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ORIGINAL RESEARCH

Characterization of a new norfloxacin metabolite monitored during a bioequivalence study by means of mass spectrometry and quantum computation

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ABSTRACT: A bioequivalence study of two formulations containing norfloxacin was used for identification and assay of the metabolite of norfloxacin in human serum samples. The bioequivalence study was based on an analytical method using liquid chromatography with fluorescence detection. The plasmatic profile of metabolite was similar to norfloxacin for both formulations. Three plasma fractions of norfloxacin metabolite from a volunteer were isolated by liquid chromatography and investigated by atmospheric-pressure chemical ionization mass-spectrometry. The structure of norfloxacin metabolite (7-aminoethylenamino-6-fluoro-4-hydroxy quinoline-3-carboxylic acid) was identified taking into account also the mass spectrometry investigations achieved for norfloxacin and ciprofloxacin (used as internal standard for the analytical method). A theoretical procedure based on quantum chemical calculations has been also used to explain the mass fragmentation in molecules of norfloxacin metabolite that differ from the molecule of norfloxacin. Copyright © 2008 John Wiley & Sons, Ltd.

KEYWORDS: norfloxacin; metabolite; human metabolization; LC/APCI-MS/MS; quantum computation; liquid chromatography

INTRODUCTION

In many circumstances, drug assay in biological fluids requires the time profile of known metabolite(s). In the case of very reactive drugs, the number of metabolites can be higher than just one. Among the metabolic mechanisms, dealkylation of amines and esters is probably the most encountered pathway. Extensive studies of these reactions revealed several predictable characteristics, which are a consequence of the catalytic mechanisms. For example, *N*-dealkylation reactions are faster for tertiary than secondary amines, and it is faster for secondary than primary amines. It is based on N basicity, decreasing from tertiary to primary amines (Thompson, 2005).

The fluoroquinolones exhibit antibacterial activity *in vitro* and in infections compared with β-lactam and aminoglycoside antimicrobial agents (Wolfson and Hooper, 1985; Venezia *et al.*, 1989). Amifloxacin, pefloxacin, enoxacin, ciprofloxacin and norfloxacin are only a few well-known examples of congeners of this class, which have been extensively studied for their absorption, distribution, metabolism and elimination in experiments in humans, but mainly in animals: mice, dogs, monkeys, etc. (Montay *et al.*, 1984; Kinzig-Schippers *et al.*, 1999).

The literature reports only a few papers focused on the determination of norfloxacin in plasma samples and application to pharmacokinetic studies. Almost all reported methods deal with plasma samples provided from different animals (chickens, pigs; Anadon *et al.*, 1992, 1995; Kowalski *et al.*, 2005), or in poultry tissue (Lim *et al.*, 2002). Recently, a liquid-chromatography method with fluorescence detection was developed, which can be applied to the reciprocal determination of furosemide or norfloxacin in plasma samples, using each of them as internal standard for the determination of the other (Galaon *et al.*, 2007). LC-coupled electrospray and atmospheric pressure chemical ionization technique has been used for the determination of seven quinolones in chicken tissues using norfloxacin as internal standard (Bailac *et al.*, 2006). Recently, an LC-MS/MS method for the determination of ulifloxacin, the active metabolite of prulifloxacin, in human plasma has been reported (Guo *et al.*, 2006). These and other papers have been also focused on the identification and determination of metabolites of norfloxacin from blood samples provided from different animals. From this

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Abbreviations used: APCI, atmospheric-pressure chemical ionization; PCM, polarizable continuum model; TEA, triethylamine.

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point of view, the number and nature of norfloxacin metabolites occurring in metabolic pathway are still debatable. The aim of this study is to propose a possible structure of a major metabolite identified during the bioequivalence study of two norfloxacin-containing formulations by means of mass-spectrometry and liquid chromatography as well as using quantum computation in order to explain the pattern fragmentation. The possibility of identifying the structure of unknown metabolites in biological samples with the aid of mass spectrometry has been emphasized recently in some other circumstances (e.g. Schanzer *et al.*, 2006; Li *et al.*, 2006; Chen *et al.*, 2006).

EXPERIMENTAL

Sample preparation of plasma samples. For the bioequivalence study a validated sample preparation procedure was used. A volume of 200 µL of plasma sample was mixed with 50 µL of aqueous solution containing 3 ppm internal standard (ciprofloxacin) and under vortex for 30 s. After that, 400 µL acetonitrile was added and vortexed for 3 min for 2000 rpm. The supernatant was isolated after centrifugation at 12,000 rpm (at 25°C) for 5 min. Then, 3400 µL solution containing 0.1% triethylamine (TEA) brought to pH = 4 with phosphoric acid was added to supernatant. After a final vortex for 3 min at 1000 rpm, a volume of 100 µL was injected into the chromatographic column.

Chromatographic method. LC separations were carried out on a monolithic column, Chromolith Performance RP-18e (Merck KGaA, Darmstadt, Germany), 100 mm length and 4.6 mm internal diameter. The column temperature was kept at 25°C. The elution was achieved in gradient mode using 1% triethylamine in aqueous solution, brought to pH = 4.0 with phosphoric acid, as the aqueous component, and methanol as the organic modifier. The elution program was set up to the following steps: 85:15 (v/v) between aqueous component and methanol for 0–9 min; a linear gradient between 9 and 10 min to a composition of 40:60; followed by a linear gradient between 10 and 11 min to the initial composition (85:15), then kept constant up to 11.5 min (final duration of the chromatographic data acquisition).

A constant flow-rate of 2.5 mL/min was applied. The fluorescence detection was achieved for 268 nm as excitation wavelength and 445 nm as emission wavelength. The photomultiplier gain was set up at 14, and the response time was optimized at 0.25 s.

Mass spectrometry experiments. The parameters controlling the APCI-MS interface were as follows: drying gas, N_2; temperature, 300°C; vaporizer temperature, 350°C; drying gas flow, 5 L/min; pressure of the nebulizer gas, 60 psi; capillary voltage, 4500 V; high voltage end plate offset, –500 V; corona discharge, 5000 V.

Computational methods. DFT calculations for norfloxacin metabolite and the possible fragments issued by mass spectrometry were performed *in vacuo* with a 6-31G(d) basis set in the frame of the GAMESS-US program (Schmidt *et al.*, 1993; Gordon and Schmidt, 2005). Preliminary semiempirical PM3 calculations were used as a semiquantitative estimation of the different possible bond breaking processes responsible for the MS. Firstly the most stable conformer of the neutral species was found by geometrical optimization and calculation of their relative energy. All the protonated species were then optimized and the corresponding transition states located, in view of estimating the activation barriers for each process. The critical points were characterized by vibrational analysis. Solvation was treated by single point calculations performed with the polarizable continuum model (PCM) (Cammi *et al.*, 2000) at the optimized geometry of the isolated molecule.

Methodology of bioequivalence study. The present study is the result of a bioequivalence project carried out for two norfloxacin-containing formulations. During the open-label, randomized, two-period, two-sequence, crossover bioequivalence study, 24 healthy male and female volunteers received one dose of norfloxacin from the tested and reference products, found on the Romanian market, with a 14 day wash-out period. The study medication was orally administered after overnight fasting. Blood samples were collected before dosing (0 h) and at 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10 and 12 h post-dose.

The pharmacokinetic parameters considered for evaluation of the bioequivalence between tested and reference products were: C_{max}, observed maximum norfloxacin plasma concentration; t_{max}, sampling time of the maximum plasma concentration; $t_{1/2}$, terminal elimination half-life time; AUD, area under plasma concentration–time curve until the last quantifiable value; and $AUC_{0-\infty}$, AUD extrapolated to infinity. Pharmacokinetic parameters were determined by means of the Kinetica software (version 3.1/2000) from Innaphase Corp., USA. The values for these parameters obtained for the two formulations proved the bioequivalence of the two studied formulations.

Materials and solvents. All solvents were HPLC grade from Merck (Darmstadt, Germany). Water for HPLC (minimum resistivity 18 MΩ and maximum content of total organic carbon of 30 ppb) was produced within laboratory with a TKA Lab HP 6UV/UF instrument and used during all experiments.

RESULTS AND DISCUSSION

Considerations on metabolism of norfloxacin

The metabolism of the fluoroquinolone drugs, such as ciprofloxacin and norfloxacin, by *Pestalotiopsis guepini* strain P-8 has already been investigated. Four major metabolites were identified from each drug by means of high-performance liquid chromatography with mass spectrometry detection and proton nuclear magnetic resonance spectroscopy. Thus, ciprofloxacin metabolites included *N*-acetylciprofloxacin, desethylene-*N*-acetylciprofloxacin, *N*-formylciprofloxacin and 7-amino-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid. Norfloxacin metabolites included *N*-

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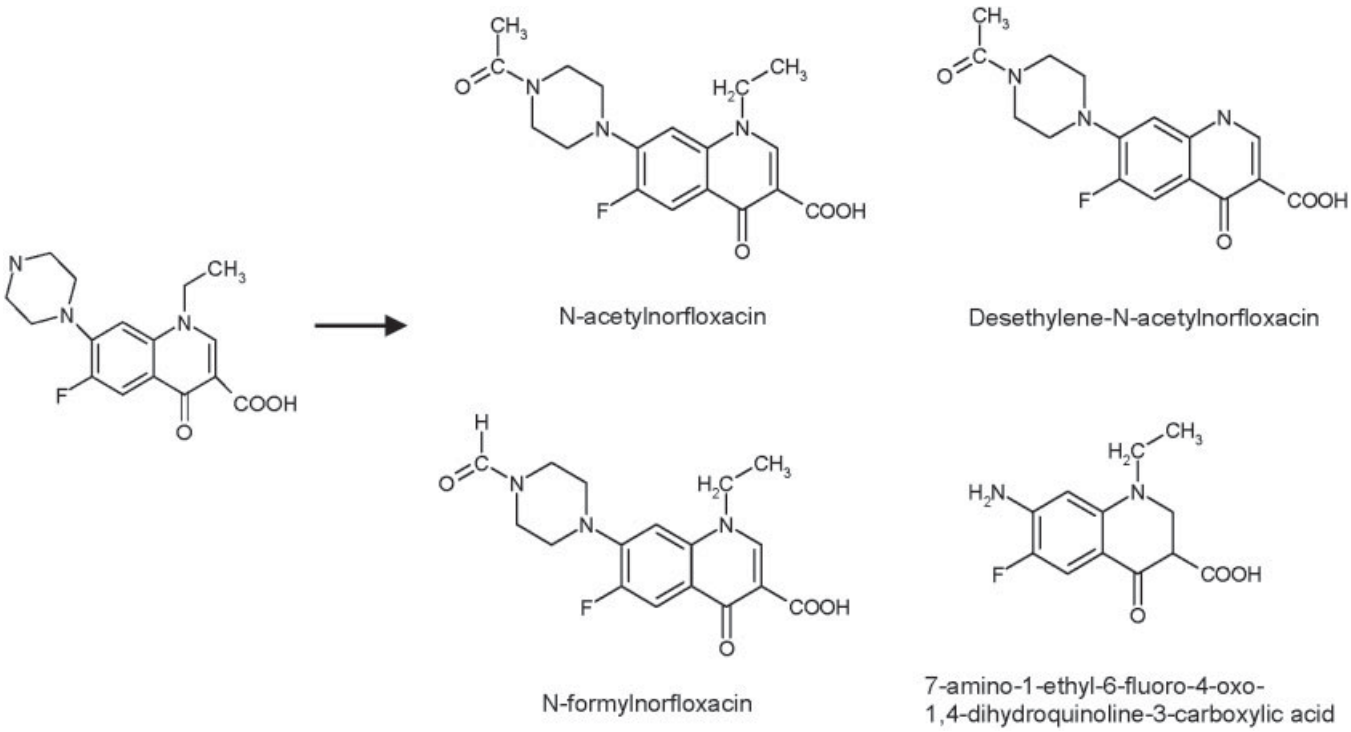

Figure 1. Structure of norfloxacin and its major metabolites.

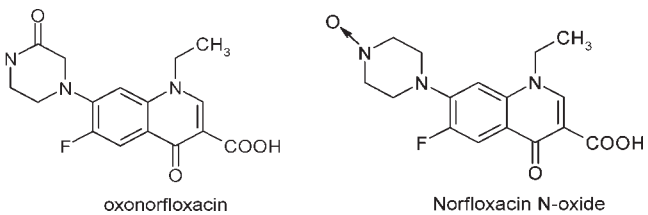

Figure 2. Structures of two identified intermediate compounds in the metabolic pathway of norfloxacin.

acetylnorfloxacin, desethylene-*N*-acetylnorfloxacin, *N*-formylnorfloxacin, and 7-amino-1-ethyl-6-fluoro4-oxo-1,4-dihydroquinoline-3-carboxylic acid. *N*-formylciprofloxacin and the four metabolites from norfloxacin are all known to be mammalian metabolites (Parshikov *et al.*, 2001). Their structures are given in Fig. 1.

An intermediate metabolite in the pathway norfloxacin → 7-amino-1-ethyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (structures given in Fig. 2) could be oxonorfloxacin or norfloxacin *N*-oxide, which have been already identified and assayed in plasma and tissue samples provided from different animals (Montay *et al.*, 1984).

The structure of norfloxacin metabolite as well as the complete human metabolic pathway has not been yet reported. It is expected that the human metabolism of norfloxacin will follow a similar pathway to that found to animals. However, some differences are likely to occur. The present study started from a bioequivalence study of two formulations containing 10 mg norfloxacin. The analytical method comprising two main stages (sample preparation and chromatographic analysis) was developed and validated using human plasma provided from healthy volunteers, who had not received any medication in the previous month. The same method was then applied to the bioequivalence study of two formulations containing norfloxacin. Unlike the plasma samples used during the development and validation of the entire analytical method, all samples provided from volunteers who participated in the bioequivalence study exhibited a new chromatographic signal, except for the plasma sample collected before dosing. For this reason, all chromatograms of plasma samples from volunteers were carefully examined and a new chromatographic peak was introduced into a new analytical protocol in order to correlate its area with the time when blood plasma had been collected from volunteers. A typical chromatogram provided from samples from volunteers is given in Fig. 3, overlapped with the chromatogram corresponding to the blank plasma sample from the same volunteer (before receiving the tablet).

During the bioequivalence study on 24 healthy volunteers, the metabolite concentrations were monitored in all samples, using the calibration response used for norfloxacin, taking into consideration the similarity between their structures. We may suppose that a slight difference between their calibration responses will shift the metabolite plasma profile upward or downward with a certain magnitude. Although this point of view is not the most objective, it can be used in this particular

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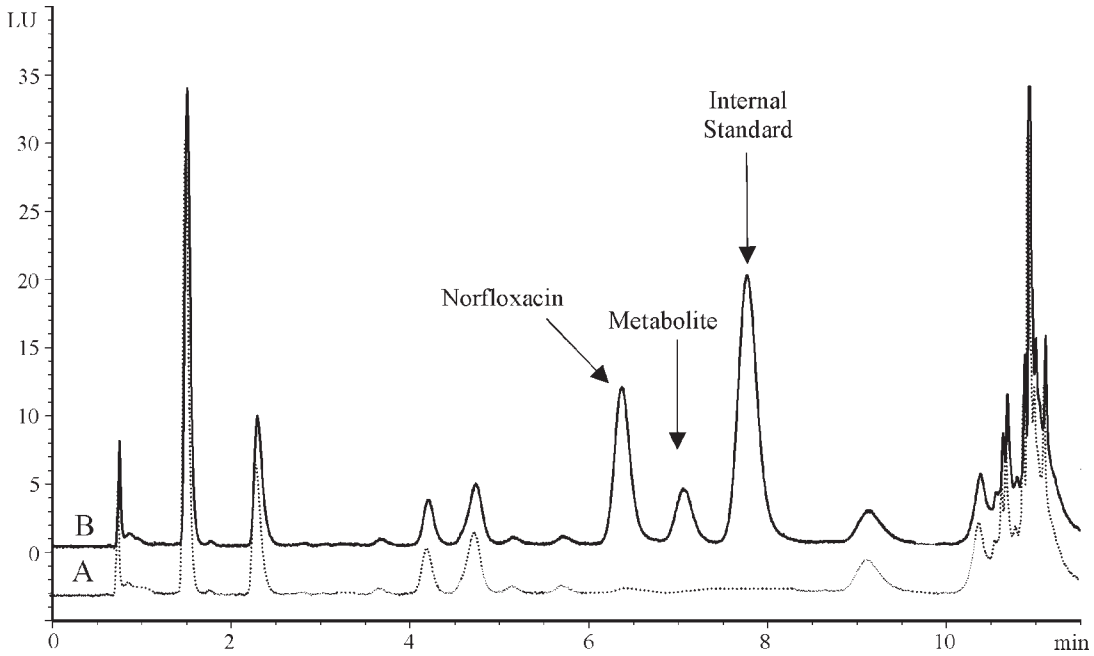

Figure 3. Two overlaid chromatograms corresponding to a blank sample (A) and a sample collected from the same volunteer at 2 h after drug administration (B).

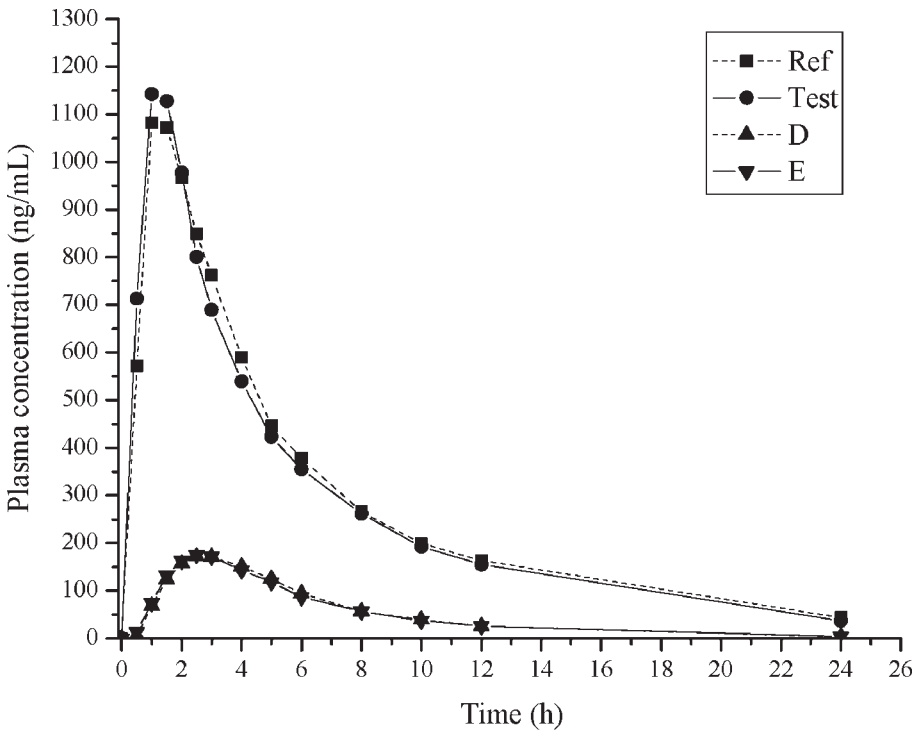

Figure 4. Plasma concentrations profiles for norfloxacin and its metabolite obtained during the bioequivalence study (Ref, reference norfloxacin formulation; Test, tested norfloxacin formulation; D, metabolite for reference formulation; E, metabolite for tested formulation).

case when the pure metabolite substance was not available. The mean concentration profiles for norfloxacin and its metabolite for both studied formulations (references and tested) are given in Fig. 4.

The main pharmacokinetic parameters (given in the Experimental section) obtained on study completion for both studied formulations proved their bioequivalence as well as the reliability of the entire analytical

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process. A plasma sample with the highest content of norfloxacin metabolite provided from a volunteer (collected at 2 h after drug administration) was chosen among all samples provided from volunteers in order to isolate this compound. This sample was prepared in the same way as all plasma samples from volunteers, and injected into the analytical column to separate the analytes of interest. The fraction eluting between 15.6 and 17.5 min was then collected; this process was repeated three times for three injected volumes, allowing having the necessary quantity of metabolite for mass-spectrometry experiments.

MS spectra interpretation

Electrospray mass spectra (ESI-MS) of seven floroquionolones other than norfloxacin have already been characterized in chicken tissue samples (Bailac *et al.*, 2005). In this study norfloxacin and ciprofloxacin in plasma samples were studied by means of APCI-MS technique. The MS spectrum of norfloxacin (M = 319) shows a spectral line at m/z = 320, which corresponds to $[M + H]^+$. Isolation of the molecular ion and collision-induced fragmentation (CID) in MS-MS produced a spectral line at m/z = 276, assigned to the loss of a carboxyl group, i.e. $[M - CO_2 + H]^+$. The same fragmentation was observed for ciprofloxacin; the APCI-MS spectrum exhibits a spectral line at m/z 332, which by isolation and fragmentation produced a MS^2 spectral line situated at m/z = 288.

The fraction of metabolite separated by liquid chromatography was studied in the same conditions by APCI mass spectrometry. The experimental APCI-MS and APCI-MS-MS spectra are given in Fig. 5.

The most intense fragment corresponding to m/z = 266 was supposed to correspond to a metabolite of norfloxacin ($[M + H]^+$). By collision-induced fragmentation, its intensity decreases and the fragment at m/z = 224 becomes the most stable among other possible fragments. This time the collision fragmentation cannot be assigned to $[M - CO_2 + H]^+$. The mass difference of the two ions (42) could be assigned to another fragmentation more favored from an energetic point of view. If the carboxyl functional group is kept in the structure of norfloxacin metabolite, then it becomes more stable than initially. In this case carboxyl can be situated on an aromatic ring, which forms a stronger bond with it than in the case of a non-aromatic ring, as it is found in the norfloxacin structure. Taking into account the previous discussion on the metabolism of norfloxacin and the already identified metabolites other than the human pathway, a possible structure of this metabolite derives from piperazinic opening and aromatization to yield the quinoline ring. The entire metabolization pathway of this drug in the human body can be proposed as that given in Fig. 6.

Thus, the final metabolite (7-aminoethylenamino-6-fluoro-4-hydroxy quinoline-3-carboxylic acid) monitored in our study by HPLC-FLD has a different APCI-MS-MS fragmentation in comparison to the initial drug structure. The elimination of the fragment with mass of 42 corresponds to the elimination of fragment C_2H_4N, according to the schema proposed in Fig. 6.

Quantum chemical calculations

A theoretical study was performed in order to explain the MS data obtained for the norfloxacin metabolite compared with the parent compound according to a usual procedure for this kind of processes (e.g. Uggerud, 1997). The absolute minimum for the norfloxacin metabolite with respect to the relative position of the two hydroxyls in the molecule was found to be 10.15 kcal/mol more stable than the other conformers (Table 1). This geometry was used in all the subsequent calculations. As the ESI-MS technique supposes that the molecules are protonated, we first optimized the equilibrium geometry for the three possible cations protonated at a nitrogen site (denoted by **1**, **2** and **3**). The most stable one *in vacuo* was proved to be **1**. We considered as possible two fragmentation processes, dealkylation and decarboxylation (as taking place in norfloxacin). For these processes we located the transition states and calculated the activation barriers with the PM3 and DFT methods (Table 1). The PM3 method was used as a preliminary step and it can be seen that it proved to be a useful tool for a semiquantitative view on the problem. The geometry of the transition states for decarboxylation does not depend to a great extent on the protonated species, whereas it does for dealkylation.

Dealkylation and decarboxylation *in vacuo* of the cation protonated at the nitrogen in the heterocycle, **1** (the most stable protonated form), issued very close activation barriers of about 75–76 kcal/mol. On the other hand, the calculated dipole moment of **1** is 1.91 D, while for **2** and **3** it is 9.39 and 21.58 D, respectively. It is thus expected that the solvation process in water stabilizes **3** to a greater extent than **1** and **2**, i.e. the most stable one would become **3**. In order to check this assumption, we performed calculations for **1**, **2** and **3** in water. The results indicate that **3** is indeed 1.27 kcal/mol more stable than **1**, such that the fragmentation of **3** is the most relevant process for the MS spectrum of norfloxacin metabolite. Isomer **2**, more unstable by 16.05 kcal/mol, was not further considered in the calculations.

In the case of **3**, the activation energy for the two bond breaking processes is 54.86 kcal/mol (dealkylation) compared with 68.06 kcal/mol (decarboxylation), meaning that the most probable bond breaking mechanism is dealkylation. This is in agreement with the MS experimental data, which, unlike norfloxacin, does not present

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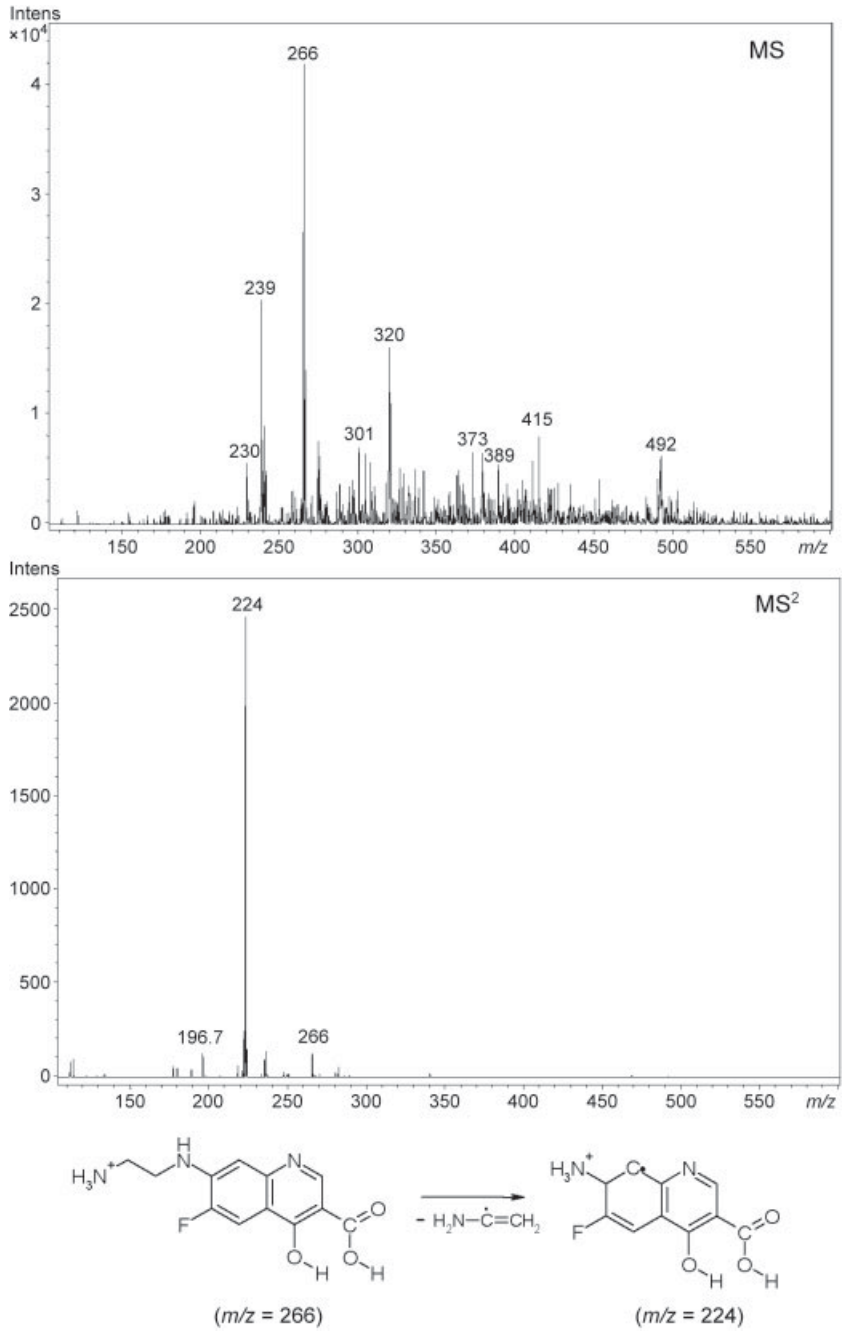

Figure 5. Mass spectra of the metabolite of norfloxacin and its MS^2 fragmentation.

an [M + 1] – 44 peak, corresponding to carbon dioxide loss, but a peak at [M + 1] – 42, corresponding to loss of a $H_2NC{\cdot}{=}CH_2$ fragment.

Chromatographic behavior

The elution order of the target analytes in reversed-phase liquid chromatography (given in Fig. 3) can be rather accurately explained by the hydrophobicity parameter, i.e. the logarithm of the octanol–water partition coefficient ($\log K_{ow}$). The proposed structure of norfloxacin metabolite is characterized by a computed value for $\log K_{ow}$ of 1.245, using the fragment methodology, in comparison with the computed value for $\log K_{ow}$ corresponding to the structure of norfloxacin of −0.306. Thus, the elution order of these two analytes can be explained. The same estimation of $\log K_{ow}$ for ciprofloxacin gives a value of about 1.701, if we do not apply a correction of −1.700 for non-alpha amino-acid type (Meylan and Howard, 1995). Its higher hydrophobicity can explain the higher retention time value of the internal standard than norfloxacin and its metabolite.

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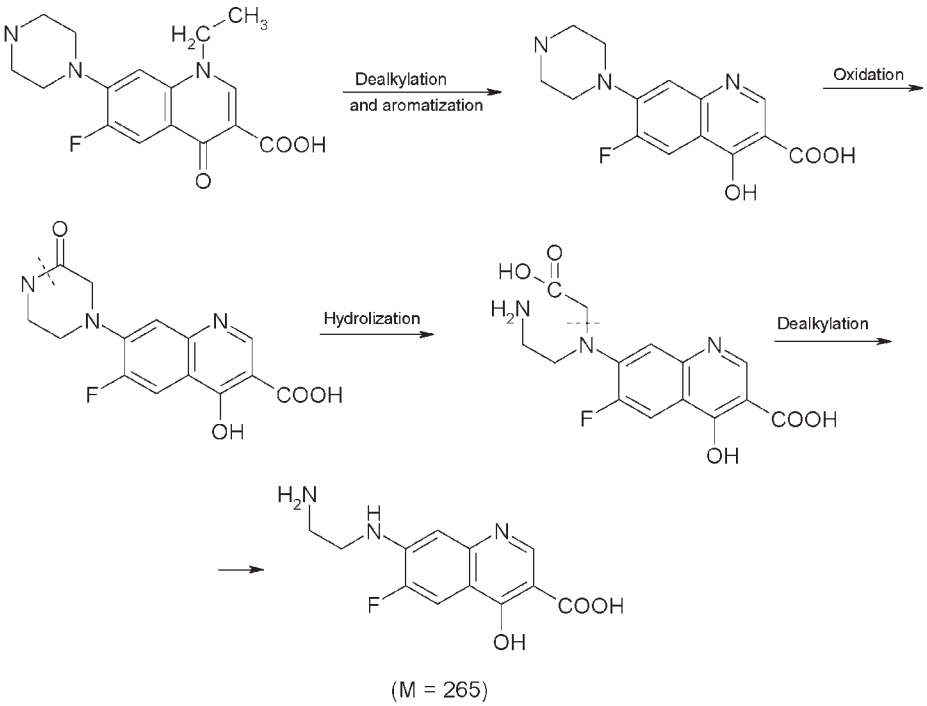
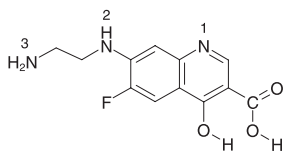
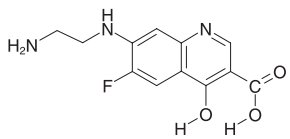
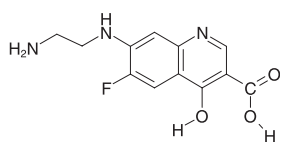


Figure 6. A possible human metabolic pathway for norfloxacin.

Table 1. Calculated results for the protonated forms: relative energies, dipole moments, activation barriers

Cation	Structure	E_{rel} (kcal/mol) DFT	E_{rel} (kcal/mol) PM3	E_{rel} solv (kcal/mol)	μ (D) DFT	μ (D) PM3	E_a (kcal/mol) Dealkylation DFT	E_a (kcal/mol) Dealkylation PM3	E_a (kcal/mol) Decarboxylation DFT	E_a (kcal/mol) Decarboxylation PM3
1	 E_{rel} = 0 kcal/mol	0.00	0.00	1.27	1.91	2.52	75.17	65.26	76.01	66.93
2	 E_{rel} = 10.15 kcal/mol	24.55	11.21	16.05	9.39	10.50	—	8.8	—	81.74
3	 E_{rel} = 10.42 kcal/mol	22.40	8.81	0.00	21.58	23.93	54.86	25.34	68.06	78.12

CONCLUSIONS

A certain metabolite of norfloxacin was detected and monitored during a bioequivalence study of two norfloxacin-containing formulations by means of liquid chromatography with fluorescence detection. The smooth profile of the pharmacokinetic curve suggests that this metabolite has a stable structure against the

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metabolization pathway of this drug. Its characterization by mass spectrometry, chromatographic behavior and quantum computation as well as with some data from literature concerning different intermediate metabolites of norfloxacin led to the conclusion that the possible structure of this metabolite is 7-aminoethylenamino-6-fluoro-4-hydroxy quinoline-3-carboxylic acid.

Acknowledgments

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