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A study of ofloxacin and levofloxacin photostability in aqueous solutions

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ABSTRACT

Introduction. The photostability is one of the most important properties of drugs. A comprehensive study of ofloxacin (OFX) and levofloxacin (LVX) photostability in aqueous solutions was performed. Ofloxacin is a chemotherapeutic agent belonging to the second generation fluoroquinolones and is a racemate of (R)-(+)-ofloxacin and (S)-(-)-ofloxacin (LVX).

Material and Methods. Samples of OFX and LVX were subjected to stress conditions of UV irradiation using a mercury-vapor lamp. The study involved development of enantioselective high-performance liquid chromatography (HPLC) and high-performance capillary electrophoresis (HPCE) methods for separation of OFX enantiomers and their degradation products. These methods were used to monitor the degradation process of OFX and LVX under irradiation and to determine the kinetics of degradation of these antibacterial agents. Moreover, the identification of photoproducts was also attempted. The structure of the main photoproducts was examined by mass spectrometry (MS).

Results and Conclusions. Using HPLC method it was possible to observe two products of OFX degradation and only one for LVX, while using HPCE method eight products of OFX degradation and six of LVX were observed. Some of the photoproducts retain character of optically active compounds. The trend of the photodegradation of both tested compounds was described by autocatalytic reaction proceeding according to the Prout-Tompkins model. Some of the products of the decomposition catalyze this reaction. The rate of degradation was similar for both enantiomers but t0.5 was slightly longer for LVX than OFX. Based on MS experiments the photodegradation products of the studied fluoroquinolones and possible pathways of UV induced decay were identified.

Keywords: fluoroquinolones, photodegradation, chiral separation.

Introduction

Ofloxacin ((±)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido [1,2,3-de][1,4] benzoxazine-6-carboxylic acid) is a second generation fluoroquinolone. This chemotherapeutic agent is often used for treatment of infections caused by Gram-negative and atypical bacteria [1–3]. Ofloxacin is a racemate of two enantiomers, (R)-(+)-ofloxacin and (S)-(-)-ofloxacin (levofloxacin). Antibacterial activity of levofloxacin is 8–128 times higher than that of R-(+)-ofloxacin and two times higher than that of racemic mixture [4]. The situation that one of the enantiomers (eutomer) is more active than the other (distomer) is relatively common in pharmacology. It is estimated that 40% of available drugs are chiral compounds and 25% are used as pure enantiomers. Administration of drugs in the form of a pure enantiomer is much more beneficial for many reasons. It is possible to use a lower dose, thus reducing the risk of side effects or overdosing. It should be noted that even if distomer is inactive it is still metabolized by the liver. Its elimination from the commercial formulation allows to relieve the organ

[5−8]. Ofloxacin is used therapeutically in its racemate form and, since 1998, additionally as the enantiopure S-(-) isomer [9].

The photostability of the drug is one of the most important properties of the substance which affects not only the stability of the original drug, but also its phototoxicity in the human body. Fluoroquinolones including ofloxacin induce photosensitivity in humans at very low incidence [10, 11]. Photosensitivity includes phototoxicity and photoallergy. The phototoxic reaction is linked to exaggerated sunburn and the action spectrum of the toxicity is thought to be included in the wavelength range of UVA. The photoallergenicity of fluoroquinolones is mainly derived from their photohaptenic moiety, and photomodification of skin epidermal cells with fluoroquinolones is thought to be an initial step for this photoallergy [12−14].

The current research presents a comprehensive study of ofloxacin and levofloxacin photostability in aqueous solutions. The study involved development of enantioselective high-performance liquid chromatography (HPLC) and high-performance capillary electrophoresis (HPCE) methods for separation of OFX enantiomers as well as their degradation products. These methods were used to monitor the degradation process of OFX and LVX under UV irradiation and to determine the kinetics of degradation of these antibacterial agents. Moreover, the identification of photoproducts was also attempted.

Experimental

Chemicals and reagents
Ofloxacin, levofloxacin, sparfloxacin (SPX, IS), pipemidic acid (PIP, IS) and 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) were obtained from Sigma Aldrich Co. (USA), L-isoleucine, $CuSO_4$, $HCOONH_4$, HPLC grade methanol and acetonitrile were obtained from Merck (Germany), H_3PO_4, NaH_2PO_4, NaOH were obtained from POCh S.A. (Poland). All chemicals used in this study were of analytical grade.

HPLC method development
An HPLC system (1200 Series, Agilent Technologies) containing a binary pump G1312A, autosampler HiP-ALS G1367B and diode array detector (DAD) G1315D (294.5 nm) was used. Separation of OFX, LVX and their photoproducts was performed using a reverse-phase column − Zorbax Eclipse XDB-C18 Analytical, 4.6x150 mm, 5-Micron (Agilent Technologies) at 20°C. Mobile phase was a mixture of: chiral mobile phase additives (CMPA):methanol 88:12 (v/v). CMPA consisted of 5 mmol/L L-isoleucine and 4 mmol/L of copper (II) sulfate. The flow rate was maintained at 0.75 mL/min.

Calibration curves were constructed in the range of 5−200 μg/mL for both OFX and LVX with addition of PIP (100 μg/mL) as the internal standard. All calibration curves solutions and mobile phase were filtered through the 0.45 μm nylon-membrane filters (Millipore).

Calibration curves of OFX and LVX were constructed for their area under the peak over IS peak area ratio (A/A_{IS}) as a function of drug concentrations. The results showed good linearity throughout the examined concentration ranges for both racemic mixture and S-(-)-enantiomer. The linear correlation equations were y = 0.1130 ± 0.0010x-0.0625 ± 0.0086 (R^2 = 0.999) for OFX and y = 0.1124 ± 0.0002x-0.0483 ± 0.0090 (R^2 = 0.999) for LVX. For validation of the method, parameters such as selectivity, limit of detection (LOD), limit of quantification (LOQ), linearity, accuracy and precision (repeatability) were investigated (**Table 1**). The proposed RP-HPLC method showed good selectivity that was proved by the fact that the mobile phase chromatogram contained no peaks at the retention times corresponding to OFX and LVX.

HPCE method development
The enantioseparation analysis of ofloxacin and levofloxacin was conducted on G1600 HPCE system (Agilent Technologies), with DAD detection. A fused silica capillary was used with a total length of 64.5 cm and an effective length of 56 cm. New capillary was con-

Table 1. The obtained values of limit of detection, limit of quantification and precision for HPLC and HPCE methods

		LOD [μg/mL]	LOQ [μg/mL]	Precision (RSD, %)
ofloxacin	HPLC	0.01	0.03	0.03
	HPCE	0.07	0.20	0.06
levofloxacin	HPLC	0.01	0.02	0.03
	HPCE	0.07	0.20	0.01

LOD − limit of detection, LOQ − limit of quantification, RSD − relative standard deviation, HPLC − high-performance liquid chromatography, HPCE − high-performance capillary electrophoresis

ditioned by rinsing with 0.1 mol/L NaOH for 15 min, methanol and deionized water for 10 minutes. Before each analysis the capillary was preconditioned according to the following schedule: 1.0 mol/L NaOH for 1.5 min., water for 1 min and finally background electrolyte (BGE) for 1.5 min. Moreover, after each run the capillary post conditioning procedure was also involved: 0.1 mol/L HCl, methanol and water for 1 min. As a background electrolyte the phosphate buffer (pH = 2.5) with 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) as a chiral separator was used. Phosphate buffer (pH = 2.5) was prepared by mixing an appropriate aliquots of orthophosphoric acid solution (50 mmol/L) with sodium dihydrogen phosphate solution (50 mmol/L). The pH of the buffer was adjusted to 2.5 by addition of orthophosphoric acid (50 mmol/L) or sodium hydroxide (0.1 mol/L) solutions. Afterwards, the amount of 2-hydroxypropyl-β-cyclodextrin was dissolved in phosphate buffer to obtain the final concentration (HP-β-CD) of 40 mmol/L. Finally the BGE solution was filtered through the 0.65µm nylon-membrane filters (Millipore) and degassed in ultrasonic bath for two minutes.

The samples of OFX and LVX were injected in hydrodynamic mode directly onto capillary under the pressure of 50 mbar for 7 seconds. The enantioseparation was performed in positive mode, in electric field of 25kV for 30 minutes and detection was set at 296.4 nm, at the maximum absorption of fluoroquinolone molecule. The HPCE studies were performed in the range of 10-150 µg/mL for both OFX and LVX in the presence of sparfloxacin (SPX) (25 µg/mL) as the internal standard. The calibration curves of OFX and LVX were plotted as peak areas ratio (A/A_{IS}) as a function of their concentration (µg/mL). It was stated that the HPCE enantioseparation method, used in presented studies exhibits good linearity in the analyzed range of concentrations and good coefficient of determination (R^2). The calibration curves were described by following equations: y = 0.03425(±0.00071)x+0.0702(±0.0587), R^2 = 0.999 for OFX and y = 0.03287(±0.0007)x-0.0917(±0.0593) R^2 = 0.999 for LVX. The method was validated and the parameters of the validation are presented in **Table 1**.

MS conditions

Hybrid triple quadrupole/linear ion trap mass spectrometer 4000 QTRAP (Sciex) with electrospray ion source (TurboIonSpray source) was used. The MS measurements were carried out in positive ionization mode. Ion spray voltage, entrance potential, declustering potential were set at 5500 V, 10 V and 30 V, respec-

tively. Nitrogen was used as a curtain gas (10 psig). In MS/MS measurements nitrogen was used as a collision gas (medium).

Photostability study

Photodegradation study of OFX and LVX was performed following the recommendations of the ICH (1QB Photostability Testing of New Drug Substances and Products). The concentration of analyzed solutions of both OFX and LVX was 100 µg/mL. The pH = 4.5 was obtained by dissolution of amounts of OFX and LVX in 2.5 mL of 0.1 mol/L HCl solution. Solutions were filtered through the 0.45µm nylon-membrane filters (Millipore) and irradiated for 210 min in a quartz cylindrical cuvette placed at a distance of 15.5 cm from a high pressure mercury lamp (HBO-50 NARVA, emission of UVB-UVA = 280–400 nm, intensity 0.119–0.125 mW/cm^2). The irradiation process was performed in triplicate.

During HPLC study of the photodegradation process of OFX and LVX a sample of 60 µL was taken every 15 min, mixed with 10 µl of PIP (714.51 µg/ml) as an IS and introduced onto the column in triplicate. For HPCE study of the degradation of OFX and LVX aqueous solutions a sample of 60 µL was taken every 15 min, mixed with 10 µl of SPX (316.4 µg/ml) as an IS and introduced into the HPCE system in triplicate. For MS analysis irradiated solutions (0, 60, 120, 180 min) were evaporated to dryness (vacuum concentrator miVac Duo, Genevac). Dry residue was dissolved in 1 mL of ammonium formate solution (0.005 mol/L in water:methanol 50:50 v/v). Before MS analysis solutions were filtered through the 0.45 µm nylon-membrane filters (Millipore). Solutions were introduced to MS system directly from a syringe pump.

Results and Disscusion

The enantioseparation of OFX and LVX and their photodegradation products were achieved by HPLC and HPCE methods.

A chiral ligand-exchange reversed-phase HPLC method for the determination of OFX- racemic mixture, LVX and monitoring of their photodegradation processes was developed. Before the irradiation three peaks were observed on the OFX chromatogram (1− pipemidic acid 10.68 min; 2− (S)-ofloxacin 15.85 min; 3− (R)-ofloxacin 19.64 min) and two on the LVX chromatogram (1− pipemidic acid 10.68 min; 2− (S)-ofloxacin 15.85 min) (**Figure 1**). The complete decomposition of both drugs occurred after 150 min of irradiation (no peaks corresponding to OFX or LVX occurred). Peaks corre-

sponding to the products of degradation (FP) on chromatograms were observed. The number of photoproducts was different for OFX and LVX. LVX decomposed with formation of only one FP (3.81 min, appeared after 90 min of irradiation) while two FPs formed in case of OFX (3.81 and 4.62 min, appeared after 120 min of irradiation) (**Figure 2**). It was found that the compounds which were formed by the decomposition of OFX were the pair of enantiomers, while in the case of LVX a single peak was observed probably with the S-(-)- configuration. Moreover, it is supposed that after 90 min of the light exposure some part of LVX molecules changed spatial configuration and peak with t_r = 19.64 min appeared on the chromatogram.
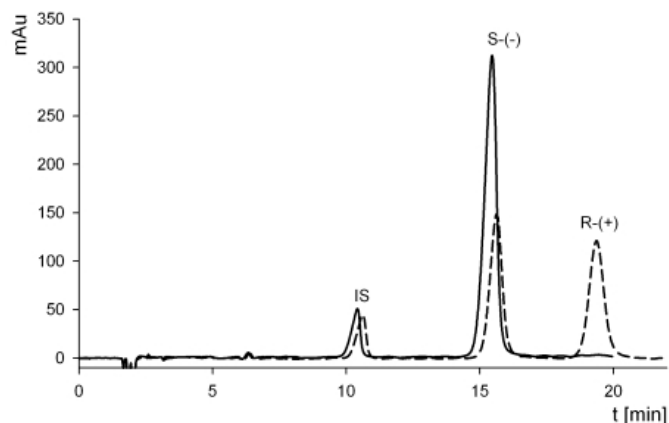
Figure 1. Chromatogram of ofloxacin (---) and levofloxacin () before the irradiation. IS – internal standard, S-(-) – (S)-(-)-ofloxacin (levofloxacin), R-(+) – (R)-(+)-ofloxacin
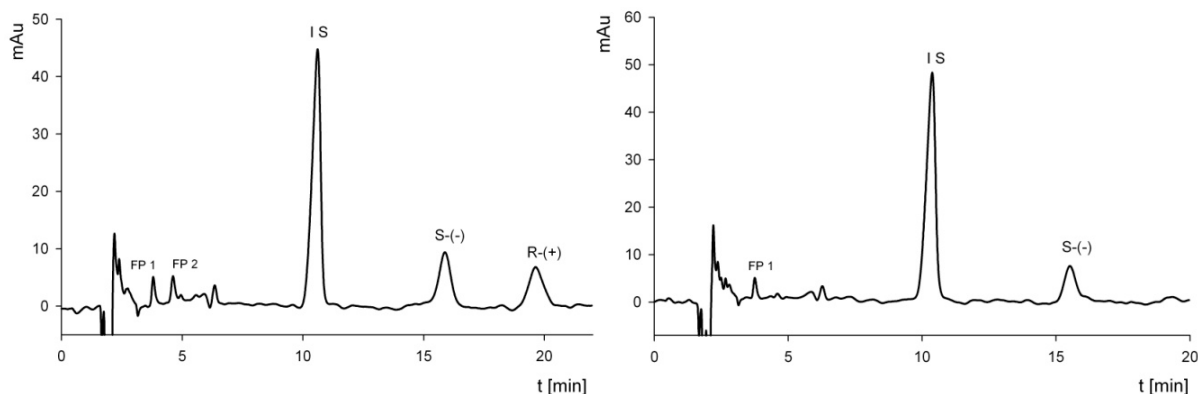
Figure 2. Chromatograms of ofloxacin (left) and levofloxacin (right) after 120 min of irradiation. FP 1 – product of degradation 1, FP 2 – product of degradation 2, IS – internal standard, S-(-) – (S)-(-)-ofloxacin (levofloxacin), R-(+) – (R)-(+)-ofloxacin
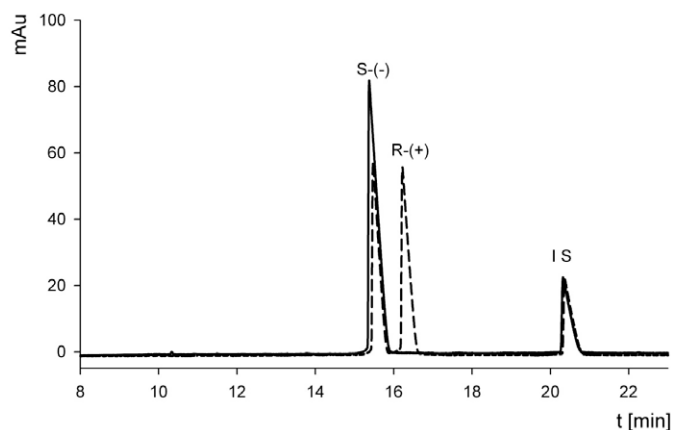
Figure 3. Electropherograms of ofloxacin (---) and levofloxacin () before the irradiation. S-(-) – (S)-(-)-ofloxacin (levofloxacin), R-(+) – (R)-(+)-ofloxacin, IS – internal standard

In HPCE method HP-β-cyclodextrin was used as a chiral selector, and allowed to separate S-(-) and R-(+) enantiomers from the racemic ofloxacin. Before the irradiation three peaks were observed on the OFX electropherogram (1– S-(-)-ofloxacin 15.25 min; 2– R-(+)-ofloxacin 15.98 min; 3– sparfloxacin as IS 20.36 min) and two on the LVX electropherogram (1– (S)-ofloxacin 15.29 min; 2- sparfloxacin as IS 20.34 min) (**Figure 3**). It was noticed that concentration of OFX and LVX decreases over the time of irradiation, while new peaks photoproducts appeared on electropherograms. Concentration of photoproducts increased with the light exposure time (**Table 2**, **Figure 4**).

The presence of six main degradation products of LVX (A-F) and eight of OFX (B, C, C', D, D', E, E', F) was observed. Six of OFX photoproducts formed three pairs of peaks with slight differences in their migration times (C and C', D and D', E and E'). On the LVX electropherogram there were observed only single peaks (C, D, E). In addition, in case of OFX each pair of peaks appeared after the same time of exposure. This means that the photodegradation products still retained properties of original chiral substances. It was also likely that during the photodegradation of LVX certain percentage of S-(-)-ofloxacin was converted into R-(+)-ofloxacin (FP A). It can be proved by the same migration times of FP A and R-(+)-ofloxacin.

Based on the obtained results (HPLC and HPCE) of decrease in the concentrations of examined compounds after increase of the irradiation time, the kinetics of the decomposition of OFX and LVX in aqueous solutions was determined. The rates of photodegradation reaction was time and product formation dependent – autocatalyzing reaction. The curves of concentration changes vs time were characterized by sigmoidal shape. For both of the tested compounds increasing of the reaction rate was observed in the initial stage of the reaction (15–30 min) and decreasing in the final stage of exposure to light (195–210 min). The course of photodegradation of the tested compounds was best described by the second-order autocatalytic reaction equation according to the Prout-Tompkins model.

The second-order autocatalytic reaction follows the model:

$$A \xrightarrow{c} C$$
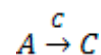
where: A – substrat, C – product and autocatalyst.

The concentration changes of the OFX and LVX during irradiation were described by the equation:

$$ln\frac{[C]}{[A]} = ln\frac{[C]_0}{[A]_0} + ([C]_0 + [A]_0)kt$$

where: $[C]$, $[A]$ – concentration of the product and substrate respectively, $[C]_0$, $[A]_0$, – the initial concentrations of the product and substrate respectively, k – the rate constant, t – time.

Table 2. Migration times [min] of ofloxacin and levofloxacin degradation products

Degradation product	A	B	C	D	E	F
Irradiation time [min]	90	90	120	135	165	165
ofloxacin	-	14.50	12.87 (C); 13.02 (C')	13.64 (D); 13.80 (D')	13.32 (E); 13.38 (E')	16.33
levofloxacin	15.98	14.53	12.86	13.63	13.29	16.38
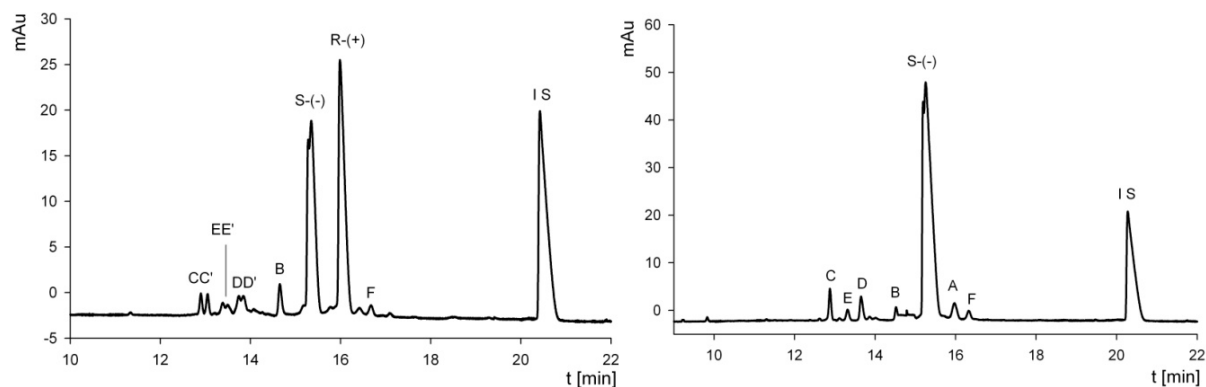
Figure 4. Electropherograms of ofloxacin (left) and levofloxacin (right) after 165 min of irradiation. S-(-) – (S)-(-)-ofloxacin (levofloxacin), R-(+) – (R)-(+)-ofloxacin, IS – internal standard, A, B, C, C', D, D', E, E', F – degradation products

Table 3. Kinetic parameters of ofloxacin and levofloxacin photodegradation reactions determined by HPLC and HPCE methods

	k [s^{-1}]	$t_{0.1}$ [min]	$t_{0.5}$ [min]
HPLC			
ofloxacin	5.43×10^{-4}	39.28	88.11
levofloxacin	6.05×10^{-4}	58.86	105.61
HPCE			
ofloxacin	5.34×10^{-6}	65.28	130.07
levofloxacin	5.76×10^{-6}	72.93	135.70

HPLC – high-performance liquid chromatography, HPCE – high-performance capillary electrophoresis

Based on the theory of the second-order autocatalytic reaction the Prout-Tompkins model was used to describe the decomposition of the substance, according to the following equation:

$$ln\frac{c_0 - c}{c} = \gamma t + const$$

where: c – percentage concentration of substrate, $(c_0 - c)$ – the difference between the initial concentration of substrate and the concentration at time t, γ – slope, $const$ – integration constant.

Calculated kinetic parameters of OFX and LVX photodegradation processes are presented in **Table 3**.

The analysis of the photodegradation products of ofloxacin and levofloxacin was also performed by mass spectrometry. The spectra of fluoroquinolones before the irradiation were compared with those after 60, 120 and 180 min of exposure to UV light. Peaks corresponding to the photoproducts were selected by comparing their intensity on the spectra of the solutions before and after irradiation and then an attempt was made to identify them. After 60 min of irradiation only one photoproduct of *m/z* 336.0 appeared. After 120 min of irradiation more degradation products were observed in the mass spectrum: *m/z* 348.0; *m/z* 346.0; *m/z* 336.0; *m/z* 322.0; *m/z* 279.0; *m/z* 103.0 and *m/z* 101.0. The number of the photoproducts decreased after 180 min of irradiation and the following peaks were detected in the spectrum: *m/z* 346.0; *m/z* 294.0; *m/z* 279.0; *m/z* 103.0 and *m/z* 101.0. It should be noted that m/z values of some FPs differed by 2 (348 and 346, 103 and 101) indicating the possibility of loss of 2 hydrogens leading to the formation of double bond. Our observations were consistent with previous literature data, which pointed formation of double bonds as characteristic for UV induced degradation of fluoroquinolones [15].

In the next step, major photoproducts of the examined fluoroquinolones were fragmented using MS/MS experiments. Fragmentation made it possible to better define the structure of photoproducts. Based on the fragmentation mass spectra an attempt was made to determine the putative degradation pathways and the chemical structure of
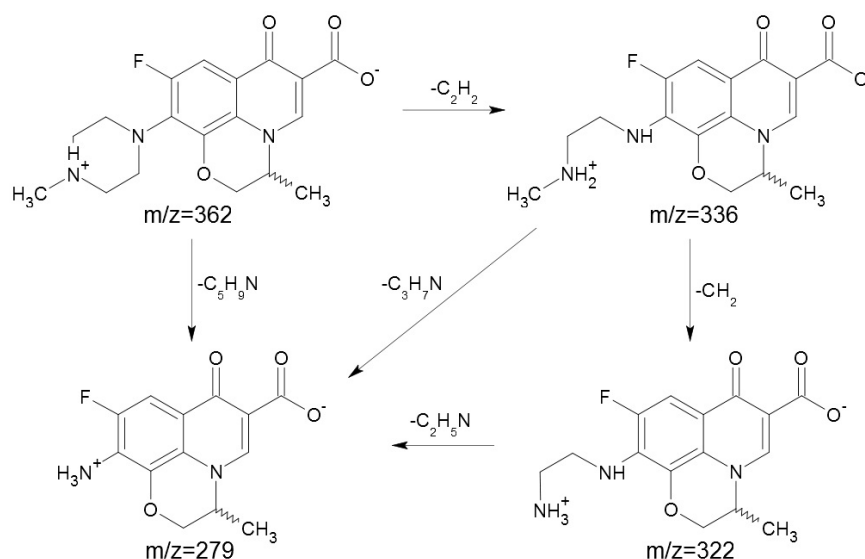
Figure 5. The diagram of ofloxacin and levofloxacin photodegradation

the formed compounds (**Figure 5**). In aqueous solutions, under the influence of UV light, the piperazine ring decomposes first. On the basis of *m/z* values of FPs it can be concluded, that disintegration proceeds by the removal of small fragments or the whole ring. In examined conditions (low pH = 4.5) there is neither fluorine atom nor carboxylic group separation observed.

Conclusions

The purpose of this study was to separate and identify products created in the photodegradation process of ofloxacin and levofloxacin and to determine the kinetics of the decomposition reactions. In order to achieve that, solutions of ofloxacin and levofloxacin were prepared and irradiated using a mercury-vapor lamp for various time periods up to 210 minutes. Photoproducts separation was carried out using two analytical techniques: a chiral ligand-exchange reversed-phase HPLC and HPCE with 2-hydroxypropylo-β-cyclodextrin as a chiral selector. It was found that numerous products appeared as a result of photodegradation. Using HPLC method it was possible to observe two products of OFX degradation and only one for LVX, while using an HPCE method eight FPs of OFX and six of LVX were observed. It was concluded that some of the photoproducts still retain character of optically active compounds. It is supposed that during irradiation some amount of (S)-ofloxacin enantiomer is converted into (R)-ofloxacin. The course of photodegradation of the tested compounds is best described by the autocatalytic reaction proceeding according to the Prout-Tompkins model. Some of the products of the decomposition are also catalyzing this reaction. Based on MS experiments it was possible to identify the photodegradation products of the studied fluoroquinolones and possible pathways of UV induced decay.

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