Electrospray Tandem Quadrupole Fragmentation of Quinolone Drugs and Related Ions. On the Reversibility of Water Loss from Protonated Ions

Pedatsur Neta, Bhaskar Godugu, Yuxue Liang, Yamil Simón-Manso, Xiaoyu Yang, and Stephen E. Stein

Chemical and Biochemical Reference Data Division, National Institute of Standards and Technology, Gaithersburg, Maryland 20899

Abstract

Selected reaction monitoring (SRM) of quinolone drugs showed[1] different sensitivities in aqueous solution *vs.* biological extract. The authors suggested formation of two singly protonated ions with different behavior, one undergoing loss of H_2O and the other loss of CO_2, so that SRM transitions might depend on the ratios of these forms generated by the electrospray. These surprising results prompted us to reexamine several quinolone drugs and some simpler compounds to further elucidate the mechanisms. We find that the relative contributions of loss of H_2O *vs.* loss of CO_2 in MS/MS experiments depend not only on molecular structure and collision energy but, in certain cases, on the cone voltage as well. We further find that many fragment ions formed by loss of H_2O can reattach a water molecule in the collision cell, whereas ions formed by loss of CO_2 do not. Since reattachment of H_2O can occur after water loss in the cone region and prior to selection of the precursor ion, this effect leads to the dependence of MS/MS spectra on the cone voltage used in creating the precursor ion, which explains the formerly observed effect on SRM ratios. Our results support the earlier conclusion that varying amounts of two ions of the same m/z are responsible for problems in the analysis of these drugs, but the origin is in dehydration/rehydration reactions. Thus, SRM's for certain complex compounds may be comparable only when monitored under equivalent ion-forming conditions, including the voltage used in the production of the protonated ions in the ESI source.

Introduction

Recent results raised questions about the validity of selected reaction monitoring (SRM) for confirming the presence of quinolone drugs in biological extracts.[1] The SRM ratios found in aqueous solutions and

in biological extracts containing difloxacin were different for some peaks. The authors explained this finding by the formation of two distinct singly protonated ions with different behavior and different stabilities at different energies, one protonated at the aliphatic amine site of the piperazine ring and one protonated at the carboxyl group.
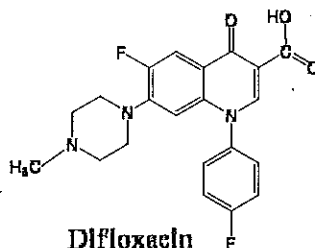
Difloxacin

This representation of difloxacin is not accurate since such compounds are known to exist in neutral solutions mainly as zwitterions.[2-6] In alkaline solutions the carboxyl group is ionized to form the anion and in acidic solutions the methylated amine group of the piperazine ring is protonated to give the cation. There is no indication of protonation at the other nitrogens.[2-6] The likelihood of protonating the carboxyl group and not the amine group in acidic solutions is negligible, since the pK_a values in solution generally are much higher for amines, while protonation of a neutral carboxyl group generally has pK_a values < 0. Is it possible then that during evaporation of the electrospray droplets this molecule can attach a proton at any of these sites and form distinct ions, which survive in the gas phase long enough to be detected in the mass spectrometer? Clearly, proton affinity in the gas phase does not follow the same trends as in aqueous solutions. The proton affinity on the surface of the droplet just before evaporation is likely to be different from either of the extreme forms and thus it is difficult to predict the relative contributions of the various protonated ions formed in the electrospray process. The authors[1] further suggested that one of these ions undergoes loss of CO_2 while the other undergoes loss of H_2O, and that the latter one is more stable at higher cone voltage while the former one fragments. To gain further insights into these important findings we decided to examine difloxacin and other quinolone drugs as well as simple model compounds which can shed light on the mechanism.

Experimental[1]


The chemicals were obtained from Sigma-Aldrich in the purest grade available. Solutions were made in 1:1 (v:v) methanol:water solvent containing 0.1 % formic acid, unless otherwise specified. The solutions were infused with a syringe into an electrspray source and the MS and MS/MS spectra were recorded with a triple quadrupole instrument (Micromass, Waters Corp., Milford, Massachusetts). To determine the sequence of fragmentation, tandem mass spectra were recorded at 20 collision voltages and fractional peak intensities were plotted as a function of collision voltage. Several experiments were also carried out using a linear ion trap instrument (LTQ, Thermo Electron Corp., Waltham, Massachusetts), where tandem mass spectra were collected at normalized collision energy settings between 0 % and 35% of the maximum energy available. Further details of the experimental conditions and data processing were as before.[7]

Results and Discussion

The mass spectrum of difloxacin in methanol:water:formic acid (50:50:0.1) shows a peak at m/z 400 of the protonated ion with maximum intensity at a cone voltage of 36 V (CV36). Increasing the cone voltage leads to in-source fragmentation, and the remaining signal at m/z 400 decreases at cone voltage of 80 V (CV80) to 10 % of the maximum. The MS/MS spectra of protonated difloxacin were recorded at 20 collision voltages, from 2 V, where no fragmentation is apparent, to 40 V, where the precursor peak has completely disappeared. Selected spectra at four of these collision voltages (Figure 1a) show the decrease in the precursor ion (m/z 400) and the formation of several ions with different voltage dependence. The main ions formed at low collision voltage are those due to loss of H_2O (m/z 382) and loss of CO_2 (m/z 356). The relative intensities of these two peaks change with collision voltage because the rates of their formation and subsequent fragmentation are different. In most cases, MS/MS spectra are expected to be independent of the cone voltage applied during formation of the precursor ion. Indeed, spectra taken at cone voltage between CV10 and CV40 are very similar. However, further increase in CV leads to a decrease in the MS^1 peak intensity and to gradual change in the MS^2 spectrum toward that CV80 (Figure 1b), where the peaks at m/z 356 and 299 have disappeared. These results are in agreement with previous findings[1] and indicate that the precursor ion produced at low CV undergoes loss of H_2O and loss of CO_2 but that fraction of the precursor ion still remaining at high CV undergoes loss of H_2O but not loss of CO_2. These results are in line with the arguments advanced by Kaufmann et al.[1] that there are two types of precursor ions and that the ion which loses CO_2 disappears at high CV.
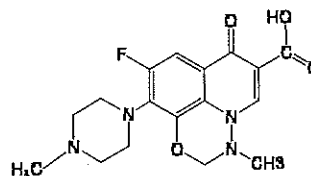
Similar results were obtained with other quinolone antibiotics: norfloxacin and enrofloxacin.
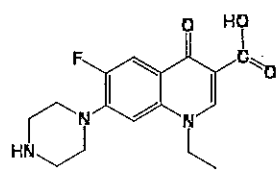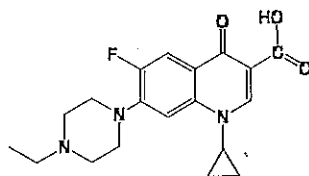
**Norfloxacin**   **Enrofloxacin**   **Marbofloxacin**

Further demonstration of these effects is seen in the collision voltage dependence of the various fragment peaks taken at different cone voltage (see Figure 2 for norfloxacin). At CV30 the H_2O loss peak (m/z 302) and the CO_2 loss peak (m/z 276) are formed in parallel, reach similar maximum intensities, but decrease at different rates. The other peaks in the figure are the subsequent fragmentation products. At CV80, however, the H_2O loss peak reaches much higher relative intensities while the CO_2 loss peak remains very small throughout the range. Marbofloxacin also showed similar behavior except that loss of CO_2 is replaced with a preferred loss of a 43 Da species (most likely $CH_3-N=CH_2$).

If indeed there are two distinct protonated ions of these drugs, one that loses H_2O and one that loses CO_2, and both processes occur approximately in parallel, i.e. both precursor ions undergo fragmentation with similar rates, how is it possible that at high CV only one of the precursor ions remains? The answer is found in MS^3 experiments on the fragment ions. The fragment ions formed by loss of H_2O and by loss of CO_2 were produced in the cone region by raising the cone voltage until the peaks reach maximum intensity. These ions were then selected into the collision cell and their CID spectra were recorded at 20 different collision energies. Representative results for norfloxacin are shown in Figure 3. The $[MH^+ - CO_2]$ ion (m/z 276) fragments to $[MH^+ - CO_2 - HF]$ (m/z 256), $[MH^+ - CO_2 - C_2H_5N]$ (m/z 233), $[MH^+ - CO_2 - C_3H_7N]$ (m/z 219), and $[MH^+ - CO_2 - C_4H_9N]$ (m/z 205). The $[MH^+ - H_2O]$ ion (m/z 302) fragments to $[MH^+ - H_2O - HF]$ (m/z 282), $[MH^+ - H_2O - CO]$ (m/z 274), $[MH^+ - H_2O - HF - CO]$ (m/z 254), and $[MH^+ - H_2O - CO - C_2H_5N]$ (m/z 231). However, in addition to these fragments, the $[MH^+ - H_2O]$ ion also adds a water molecule in the collision cell to form the original MH^+ ion (m/z 320). This process is most pronounced at the lowest collision energies while at higher energies the MH^+ ion undergoes fragmentation. No such addition is observed for the $[MH^+ - CO_2]$ ion. Addition of a water molecule in the collision cell has been reported for other compounds[8] and is explained by the presence of small amounts of water in the collision cell which is difficult to remove by standard pumping. Furthermore, if norfloxacin is dissolved in methanol with no added water, MS^3 experiments on the $[MH^+ - H_2O]$ ion (m/z 302) show addition of H_2O (m/z 320) as well as addition of CH_3OH (m/z 334). Thus, loss of CO_2 is an irreversible process whereas loss of H_2O is reversible under our experimental conditions; and these processes can take place in the collision cell as well as in the cone

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region (in-source). As a result, increasing the cone voltage leads to in-source fragmentation of all types of MH$^+$ ions but only those that decompose by losing water are partly recovered by gaining a water molecule. This differentiation is reasonable since loss of CO_2 leads to replacement of the CO_2H group with H, forming a relatively stable species, but loss of H_2O leads to formation of an acyl species, which has high affinity for water (or alcohols).

These results were further confirmed by experiments with norfloxacin and difloxacin in a linear ion trap mass spectrometer (LTQ). MS/MS spectra showed essentially the same fragment ions as in the triple quadrupole instrument discussed above. MS^3 spectra were recorded for the [MH$^+$ - H_2O] ions using collision energy settings between 0 % and 30 % of the maximum. At low energy settings, a peak for the MH$^+$ ion, clearly formed from the [MH$^+$ - H_2O] ion, was observed for both quinolones. The intensity of the MH$^+$ product at 0 % energy setting was 10 times smaller than that of the precursor [MH$^+$ - H_2O] ion. At higher collision energies the relative intensity of the MH$^+$ product was smaller and at 30 % or higher settings it was not possible to monitor this rehydration process because the [MH$^+$ - H_2O] ion peak was very small due to further fragmentation. In fact, in such cases it was possible to observe rehydration of [MH$^+$ - H_2O - HF] in MS^4 experiments. Similar MS^3 experiments on the [MH$^+$ - CO_2] ion showed no addition reactions.

It is reasonable to assume that loss of H_2O from the carboxyl group results from addition of a proton to that site, suggesting that loss of CO_2 occurs when the proton is added away from this site, possibly at the most basic piperazine nitrogen. To test this assumption we examined three related compounds lacking the piperazine ring:

Flumequine   Nalidixic acid   HTQC
4-Hydroxy-7-triFluoromethyl-
3-quinolinecarboxylic acid
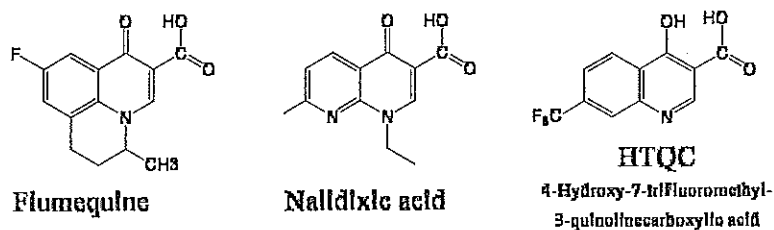
Indeed, the protonated ions of these three compounds undergo rapid loss of H_2O, and subsequent loss of CO or other species, but no detectable loss of CO_2. Furthermore, their MS/MS spectra were independent of the cone voltage and their [MH$^+$ - H_2O] ions undergo substantial addition of water in the collision cell to reform MH$^+$. In another test, we used enrofloxacin and alkylated the piperazine nitrogen with n-butyl group.
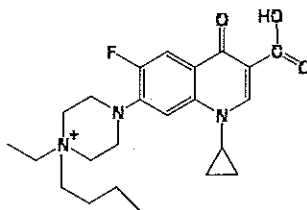
n-Butyl-enrofloxacin cation

The reaction was carried out with n-butyl bromide in ethanol at 70° C. To avoid alkylation of the other two nitrogens, a less favored process due to their lower basicities, the reaction was carried out to only ~ 10 % conversion. The resulting quaternary ammonium ion (m/z 416) does not protonate on the carboxyl group (unless it forms a 2+ ion). Its fragmentation in the collision cell shows loss of CO_2 (m/z 372) but no loss of H_2O (m/z 398, < 0.1 %). These results indicate that when the positive charge or the proton are on the piperazine ring the ion undergoes loss of CO_2 and when the charge is elsewhere on the quinolone molecule the ion undergoes loss of H_2O. It is not clear whether loss of H_2O occurs only when the proton is attached to the carboxyl group during the electrospray process, or whether it is possible for a proton attached to the oxygen or nitrogen of the quinolone ring to be collisionally activated and transferred to the carboxyl group.

To shed light on the competition between loss of H_2O and loss of CO_2 and its relation to the site of protonation, and on the process of water attachment in the collision cell, we examined several simple compounds (Table 1). Protonated phenol readily undergoes water loss to form the phenyl cation, which can attach a water molecule and revert to the precursor ion. The three isomeric dihydroxybenzenes undergo similar processes to varying degrees. The only other process occurring in these phenols is partial loss of CO leading to further fragmentation of the opened ring. Protonated benzoic acid undergoes mainly loss of CO_2 and only ~ 10 % loss of H_2O. The ion formed by loss of water can gain water in the collision cell but the contribution of this process is minimal because a preferred route is loss of CO from this ion, which is more evident at higher collision voltage. When the benzene ring bears both a carboxyl and a hydroxyl group, the preferred route depends on the relative positions of these groups. 2-Hydroxybenzoic acid undergoes loss of H_2O but no detectable loss of CO_2. Presumably, the vicinity of the two functional groups and the expected hydrogen bonding between them causes all protonated ions to undergo H_2O loss. On the other hand, protonated 3- and 4-hydroxybenzoic acids undergo both loss of H_2O and loss of CO_2 with the latter being the predominant one. In these compounds, the ions formed by loss of H_2O also lose CO rapidly and have little chance of gaining a water molecule. It is interesting to note that protonated 2-aminobenzoic acid undergoes loss of H_2O with

no detectable loss of CO_2 or NH_3. Protonated 4-aminobenzoic acid undergoes loss of both H_2O and CO_2 and the 3-aminobenzoic loses H_2O, CO_2, and NH_3 to various degrees. In these cases again loss of H_2O is followed by loss of CO so that addition of water to reform the precursor is barely detectable. By comparison with the above phenolic compounds, protonated benzyl alcohol undergoes very rapid loss of water to form the benzyl cation, which is not found to gain a water molecule, presumably because it is rather stable under the experimental conditions. Thus, the process of attaching a water molecule in the collision cell is observable when the ion formed by the initial loss of water is sufficiently stable toward further fragmentation, such as loss of CO, but not highly stable to reach the detector without reacting.

By comparison with the benzene derivatives discussed above, pyridine derivatives are much more basic; they attach the proton more strongly and require higher collision energies to undergo fragmentation, as reflected in their relatively higher $V_{1/2}$ values (the collision voltage required to fragment half the precursor ions, Table 1). Despite these differences, the behavior of the three isomeric hydroxypyridines (or pyridones, the more prevalent keto forms) is qualitatively similar to that of phenol and dihydroxybenzenes. The behavior of the three isomeric carboxypyridines, however, depends on their structure. Protonated picolinic acid (the 2-isomer) undergoes rapid loss of H_2O but no loss of CO_2. The product ion then undergoes loss of CO but also can attach a water molecule in MS^3 experiments. Protonated nicotinic acid (the 3-isomer) undergoes loss of H_2O much more slowly and the dominant process is loss of CO_2. The ion formed by loss of H_2O can also lose CO as well as gain a water molecule. Protonated isonicotinic acid (the 4-isomer) undergoes mainly loss of CO_2, and most of the ions formed by H_2O loss undergo subsequent loss of CO with minimal water gain.

The results discussed above (Table 1) indicate that both loss of H_2O and loss of CO_2 can take place in protonated ions with a single aromatic or heterocyclic ring, where presumably the site of protonation is very near or at the carboxyl group. Thus the two neutral losses are competitive processes, but they have different mechanisms. Loss of water is generally acid- or base-catalyzed and thus expected to take place when a proton is attached to the oxygen of an OH group (alcoholic, phenolic, carboxylic). Decarboxylation takes place via thermal decomposition or oxidation and may be inhibited by protonation and facilitated by deprotonation. In CID, decarboxylation may occur by moving the COOH proton to the ring or to another functional group, or even partly removing it by hydrogen bonding with an adjacent functional group. This removal of the COOH proton is more facile when the site of protonation of the examined ion is further away from this group, as is the case with the ions of the quinolone drugs which have a piperazine ring. In norfloxacin, loss of CO_2 accounts for one third of the

initial dissociation and in difloxacin and enrofloxacin it accounts for two thirds (Table 1). This loss occurs when the ion is protonated at the piperazine ring, but when the ion is protonated on the quinolone ring or the carboxyl group it undergoes loss of water, and its product can regain water and revert to the original precursor (Table 1). Most likely, the proton on the piperazine ring does not move to the carboxyl group following vibrational excitation because of the distance between those sites and the lack of conjugation or bridging sites. Therefore, protonation appears to occur initially at the different sites and to lead to the different products. To gain further support for this conclusion we used the separate parts of such molecules in a mixture. We combined equimolar (5×10^{-6} mol L^{-1}) concentrations of piperazine and flumequine in methanol/water/formic acid and recorded the mass spectrum at several cone voltages. Both protonated ions were observed: [piperazine + H^+], m/z 87, and [flumequine + H^+], m/z 262. Their peak intensity ratio varied between 1.6 and 1.9 (flumequine/piperazine) at cone voltage between 20 V and 40 V and the intensities were similar to those observed in separate solutions of each individual component. This result indicates that the ease of protonation of the quinolone and the piperazine moieties in the quinolone drugs are not very different and that both of these sites are likely to be protonated.

If two protonated ions differ solely in the site of protonation but have identical structures, can they have different mobilities as claimed by Kaufmann et al.[1]? Possibly, the piperazine protonated ion has weak hydrogen bonding between the neutral carboxyl and carbonyl groups (Structure 1),

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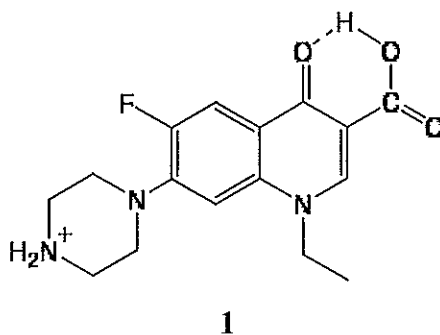
but the ion protonated on the carboxyl group is resonance stabilized and has a stronger hydrogen bond (Structure 2),
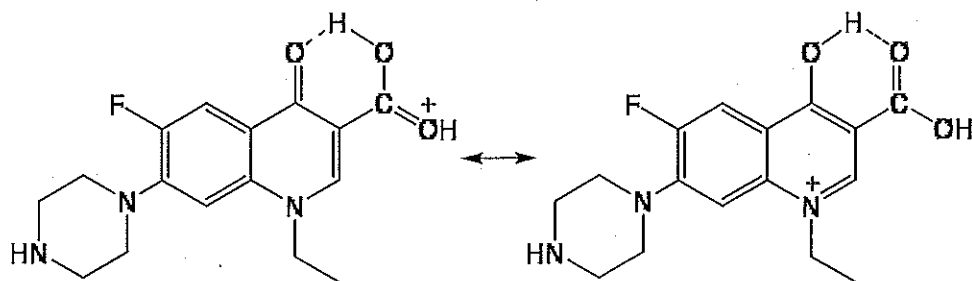
2

which may lead to slightly different geometry and thus different mobility.[9-11] Another difference expected between structures **1** and **2** is in their dipole moment, which has been suggested to affect mobility.[10] It should be mentioned that if the molecule is protonated on one of the other two nitrogens, the resulting ions do not have stronger hydrogen bonding around the carboxyl group, but if they lose H_2O to form an acyl species, the subsequent gain of H_2O will take place at the acyl site and lead to formation of structure **2**.

To estimate the proton affinities of the most favored protonation sites in norfloxacin, we used density functional theory (DFT) calculations to compare the two nitrogens in the piperazine moiety, the nitrogen in the quinolone moiety and the oxygen in the carboxyl group. The calculations were performed using the hybrid density functional method B3LYP[12] in conjunction with Pople's basis set 6-311+g (d,p) as implemented in Gaussian 03,[13] and as outlined before.[14] For all the optimized structures, frequency analyses at the same level of theory were used to identify them as real minima on the potential energy surface. These calculations include thermal and zero-point corrections. The enthalpies at 298 K and the proton affinities (PA's) of the optimized structures (Table 2) show that the two nitrogens in the piperazine ring are similar, while N1 in the quinolone ring is the least favored site for protonation. The behavior of the piperazine nitrogens is not surprising because the PA difference between piperazine and dimethylaniline is less than 3 kJ/mol, 943.7 kJ/mol for piperazine and 941 for DMA.[15] In contrast with the condensed phase behavior (pK_a), protonation at the carboxyl site is very favored due to hydrogen bonding with the carbonyl group on the quinolone ring. We cannot find experimental PA values for similar systems, but a comparison between 2,4-dihydroxybenzoic acid (863 kJ/mol) and 3,4-dihydroxybenzoic acid (847 kJ/mol) shows a similar stabilizing effect due to hydrogen bonding between ortho hydroxyl and carboxyl groups.[16] Relaxed scans of the bond distances C3-C13 and C13-O15 suggest a higher energy barrier for the loss of CO_2 than for the loss of water (by ~ 90 kJ/mol) for the species protonated at this site. Therefore, only species protonated on the piperazine ring undergo decarboxylation as the dominant pathway.

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The theoretical calculations also predict a dipole moment for the ion protonated on N21 (~ 30.7 D) ten times larger than that for the ion protonated on the carboxyl group (~ 3.1 D). Although, the dependence of mobility on ion-molecule collision does not include explicitly structural information of the ion other than ion size, the alignment of the dipole with the electric field affects the collision cross section. This empirical dependence has been suggested earlier[10] and recently used to develop regression models for predicting gas-phase ion mobility constant of diverse compounds.[17] Thus, the different dipole moments calculated for structures **1** and **2** are another reason for their different mobilities.

In summary, the question raised[1] about the reliability of SRM data in identifying quinolone drugs should be raised for other compounds if their protonated ions can undergo "reversible" water loss and if their structures are sufficiently large to accommodate two types of precursors with the same m/z value. The present results for such cases indicate dependence of MS/MS spectra on the cone voltage used in production of the precursor ions, in line with the previous findings.[1] No such dependence was detected for any of the small molecules used in this study (all compounds in Table 1 except for the four fluoroquinolone drugs). The present findings suggest that one must take precautions when monitoring SRM's for complex compounds because, in certain cases, particularly those involving dehydration/rehydration reactions, these can be comparable only when monitored under strictly identical conditions. For such cases, the "cone voltage" used in the production of the protonated ions in the ESI source has a strong effect, and since the dehydration/rehydration process becomes more pronounced at increased cone voltage, problems with SRM comparability may be alleviated by using low cone voltage.

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Table 1. Intensities of neutral loss peaks (% of total intensity) at a collision voltage ($V_{1/2}$) where half the precursor ion (p) remains and the maximum value at the corresponding collision voltage (given in parentheses). The last column is percent intensity of MH^+ ion formed from $[MH^+-H_2O]$ by attachment of a water molecule in the collision cell

Compound	$V_{1/2}$	p-H$_2$O at $V_{1/2}$	p-H$_2$O max	p-CO at $V_{1/2}$	p-CO Max	p-CO$_2$ at $V_{1/2}$	p-CO$_2$ max	p-HCO$_2$H at $V_{1/2}$	p-HCO$_2$H max	+H$_2$O in MS^3
Phenol	13.7	43.2	62.6 (18)	2.5	2.8 (20)	0.25	33 (36)			5
Catechol	9.2	35.2	42.7 (12)	1.3	2.8 (12)	0.1	0.1	6.2	74 (22)	10
Resorcinol	14.2	24.4	24.4 (14)	1.7	1.8 (16)	0.03	0.2 (36)	20.6	65 (24)	18
Hydroquinone	13.1	22.5	24.3 (14)	2.0	3.2 (14)	0.3	0.3 (12)	17.0	60 (22)	12
benzoic acid	8.4	3.7	12.8 (14)	0.04	4.4 (28)	42	70 (12)	0.4	67 (27)	0.01
2-hydroxybenzoic acid	8.5	46	89 (14)	0.01	1.8 (22)	0.05	0.2 (16)	0.06	21 (22)	0.03
3-hydroxybenzoic acid	7.4	4.1	12.7 (14)	0.03	0.8 (32)	41	66 (10)	0.07	9.0 (22)	0.06
4-hydroxybenzoic acid	9.2	11.7	27.5 (15)	0.01	1.2 (34)	33	49 (12)	0.1	14 (23)	0.04
2-aminobenzoic acid[a]	5.8	52	95 (12)	0	0.3 (25)	0.01	0.04 (14)	0.06	53 (22)	0
3-aminobenzoic acid[b]	14.5	4.1	4.7 (16)	0.2	0.4 (20)	17.6	19.2 (16)	3.4	8.7 (20)	0
4-aminobenzoic acid[c]	10.0	22.6	41 (14)	0.01	0.7 (24)	26.1	37 (14)	0.2	27 (24)	0
3-pyridylcarbinol[d]	18.6	2.4	9.2 (38)	0.9	0.9 (18)	0.03	6.4 (36)	~0		0.4
2-hydroxypyridine	14.3	48.5	70 (20)	0.04	0.07 (22)	~0	1.8 (40)	~0	5.2 (40)	12
3-hydroxypyridine[e]	23.3	8.1	9.1 (28)	19.5	20.2 (22)	0.54	10.8 (40)	~0		33
4-hydroxypyridine[f]	22.9	8.6	8.7 (24)	10.9	11.3 (21)	0.04	6.8 (40)	~0	1.1 (40)	17
picolinic acid	9.3	38.8	43 (10)	2.3	17.6 (18)	~0		6.8	76 (24)	40
nicotinic acid	18.0	4.0	4.0 (18)	3.4	5.0 (24)	23.4	36 (24)	16.7	30 (28)	42
isonicotinic acid	16.4	0.24	0.26 (18)	18.7	21.5 (18)	26	44 (22)	2.0	9.3 (28)	1
6-hydroxynicotinic acid	18.1	30.1	30.1 (18)	1.7	2.8 (22)	0.5	0.9 (28)	5.3	10.6 (24)	47
N-methylnicotinic acid	18.0	1.05	1.1 (16)	2.4	3.5 (23)	19.2	25 (22)	19.4	27 (22)	48
Nicotinamide[g]	15.9	~0.1	0.4 (22)	0.2	0.3 (22)			0.04	0.7 (40)	
N-methylnicotinamide[h]	17.3	0.38	0.54 (20)	0.2	0.23 (18)			0.01	0.3 (40)	
Norfloxacin	15.1	28.5	51 (22)	~0		15.5	17.7 (18)	0.23	1.2 (32)	67
Difloxacin	16.3	16.7	31 (22)	0		28.9	39 (20)	0.27	2.0 (24)	71
Enrofloxacin	15.4	15.5	30 (22)	0		29.9	43 (18)	0.4	3.0 (24)	49
Marbofloxacin[i]	14.2	2.8	7.6 (21)	0		~0		0		66
Flumequine[j]	12.4	48.4	81 (20)	~0						25
Nalidixic acid	10.3	48.5	72 (15)	~0		0.02		0.3	58 (26)	31
HTQC	11.2	47.8	78 (20)					~0	18 (30)	

Footnotes to Table 1

[a]No detectable loss of NH$_3$. [b]Loss of NH$_3$ was 3.3 (V$_{1/2}$) and 6.5 (18). The ion formed by loss of NH$_3$ partially gained H$_2$O in the collision cell. [c]Loss of NH$_3$ very minimal. [d]Significant loss of CH$_2$O from the side chain: 20.9 (V$_{1/2}$) and 27 (24). [e]Loss of NH$_3$ was 0.5 (V$_{1/2}$) and 2.3 (38). [f]Loss of NH$_3$ was 0.7 (V$_{1/2}$) and 4.2 (34). [g]Loss of NH$_3$ was 3.5 (V$_{1/2}$) and 3.5 (16). The ion formed by this loss gains water in the collision cell. There were also losses of HCN 4.4 (V$_{1/2}$) and 4.8 (18), CONH 34.5 (V$_{1/2}$) and 58 (22), and HCONH$_2$ 5.9 (V$_{1/2}$) and 18.5 (28). [h]Loss of NH$_3$ was 0.8 (V$_{1/2}$) and 0.8 (16). There were also losses of HCN 2.0 (V$_{1/2}$) and 2.9 (22), CONH 26.7 (V$_{1/2}$) and 37 (22), and HCONH$_2$ 8.8 (V$_{1/2}$) and 14 (22). [i]There was also a loss of a 43 Da species, which is not CONH, but most likely CH$_3$-N=CH$_2$, 22.2 (V$_{1/2}$) and 23.5 (16). [j]Loss of C$_3$H$_6$ was 0.01 (V$_{1/2}$) and 4.8 (32).

Table 2. Enthalpies and proton affinities of the different minima of norfloxacin at 298 K

Protonation site	Enthalpy (au.)	Proton affinity (kJ/mol)
N21	-1110.639131	905.8
N18	-1110.638190	903.4
N1	-1110.588285	772.3
O	-1110.667525	980.4
Neutral	-1110.294121	
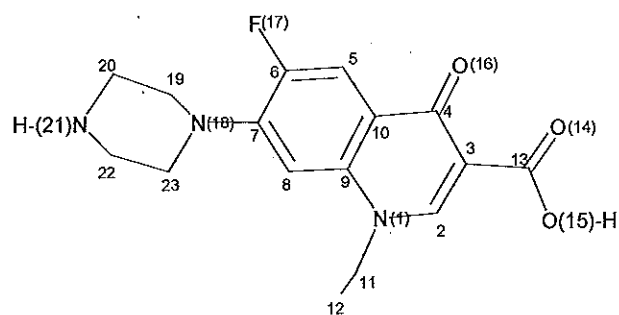
Figure Captions

Figure 1. MS/MS spectra of protonated difloxacin ion formed at cone voltage 30 V (a) and 80 V (b) at five selected collision voltages (as marked on the figure).

Figure 2. Relative intensities of the ions formed by fragmentation of protonated norfloxacin, formed at cone voltage 30 V and 80 V, as a function of collision voltage in the range of 5 V to 40 V.

Figure 3. MS^3 spectra of the ions formed from protonated norfloxacin by loss of H_2O (m/z 302) and by loss CO_2 (m/z 276). The neutral-loss ions were produced in the source at increased cone voltage and were selected into the collision cell for further fragmentation. Only the ion formed by loss of water (m/z 302) shows attachment of water (back to m/z 320) in the collision cell.
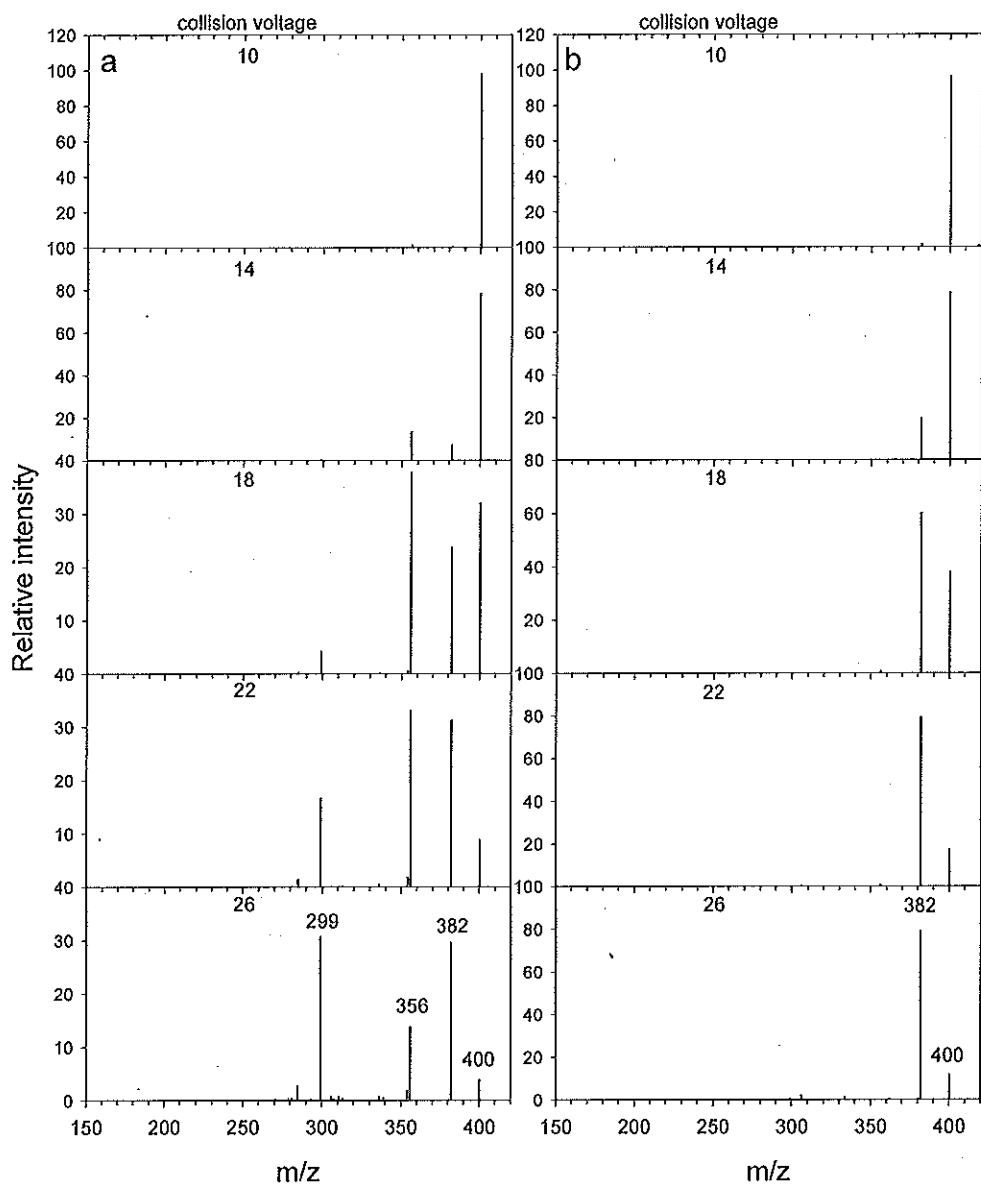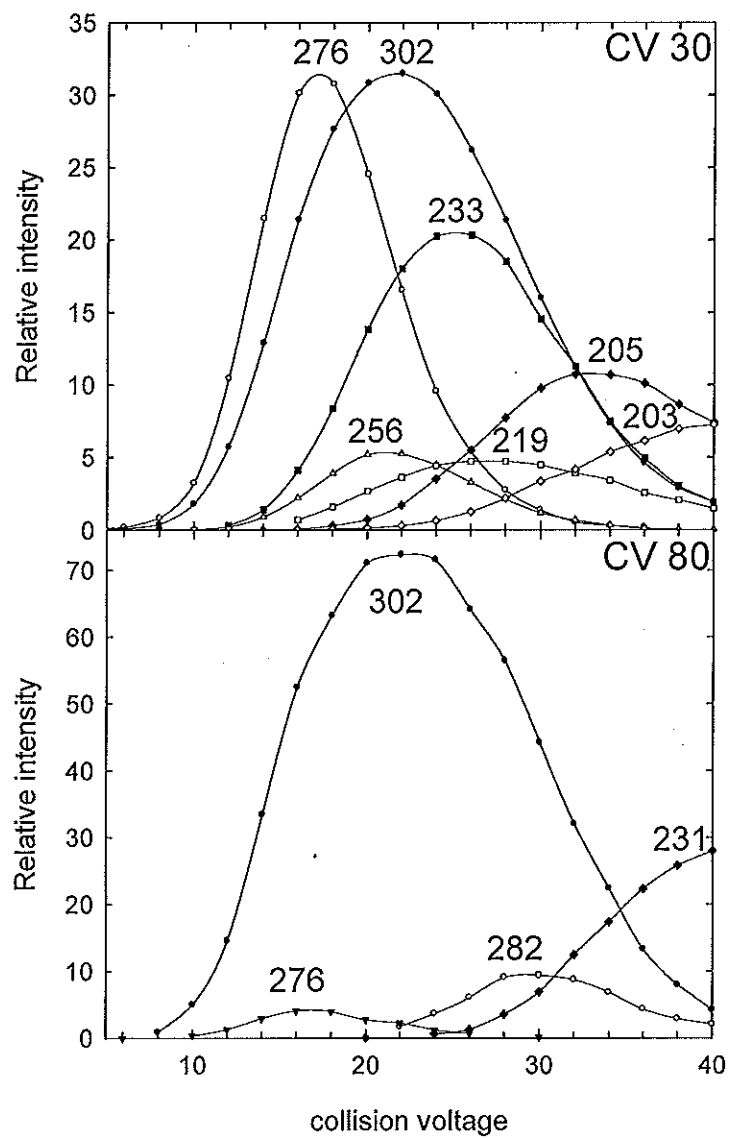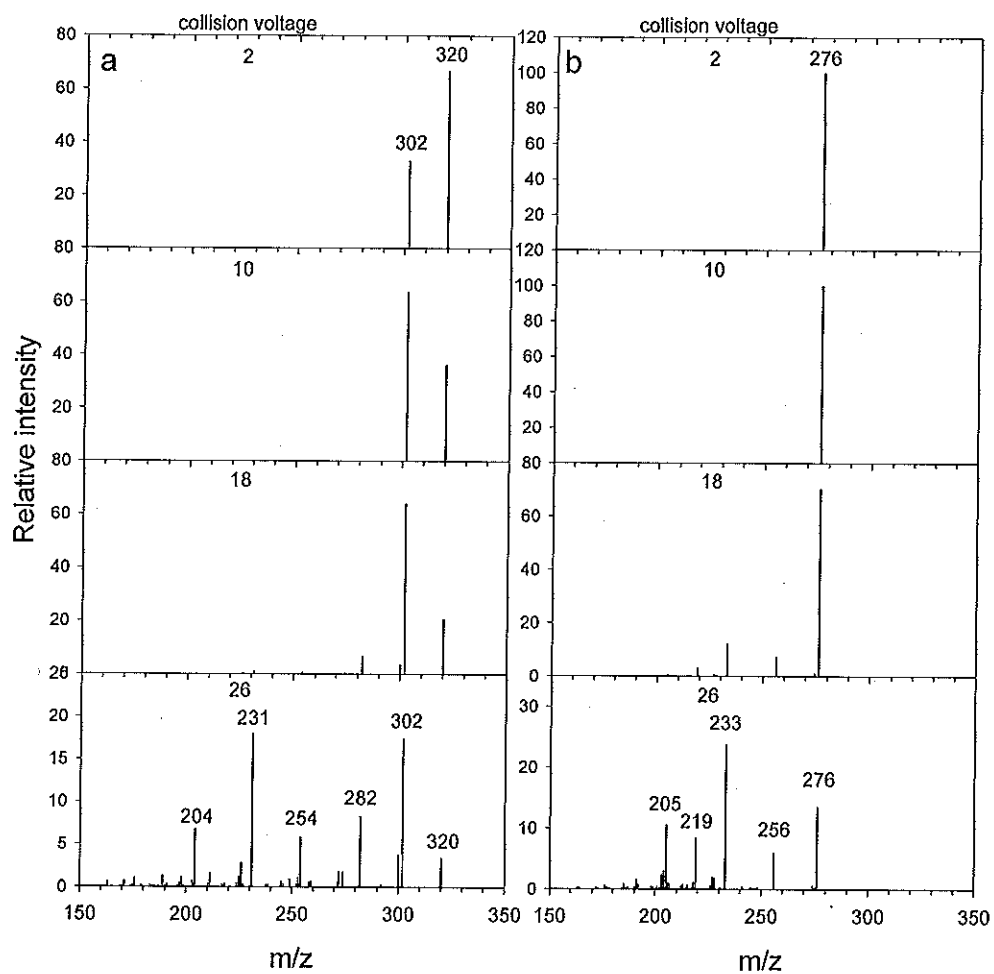
Figure 1

Figure 2

Figure 3