresponds to a drug-free urine spiked only with IS and working in full scan mode. Dashed lines correspond to drug-free urine spiked with the studied quinolones, working in SIM mode: norfloxacin (1); ofloxacin (2); and ciprofloxacin (3).

OFL and 17 $\mu g\,l^{-1}$ for CIP and then the developed method is sensitive enough to permit routine analysis of quinolones in these biological samples. Quality parameters for each fluoroquinolone are summarized in Table 3.

4. Conclusions

Solid-phase extraction and LC–ESI–MS methods provide a fast, sensitive and selective procedure for the identification and simultaneous determination of a fluoroquinolone mixture in human urine samples. The mass spectra obtained provide adequate information for identification purposes that is difficult to obtain by other methods. The LC–MS proposed method uses a mobile phase acetonitrile–ammonium-acetate buffer adjusted at pH 2.5 with formic acid (20:80 v/v) using an isocratic elution. The operational parameters of the mass spectrometer were optimized in order to obtain more sensitivity. To validate the proposed method quality parameters were determined in order to know the possibility to apply the methodology the routine analysis of urine samples in future pharmacokinetic and clinical studied. Likewise, the method detection limit was about 20 $\mu g\,l^{-1}$ for most of the quinolones studied.

Acknowledgements

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