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ELECTROSPRAY MASS SPECTROMETRIC CHARACTERIZATION OF FLUOROQUINOLONE ANTIBIOTICS: NORFLOXACIN, ENOXACIN, CIPROFLOXACIN AND OFLOXACIN

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# Electrospray Mass Spectrometric Characterization of Fluoroquinolone Antibiotics: Norfloxacin, Enoxacin, Ciprofloxacin and Ofloxacin

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**We report the electrospray (ESI) mass spectra of four fluoroquinolones, enoxacin, norfloxacin, ofloxacin and ciprofloxacin, a group of highly potent antibiotics that have not been previously characterized by this ionization technique. At low sampling cone voltage settings, 50 and 100 V, the electrospray mass spectra contained primarily molecular adduct ions. At a sampling cone voltage setting of 150 V, collisionally activated dissociation mass spectra containing structurally significant product ions were produced and accurately mass measured at a magnetic sector resolution of 3000. Gradient separation and characterization of these compounds was demonstrated by analysis using packed capillary column liquid chromatography with UV and ESI-MS detection. UV detection limits in the 1 to 2 ng range ((signal-to-noise ratio S/N) = 10:1) were estimated and interpretable mass spectra with S/N ratios in excess of 100:1 were observed for 1.5 ng of analyte during electrospray mass spectrometric analysis.**

The quinolones (also called fluoroquinolones, 4-quinolones and quinolone carboxylic acids) represent a highly potent group of antibiotics, the first of which, nalidixic acid, was isolated during chloroquine synthesis in the 1960s.[1] A second-generation of more-effective quinolones, including norfloxacin, enoxacin, ciprofloxacin and ofloxacin, was discovered in the 1980s. Norfloxacin was the first clinically available fluoroquinolone, with ciprofloxacin and ofloxacin being approved for clinical use in 1987 and 1990, respectively[1]. These new fluoroquinolones, containing a fluorine substituent at the C6 position and a piperazinyl (or piperazine derivative) at the C7 position (Fig. 1), are more potent *in vitro*, broad spectrum antibiotics than the first-generation quinolones. The fluoroquinolones exhibit varying degrees of potency against enteric and non-enteric gram-negative bacilli and staphylococci, some activity against aerobes and streptococci and offer treatment against pathogens such as rickettsiae and mycobacteria .[1] Clinically, these fluoroquinolones are useful as therapeutic agents for a wide variety of serious infections including bone, joint, urinary and respiratory-tract infections, skin infections, prostatitis and some sexually transmitted diseases. The Canadian defence science community is particularly interested in the encapsulation of one of the most effective fluoroquinolones, ciprofloxacin, as targeted delivery of ciprofloxacin may provide a more effective means to reduce microbial infections.[2]

The spectrometric characterization of chemotherapeutic drugs and their metabolites is vital to the discovery and development of new pharmaceutical products. Mass spectrometry has contributed significantly in this role, particularly for applications which require on-line chromatographic separation and characterization. LC/thermospray-MS has been used to characterize quinolone sulfamates[3] and for the confirmation of nalidixic acid and other analogues in fish.[4] Both ciprofloxacin[5] and ofloxacin[6] have been characterized by electron impact ionization and their metabolites have been identified by fast-atom bombardment (FAB) mass spectrometry following LC fraction collection.[5,6] LC/FAB-MS was not employed in these metabolite studies, but has been recently demonstrated for the packed capillary column LC/FAB-MS analysis of a number of quinolones in human plasma.[7] Molecular adduct ions were obtained along with fragment ions, due to loss of $H_2O$ and $CO_2$, from the $[M+H]^+$ ion. Losses of both the neutral species $H_2O$ and $CO_2$ are commonly observed for many compounds and as such provide minimal structural data for the quinolones studied but no information on the substitution at the N1, C7 and C8 ring positions.

Most recently, chemical ionization (CI) mass spectra and tandem mass spectra were reported for several quinolones including norfloxacin, enoxacin and ciprofloxacin, in a combination of collisionally–activated dissociation (CAD) and ion/molecule reactions in a quadrupole ion trap.[8] The acquired mass spectra contained molecular adduct ions but structurally significant product ions were limited to loss of $H_2O$ and

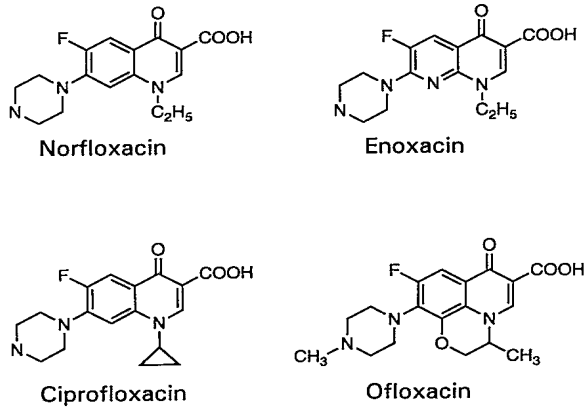


**Figure 1.** Fluoroquinolone structures.

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$(H_2O + C_2H_4 + C_2H_5N)$ from enoxacin, to loss of $H_2O$, $(H_2O + HF)$ and $(H_2O + 28 + C_2H_5N)$ from norfloxacin and to loss of $H_2O$ and $(H_2O + 2CO + C_2H_4)$ from ciprofloxacin.

The useful range of mass spectrometry for biomolecules increased dramatically with the demonstration by Fenn and co-workers that electrospray ionization (ESI), an atmospheric-pressure ionization technique, can be used to ionize large biomolecules.[9,10] In the ensuing years, the number of high-mass biomolecule applications increased dramatically, particularly with the commercial availability of chromatographic interfaces that allowed LC/ESI-MS analyses.[11–13] Early applications of this technique for low-molecular-weight environmental and pharmaceutical compounds have been described by Henion and co-workers,[14] but there are relatively few reports of the use of this new technique for the analysis of polar, low molecular weight compounds.[13]

The ESI mass spectra of $\beta$-lactam antibiotics, including penicillin G and ampicillin, were acquired by Voyksner and co-workers, and an LC/ESI-MS method was developed for $\beta$-lactam residue analysis.[15,16] However, no reports of the use of ESI-MS for the detection and identification of fluoroquinolones have been found in the literature. We report the first ESI-MS data for these important broad spectrum antibiotics and demonstrate on-line separation and characterization of them by packed capillary column LC/ESI-MS.

## EXPERIMENTAL

### Samples

A ciprofloxacin salt was provided by Bayer Leverkusen (Germany), and norfloxacin, enoxacin and ofloxacin were all purchased from Sigma Chemical Co. Ciprofloxacin standards were prepared in distilled water while the other three fluoroquinolones were initially dissolved at 0.5 mg/mL in distilled water containing 0.05% trifluoroacetic acid to enhance solubility. Further dilutions of all initial standards were made with distilled water and all samples were stored at 4 °C prior to use.

### Instrumental

All electrospray mass spectra were acquired using a VG (Fisons, Manchester, UK) Autospec-Q mass spectrometer equipped with the recently released VG (Fisons) Mark II electrospray interface. The electrospray needle was operated at 7.6 kV and ions were accelerated into the mass spectrometer at 4 kV. The effect of sampling cone voltage was investigated by varying this parameter from 50 to 150 V. Nitrogen (Very Dry, Liquid Carbonic Inc.) bath gas was introduced into the interface (80 °C) at a flow rate of 500 L/hr. Nitrogen nebulizer gas was introduced at a flow rate of 14 L/h. The electrospray interface was pumped with both a rotary and a turbomolecular pump, which enabled maintenance of a $3 \times 10^{-6}$ Torr and $4 \times 10^{-8}$ Torr within the source and analyser regions of the instrument, respectively.

Electrospray data were acquired in the continuum mode by scanning the magnet from $m/z$ 500 to 100 or $m/z$ 370 to 310 exponentially at a scan rate of 15 or 25 s/decade, respectively. Five to ten scans were typically averaged to enhance the signal-to-noise ratio and the data were smoothed using VG (Fisons) OPUS software, with a smooth-number of 2 and a window of 5. A resolution of 3000 (10% valley definition) was employed during $m/z$ 500 to 100 scans to facilitate accurate mass measurement of the molecular adduct and product ions formed during ESI-MS characterization. A resolution of 1200 was employed during LC-UV/ESI-MS analysis of standards and tablet extracts. External calibrations were performed with a solution of polyethylene glycol 200 (Aldrich) in distilled water.

LC separations were performed with an Applied Biosystems Model 140B dual syringe pump, with a Model 759A variable wavelength UV detector (210 nm) located prior to the ESI interface. Injections (5 µL) were made onto a 15 cm × 0.53 mm i.d. $C_{18}$ (5 µm) packed, J&W DB-1 coated fused–silica capillary column (NRC, Saskatoon, Canada). Water was distilled in glass and filtered through a Millipore 0.45 µm filter prior to use. Acetonitrile (UV grade) was obtained from Burdick and Jackson and HPLC grade trifluoroacetic acid (TFA) was purchased from Pierce. The following solvent compositions were prepared for LC analyses: Solvent A (0.005% TFA in water) and Solvent B (0.05% TFA in acetonitrile + water (80:20)). Gradient separations were obtained by prepressurizing at 20% B and programming from 20% B to 60% B at a rate of 0.25% B/min.[17] Loop injections of 0.1 mg/mL standards, for accurate mass analysis, were made under isocratic conditions with 30% B. Both isocratic and gradient separations were made with a flow rate of 20 µL/min.

Two Rheodyne 8125 sample injectors were placed in series so that the LC column was located after the first injector and prior to the second. The principal advantage of this LC configuration was that it allowed the analyst the flexibility of sample introduction either through the first injector and column or direct loop injection of standards or calibrants.

## RESULTS AND DISCUSSION

Electrospray ionization is a gentle ionization technique that can provide molecular ion information, in the form of $[M + nH]^{n+}$ adduct ions for higher mass compounds, such as peptides and proteins. Lower molecular weight polar compounds typically produce $[M+H]^+$ ions and/or molecular adduct ions depending on the mobile phase. For example, the presence of $[M + H + \text{methanol}]^+$ adduct ions was found to increase with increasing methanol strength during $\beta$-lactam antibiotic LC/ESI-MS analyses.[15]

Molecular weight information, while crucial for identification, is insufficient for confirmation of a target compound and on its own would offer limited insight during identification of unknowns or metabolites. Additional structurally significant product ions would increase certainty during target compound detection and would be beneficial during structural elucidation of unknowns. Product-ion production may be enhanced by increasing the likelihood of CAD in the electrospray interface prior to mass analysis. Experimentally this is done by increasing the sampling cone voltage, or potential between the sampling cone and the skimmer plate. Typically, at low sampling-cone voltages, 50 V, only

molecular-ion information would be obtained, while at higher voltages, 150 V, the residence time increases giving ample opportunity for collision or near-collision with neutrals in the ESI interface. The resultant CAD mass spectra generally contain a number of structurally significant product ions resulting from loss of neutral species from the molecular adduct ions. This approach has been used by Straub and Voykner for the characterization of another group of antibiotics, the $\beta$-lactams, where significant product-ion information was accessed by increasing the voltage from 80 to 160 V.[15]

The fluoroquinolone antibiotics, norfloxacin, enoxacin, ciprofloxacin and ofloxacin, have not yet been characterized by ESI-MS. Atmospheric pressure ionization, including ESI-MS, has become the method of choice for a wide variety of LC/MS applications and characterization of these antibiotics would have application in the pharmaceutical community. The present study was initiated with the primary aims being: the electrospray mass spectrometric characterization of the fluoroquinolones by ESI-MS under a number of different cone voltage settings and, demonstration of packed capillary column LC separation of these compounds.

The four fluoroquinolones (Fig. 1) are structurally similar with differences due to:

(i) the nature of the R-group attached at the N1 ring position;
(ii) the nature of the piperazinyl, or piperazine derivative at the C7 ring position and;
(iii) the presence of either a nitrogen or carbon atom at ring position 8.

Table 1 lists the ESI-MS data obtained at 3000 resolution (10% valley definition) during loop injection of each of the four fluoroquinolones at sampling cone voltages of 50, 100 and 150 V. A number of possible ion structures and pathways were postulated based on this data. The theoretical masses of postulated product ions

**Table 1. Electrospray mass spectra obtained at three sampling cone voltages (150, 100 and 50 volts) and a sector resolution of 3000 (10% valley)**

| Possible ion structure | Observed mass (u)[a] | Theoretical mass (u) | Difference (u) | % Relative intensity 150 V | 100 V | 50 V |
|---|---|---|---|---|---|---|
| (*a*) *Norfloxacin* | | | | | | |
| $[M+CH_3CNH]^+$ | 361.1712 | 361.1676 | +0.0036 | 3 | 8 | 11 |
| $[M+H]^+$ | 320.1427 | 320.1410 | +0.0017 | 100 | 100 | 100 |
| $[M+H-HF]^+$ | 300.1394 | 300.1348 | +0.0046 | 3 | | |
| $[M+H-C_2H_4]^+$ | 292.1180 | 292.1097 | +0.0083 | 2 | | |
| $[M+H-CO_2]^+$ | 276.1542 | 276.1512 | +0.0030 | 38 | 5 | |
| $[M+H-CO_2-HF]^+$ | 256.1490 | 256.1450 | +0.0040 | 3 | | |
| $[M+H-C_2H_4-C_2H_5N]^+$ | 249.0680 | 249.0675 | +0.0005 | 3 | | |
| $[M+H-CO_2-C_2H_5N]^+$ | 233.1093 | 233.1090 | +0.0003 | 43 | | |
| $[M+H-C_2H_4-C_2H_5N-H_2O]^+$ | 231.0653 | 231.0570 | +0.0083 | 11 | | |
| $[M+H-CO_2-C_3H_7N]^+$ | 219.0945 | 219.0934 | +0.0011 | 11 | | |
| $[M+H-CO_2-C_2H_5N-C_2H_4]^+$ | 205.0804 | 205.0777 | +0.0027 | 16 | | |
| (*b*) *Enoxacin* | | | | | | |
| $[M+CH_3CNH]^+$ | 362.1617 | 362.1628 | −0.0011 | 3 | 10 | 11 |
| $[M+H]^+$ | 321.1354 | 321.1363 | −0.0009 | 100 | 100 | 100 |
| $[M+H-HF]^+$ | 301.1298 | 301.1301 | −0.0003 | 1 | | |
| $[M+H-C_2H_4]^+$ | 293.1052 | 293.1050 | +0.0002 | 5 | | |
| $[M+H-CO_2]^+$ | 277.1478 | 277.1465 | +0.0013 | 18 | 4 | |
| $[M+H-CO_2-HF]^+$ | 257.1368 | 257.1402 | −0.0034 | 4 | | |
| $[M+H-C_2H_4-C_2H_5N]^+$ | 250.0631 | 250.0628 | +0.0003 | 14 | | |
| $[M+H-CO_2-C_2H_5N]^+$ | 234.1056 | 234.1043 | +0.0013 | 51 | | |
| $[M+H-C_2H_4-C_2H_5N-H_2O]^+$ | 232.0620 | 232.0522 | +0.0098 | 2 | | |
| $[M+H-CO_2-C_3H_7N]^+$ | 220.0875 | 220.0886 | −0.0011 | 2 | | |
| $[M+H-CO_2-C_2H_5N-C_2H_4]^+$ | 206.0751 | 206.0730 | +0.0021 | 20 | | |
| (*c*) *Ciprofloxacin* | | | | | | |
| $[M+CH_3CNH]^+$ | 373.1656 | 373.1676 | −0.0020 | 2 | 5 | 7 |
| $[M+H]^+$ | 332.1432 | 332.1410 | +0.0022 | 100 | 100 | 100 |
| $[M+H-HF]^+$ | 312.1371 | 312.1348 | +0.0023 | 2 | | |
| $[M+H-CO_2]^+$ | 288.1503 | 288.1512 | −0.0009 | 30 | 4 | |
| $[M+H-CO_2-HF]^+$ | 268.1422 | 268.1450 | −0.0028 | 2 | | |
| $[M+H-C_3H_4-C_2H_5N]^+$ | 249.0684 | 249.0675 | +0.0009 | 10 | | |
| $[M+H-CO_2-C_2H_5N]^+$ | 245.1037 | 245.1090 | −0.0053 | 25 | | |
| $[M+H-C_3H_4-C_2H_5N-H_2O]^+$ | 231.0618 | 231.0570 | +0.0052 | 19 | | |
| $[M+H-CO_2-C_2H_5N-C_3H_4]^+$ | 205.0760 | 205.0777 | −0.0017 | 2 | | |
| (*d*) *Ofloxacin* | | | | | | |
| $[M+CH_3CNH]^+$ | 403.1796 | 403.1782 | +0.0014 | 1 | 3 | 2 |
| $[M+H]^+$ | 362.1545 | 362.1516 | +0.0029 | 50 | 100 | 100 |
| $[M+H-CO_2]^+$ | 318.1665 | 318.1618 | +0.0047 | 100 | 7 | |
| $[M+H-CO_2-C_3H_7N]^+$ | 261.1057 | 261.1039 | +0.0018 | 90 | | |
| $[M+H-CO_2-C_4H_9N]^+$ | 247.0895 | 247.0883 | +0.0012 | 4 | | |
| $[M+H-CO_2-C_3H_7N-C_3H_4O]^+$ | 205.0763 | 205.0777 | −0.0014 | 2 | | |

[a] Data obtained during loop injection of a 100 ng/μL standard with a sampling cone voltage of 150 V.

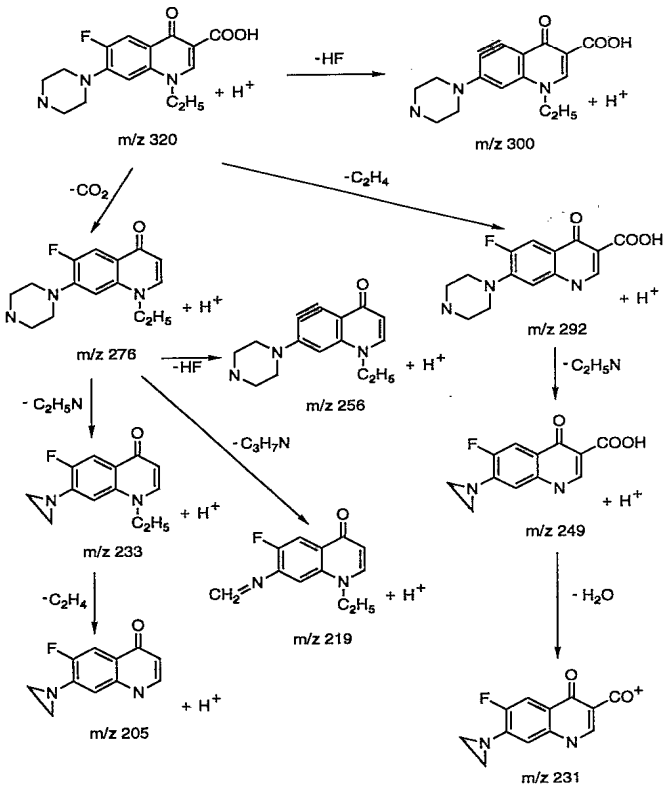


**Figure 2.** Possible structures and pathways for product ions observed during ESI-MS analysis of norfloxacin. (Sector resolution: 3000 with a 10% valley; sampling cone voltage: 150 V).

were calculated and compared to the observed data. Errors between observed and theoretical masses were consistently low, with errors typically $0.003 \pm 0.002$ u. At a sampling cone voltage of 50 V the ESI mass spectra exhibit only molecular ion adducts due to attachment of a proton or protonated acetonitrile. Minor product ion formation, $[M+H-CO_2]^+$, was observed by increasing the sampling cone voltage to 100 V. Structurally significant product ions, the result of CAD, were observed for all four fluoroquinolones at a sampling cone voltage of 150 V.

Norfloxacin and enoxacin are two similar compounds differing only at position ring 8, with norfloxacin containing a carbon atom and enoxacin a nitrogen atom. Product ions (sampling cone voltage 150 V) due to loss of the same neutral species were observed since the quinolone ring remained intact. The major product ion pathway for both compounds involved loss of $CO_2$ from the carboxylic acid substituent at C3 followed by loss of $C_2H_5N$ from the piperazinyl group and $C_2H_4$ from the ethyl R-group at N1. An alternative pathway involved an initial loss of $C_2H_4$ from the R-group at N1, followed by loss of $C_2H_5N$ from the piperazinyl group and $H_2O$ from the carboxylic acid substituent. Weak product ions, due to loss of HF from $[M+H]^+$ and $[M+H-CO_2]^+$ ions, were also observed along with loss of $C_3H_7N$ from $[M+H-CO_2]^+$ ions for each compound. Figure 2 illustrates possible product-ion structures, masses and pathways observed during CAD-MS analysis of norfloxacin.

Ciprofloxacin was similar to other fluoroquinolones, the principal product-ion pathway involving loss of $CO_2$ for the $[M+H]^+$ ion, followed by sequential losses of $C_2H_5N$ from the piperazinyl group at C7 and of $C_2H_4$ from the cyclopropyl R-group at N1. An alternative pathway involved sequential losses of $C_5H_9N$ and $H_2O$ from the $[M+H]^+$ ion. Product ions due to loss of HF were weak. Most importantly, evidence for differences in R-groups between ciprofloxacin (cyclopropyl) and norfloxacin (ethyl) was obtained. A neutral loss of $C_3H_4$ (R-group less hydrogen) was observed for ciprofloxacin, while neutral loss of $C_2H_4$ (R-group less hydrogen) was evident in the CAD mass spectrum of norfloxacin.

The CAD mass spectrum obtained for ofloxacin contained fewer product ions, probably due to the extra ring in its structure due to an R-group bridging the N1 to C8 positions. The principal product-ion pathway involved loss of $CO_2$ from the $[M+H]^+$ ion, followed by sequential neutral losses of $C_3H_7N$ from the methylated piperazinyl group at C7 and of $C_3H_4O$ from the R-group. Product ions due to loss of HF were not observed. An additional product ion, due to neutral loss of $C_4H_9N$ from the $[M+H-CO_2]^+$ ion, and characteristic of the methylated piperazinyl substituent, was also detected.

In general, the CAD-MS data obtained following electrospray sample introduction were richer in structurally significant product ions than CI-MS/MS[8] and FAB-MS[7] and were similar to those obtained under electron impact ionization.[5,6] FAB-MS fragmentation ions were limited to loss of $H_2O$ and $CO_2$, while the CI-MS/MS data, obtained with a Finnigan (San Jose, CA, USA) ion-trap mass spectrometer (ITMS), illustrate typical tandem mass spectrometric results for this relatively gentle MS/MS technique. The CI-MS/MS conditions employed represent optimum multiple collision conditions with a low molecular weight target gas, helium.[8] Fewer product ions result, because the experimental conditions favour the pathway with the lowest activation energy barrier as opposed to multiple higher energy routes. The electron impact ionization mass spectra were obtained following batch introduction from a heated solids probe, a technique that requires LC fraction collection for mixture analysis. Clearly an on-line method, such as LC/ESI-MS, would be beneficial for the characterization of the fluoroquinolones and their metabolites in more complex samples.

Prior LC-UV/ESI-MS analyses were conducted with microbore 1 mm i.d. LC columns that operate most efficiently with flow rates in the 50 to 100 μL/min range. Flow rates of 50 μL/min, used during LC-UV/ESI-MS analysis, resulted in the acquisition of ESI mass spectra with good molecular ion information. However with increasing sampling cone voltage, the characteristic product ions generated during loop injection at flow rates of 10 to 20 μL/min, were not evident.[18] Inefficient desolvation was suspected. Neither increasing the bath gas temperature to the maximum (100 °C) for the interface or increasing the sampling cone voltage remedied this situation. A packed capillary column (0.53 mm i.d.) was therefore investigated for LC-UV/ESI-MS applications, because columns of this internal diameter should provide efficient separation in the 10 to 20 μL/min range. At these flow rates ESI-MS data containing primarily molecular adduct ion information (sampling cone voltages of 50 to 100 V) or molecular adduct and product ions (sampling cone voltages of 150 V) were possible during LC/ESI-MS analyses.

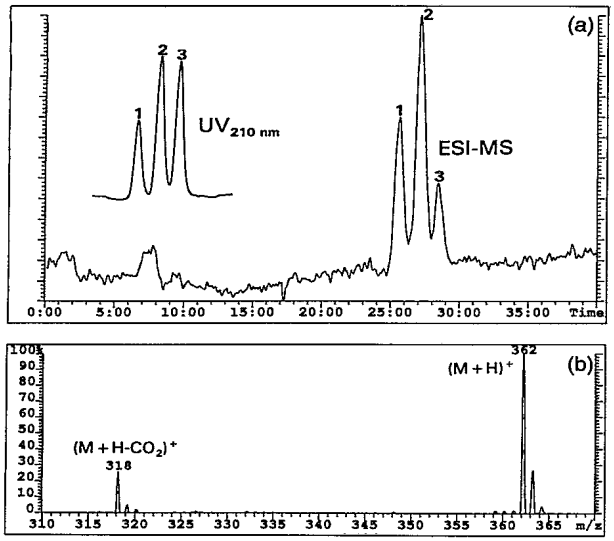


**Figure 3.** LC/ESI-MS total-ion-current ($m/z$ 370 to 310) chromatogram obtained during packed capillary column LC-UV/MS analysis of (a) a standard containing enoxacin, 17 ng (peak 1) + ofloxacin 17 ng (peak 2) + ciprofloxacin, 14 ng (peak 3). (Sector resolution: 1200 with 10% valley; sampling cone voltage: 100 V). LC-UV chromatogram is inset. (b) Electrospray mass spectrum obtained for ofloxacin.

Figure 3 illustrates a typical packed capillary column LC-UV/ESI-MS separation of enoxacin, ofloxacin and ciprofloxacin. All components were readily detected at the 14–17 ng level, with only a slight loss in chromatographic resolution due to the dead volume between the UV cell and ESI interface. UV detection limits for enoxacin, ofloxacin and ciprofloxacin were estimated to be 2.2 ng, 1.3 ng and 1.1 ng (S/N = 10:1), respectively. All three compounds were detected in the total-ion-current ($m/z$ 370 to 310) chromatogram with S/N ratios in the 5:1 to 15:1 range. More importantly, the acquired ESI-MS mass spectra, after background subtraction, were essentially free of interference. S/N ratios of greater than 100:1, based on the somewhat arbitrary definition of an interpretable mass spectrum, were estimated during acquisition of a single mass spectrometric scan (corresponding to about 1.5 ng of analyte).

Loop injections were performed with 0.1 mg/mL standards to ensure accurate mass measurement of ions with relative intensities as low as 1%, while LC analyses of the fluoroquinolones were conducted with more dilute standards (0.03 and 0.003 mg/mL). The acquired data were similar, a difference being noted in the relative intensities of the protonated acetonitrile molecular ion adduct ions. Relative intensities approaching the intensity of the protonated molecules were observed at lower concentration for enoxacin, norfloxacin and ciprofloxacin. A dependence on concentration was suspected, since the mobile-phase compositions were similar for both analyses.[15]

## CONCLUSIONS

The fluoroquinolones, including enoxacin, norfloxacin, ofloxacin and ciprofloxacin, represent a group of highly potent antibiotics that have not been previously characterized by electrospray mass spectrometry. We report the electrospray mass spectra for these antibiotics, making use of three different sampling cone voltage settings. At low sampling cone voltage settings, 50 and 100 V, the electrospray mass spectra contained primarily molecular adduct ions. At a higher sampling cone voltage setting, 150 V, informative collisionally–activated dissociation mass spectra, containing structurally significant product ions were produced and accurately mass measured at a magnetic sector resolution of 3000.

On-line gradient separation and characterization of these compounds was demonstrated by packed capillary column LC-UV/ESI-MS analysis. UV detection limits in the 1–2 ng range (S/N = 10:1) were estimated and interpretable mass spectra with S/N ratios in excess of 100:1 were observed for 1.5 ng of analyte during electrospray mass spectrometric analysis.

The electrospray data reported are sufficient for the detection and identification of these compounds in samples of interest to the pharmaceutical community. The structurally informative nature of the collisionally–activated dissociation mass spectra, along with the ease of chromatographic interfacing, make this technique particularly valuable for fluoroquinolone identification and should aid in the structural elucidation of metabolites or related unknowns.

## Acknowledgements

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