Journal of Photochemistry and Photobiology A: Chemistry 198 (2008) 268–273

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology A: Chemistry

journal homepage: www.elsevier.com/locate/jphotochem

UV light induced photodegradation of liposome encapsulated fluoroquinolones: An MS study

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ARTICLE INFO

Article history:
Received 19 October 2007
Received in revised form 29 March 2008
Accepted 4 April 2008
Available online 14 April 2008

Keywords:
Fluoroquinolone
Liposome
Photodegradation
Mass spectrometry
UV

ABSTRACT

Fluoroquinolone antibacterial agents are among the drugs most commonly causing phototoxic side effects. The phototoxicity may be originated in formation of reactive oxygen species upon ultraviolet exposure. Researches aiming the liposomal encapsulation of fluoroquinolones, expecting an increase in their therapeutic index, enhance the importance of studies on physicochemical properties and photostability of liposomal preparations. We studied the photodegradation of ciprofloxacin, ofloxacin and lomefloxacin by mass spectrometry upon various doses of UV irradiation. Lomefloxacin, the most phototoxic fluoroquinolone among them, was encapsulated into small unilamellar and multilamellar liposomes. Impact of vesicle structure and lipid composition – the presence of unsaturated fatty acid containing dioleoylphosphatidylcholine in dipalmitoyl-phosphatidylcholine liposomes – on the lomefloxacin photolysis was investigated; the structure of the main photoproducts was identified by mass spectrometry. It was found that the presence and type of lipids influence the ways of photodegradation process.

1. Introduction

Fluoroquinolones are the only synthetic antibacterial agents to rival β-lactams for impact and usage in the antibacterial field. In two decades, they moved from a small group of drugs used predominantly in urinal tract infection to agents with a remarkably broad spectrum of activity and excellent pharmacokinetics [1]. Among side effects induced by fluoroquinolones – in the presence of light – acute phototoxic reactions are by far the most common, and are generally characterized by erythema and oedema followed by hyperpigmentation and desquamation [2]. The fluoroquinolone antibacterials possess phototoxic properties both in human and animal subjects [3,4]. Their phototoxicity order reported is lomefloxacin (LMFX), pefloxacin (PEFX) ≫ ciprofloxacin (CPFX) > enoxacin (ENOX), ofloxacin (OFLX) [5], while others report LMFX > OFLX > CPFX [4]. Evaluating the molecular background, the formation of reactive oxygen species (ROS) appears to be responsible for the light induced adverse effects [6,7]. Quinolones can undergo a variety of photochemical processes such as generation of singlet oxygen, production of superoxide, decarboxylation, defluorination and oxidation of an amino substituent at C7 [8]. According to some recent studies, beside ROS, the presence of highly reactive species arising from direct photolysis of fluoroquinolones (e.g. LMFX) can justify the photogenotoxic properties associated with fluoroquinolones likely due to direct reaction of their cations with DNA [9,10]. A notable feature, particularly of C8 fluorinated fluoroquinolones such as LMFX is its ability to undergo photolysis and defluorinate upon UV-light exposure. UVA irradiation of 10 μg/ml of LMFX in phosphate buffer (pH 7.4) lead to more than 50% decomposition within 10 min [11]. Loss of the fluorine atom at C8 and partial breakdown of the piperazine ring occur. The only two photoproducts formed under these conditions are 1-ethyl-6-fluoro-1,4-dihydro-7-(2-aminoethyl-amino)-4-oxo-3-quinolinecarboxylic acid and 1-ethyl-6-fluoro-1,4-dihydro-7-(2-aminopropyl-amino)-4-oxo-3-quinolinecarboxylic acid [11]. The mechanism of photo-induced defluorination of LMFX in aqueous media is published [11–15] but there are no data on the loss of fluorines in other media including the circumstances of nano-delivery systems, e.g. liposomes.

Innovative technologies aim to increase the bioavailability of fluoroquinolones by means of liposomes. It seems clear that a well-designed lipid carrier system allows increased drug concentration at the sites of action but reduces drug toxicity [16]. However, it is necessary to investigate the effects of liposomal encapsulation not only on the therapeutic efficacy of the encapsulated drug, but also on the extent of ROS generation and photodegradation of the encap-

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doi:10.1016/j.jphotochem.2008.04.004

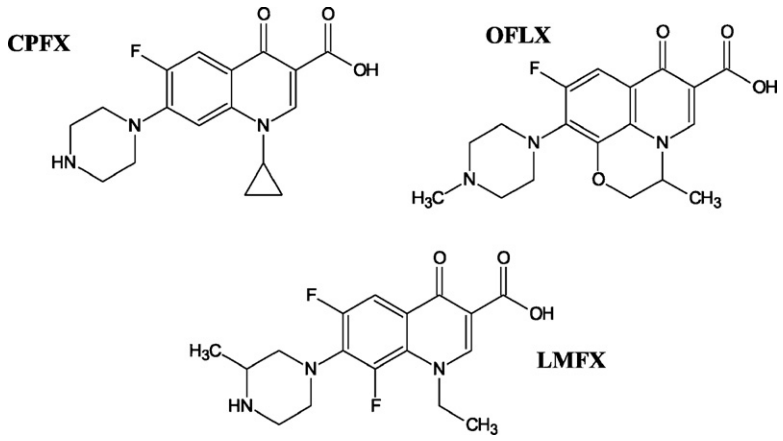

Fig. 1. Chemical structures of ciprofloxacin (CPFX), ofloxacin (OFLX) and lomefloxacin (LMFX).

sulated fluoroquinolones upon UV-irradiation. This latter informs about the photostability of liposomal fluoroquinolones in the presence of UV-exposure that is of prominent importance in case of their UV-sterilization, storage and topical administration.

In our earlier work we investigated a prominent member of the quinolones, the nalidixic acid, and observed increased free radical formation in the presence of α-L-dipalmitoyl-phosphatidylcholine (DPPC) [17]. In the present work we present a mass spectrometry (MS) study carried out on fluoroquinolone (CPFX, OFLX and LMFX) containing aqueous and liposomal samples exposed to various UVB doses. We address the question whether the presence of liposomes alters the extent and the ways of fluoroquinolone photodegradation and whether the liposome structure influences the LMFX degradation. LMFX containing small unilamellar vesicles (SUV) and multilamellar vesicles (MLV) composed of DPPC were examined. In order to gain further pieces of information on the role of lipid composition and the effect of unsaturated fatty acid containing lipids on the rate of LMFX photodegradation, SUVs prepared from a mixture of DPPC and dioleoyl-phosphatidylcholine (DOPC) (70/30 mol/mol%) encapsulating LMFX were also studied.

In each case the degradation constant for the encapsulated agent and the structure of photoproducts formed as a result of irradiation were determined using MS or MS–MS technique.

2. Materials and methods

2.1. Fluoroquinolone containing solutions

Non-irradiated and irradiated samples of 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, OFLX) (Sigma Chem. Co.), ciprofloxacin (1-cyclopropyl-6-fluoro-4-oxo-7-piperazin-1-yl-quinoline-3-carboxylic acid, CPFX) (Sigma Chem. Co.) and lomefloxacin (1-ethyl-6,8-difluoro-7-(3-methylpiperazin-1-yl)-4-oxo-quinoline-3-carboxylic acid, LMFX) (Sigma Chem. Co.) dissolved in distilled water in a concentration of 0.2 mM were used for MS and MS–MS measurements.

2.2. Preparation of liposomes

2.2.1. Multilamellar vesicles (MLVs)

These were prepared using the thin-film hydration method. 10 mg lipid, α-L-dipalmitoyl-phosphatidylcholine (DPPC) (Sigma Chemical Co.) or a mixture of DPPC and dioleoyl-phosphatidylcholine (DOPC) (Sigma Chemical Co.) (∼7.0 and ∼3.0 mg, respectively) were dissolved in absolute ethanol (Merck). The mixture was dried to thin-film under nitrogen stream. The rest of the solvent was removed from the lipid film in 30 min using a vacuum-line and the samples were stored in a desiccator overnight. Thin lipid films were hydrated above the main-transition temperature of DPPC, at ∼50 °C. 5.0 ml of aqueous solutions of ofloxacin, ciprofloxacin or lomefloxacin in concentrations of 0.2 mM were used for hydration, resulting in a final lipid concentration of 2 mg/ml. In case of 0.2 mM final fluoroquinolone concentration, the liposomes contained the drug in a lipid/fluoroquinolone molar ratio of ∼13:1. Control liposomes were hydrated with distilled water. The liposomes neither were extruded, nor were the non-entrapped fluoroquinolone fractions removed.

2.2.2. Small unilamellar vesicles (SUVs)

These were prepared from the MLV suspensions by sonication in a Soniprep 150 MSE device – above the main phase transition temperature – with two times 10 min sonication (frequency of 20 kHz and wave amplitude of 8.5 μm), and 10 min pause between sonications.

2.3. Irradiation procedure

FS-20 lamp (with a nominal power of 40 W) emitting mainly in UVB region (λ = 280–320 nm) was used for irradiation experiments. Aqueous or liposomal preparations of fluoroquinolones in uncovered Petri dishes were exposed to UVB light at room temperature. Irradiation intensity and power were measured with a WLX-3W radiometer (Vilbert-Lourmat, France) equipped with a CX-312 sensor of interference type. During irradiation the UVB-intensity was kept at 1.1 ± 0.1 mW/cm^2 at the samples' level. The irradiance was between 0 and 22 kJ/m^2.

2.4. Analytical method

Agilent 1100 series LC-MSD SL type mass spectrometer with electrospray ion source (ESI/MS) (Germany, HP ChemStation software) was used to perform MS measurements in positive mode. 15 μl samples were injected. Capillary voltage was 4000 V and fragmentor was 50. Quadrupole scanned over the range m/z 50–1000. The capillary temperature was kept at 350 °C. As nebuliser and drying gas nitrogen was applied with 35 psi and 13 l/min, respectively.

MS/MS measurements were carried out with a PE Sciex API 2000 mass spectrometer with TurboIonSpray source in positive mode. The temperature of the ion source was kept at 300 °C. The orifice voltage was optimized between 30 and 60 V in order to achieve optimal fragmentation of the ions. Nebuliser, curtain and collision

gas was nitrogen. Nebuliser and drying gases were applied with 35 psi and 13 l/min, respectively.

3. Results and discussion

For aqueous solutions of OFLX, LMFX and CPFX (Fig. 1) in a fluoroquinolone concentration of 0.2 mM, pH values of 6.38; 5.53 and 5.78 were measured, respectively. Compared to the liposome-free aqueous LMFX solution (pH 5.53) the presence of DPPC or DPPC/DOPC (which are mostly in zwitterionic form under the conditions studied) do not significantly influence the pH of the liposomal LMFX suspensions (data not shown here). In preliminary measurements it was checked that the UV-dose (max. 22 kJ/m^2) applied in our experiments does not lead to a degradation of DPPC and DOPC lipids resulting in formation of products with lower or higher m/z values, than m/z values determined for standard DPPC and DOPC. m/z values for lipids were 735 and 787, respectively. Furthermore, it was proven that the preparation procedure of SUVs – the sonication of MLVs – does not lead to the formation of lipid fragments; DPPC and DOPC are stable under the conditions studied.

Literature data give evidence that the photochemical degradation of quinolones in aqueous solution proceeds in two main steps [18,19]. The first step is the degradation of the piperazine substitution forming 7-amino compounds which are more stable against photolysis than the corresponding parent compound. The next step is the photochemical degradation down to CO_2 formed intermediate polar substances.

Many factors have been found to affect CPFX photodegradation, including light intensity, presence of organic compounds, pH, CPFX and phosphate levels, although unified conclusions have not been made on its fate [19–21]. CPFX degrades photochemically in acidic solutions, under irradiation with UV-light ($\lambda > 300$ nm) forming two main degradation products. The formation of 7-[(2-aminoethyl)amino]-1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-3-quinoline carboxylic acid (m/z 306) is dominant in aqueous acidic (pH < 5) solutions, whereas 7-amino-1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-3-quinoline carboxylic acid (m/z 263) is the main degradation product when the solvent contains water-miscible organic solvent [22].

According to our measurements 22 kJ/m^2 UVB irradiation of 0.2 mM aqueous CPFX (m/z 332.2) solution leads to the formation of photoproducts with the following typical m/z values: 330 [23], 306 [22,24,25] and 288 [23]. The relative intensity of photoproduct peaks is ~1–3% if CPFX's m/z peak intensity is taken as 100%.

Since irradiation of CPFX results in a loss of antibacterial activity and since all substances, parent drug as well as their photoproducts, are potential candidates for undesired side effects, quinolone drugs should be strictly protected from all light during storage and administration. As little as 1 h of exposure to room light is enough for the formation of detectable amounts of CPFX photoproducts [26].

The introduction of a methoxy-group into the eight position of quinolones plays an important role in the stability of fluoroquinolones against irradiation by UV light. LMFX exposed to 3 kJ/m^2 irradiation, or OFLX and CPFX exposed to 10 kJ/m^2 irradiation underwent absorption spectrum changes, an accompanying decrease in antibacterial activity and an increase in cytotoxic activity [27].

On the basis of MS peak intensities, degradation constants for the examined fluoroquinolones were determined in aqueous solutions (Table 1). For the determination of the degradation rate as a result of 3 kJ/m^2 irradiation, all detected peaks – not lower than 3% of the highest peak intensity – were taken into consideration. The sum of all considered relative peak intensity was taken as unity and divided into proportional parts among all considered peaks in order to gain comparable values to describe the degradation process. The photodegradation products of the examined fluoroquinolones – as standards – are not commercially available, thus their ionization rate under the MS conditions applied can not directly been determined. However, using the same circumstances (fragmentor voltage, nebulizer and curtain gas flow rate, capillary voltage and temperature, etc.) during all MS experiments, it can be supposed that all degradation products – possessing the same quinolone ground structure – show similar rate of ionization as the fluoroquinolones studied (LMFX, OFLX and CPFX). Photodegradation of LMFX and OFLX can be well described with exponential functions. The R^2 values determined for the exponential functions used to describe the degradation process are in the range between 0.892 and 0.996 (Table 1), reflecting good correlations between UV dose and photodegradation. Furthermore, the R^2 values can confirm the assumption that both cromogenic properties and ionization rate of the examined fluoroquinolone molecules and that of their photodegradation products are near to each other. For the aqueous LMFX solution the degradation constant is ~26 times higher than for the aqueous solution of OFLX, while for CPFX 3 kJ/m^2 irradiation does not result in the formation of detectable amount of photoproducts (22 kJ/m^2 UVB-irradiation was required to be able to determine the main photoproducts, see above).

Table 1
Degradation constants and correlation constants (R^2) determined for OFLX and LMFX in aqueous and liposomal media

Fluoroquinolone	Medium	Degradation constant	R^2
OFLX	Aqueous	−0.019	0.892
LMFX	Aqueous	−0.503	0.996
	DPPC SUV	−0.401	0.967
	DPPC MLV	−0.510	0.990
	DPPC/DOPC (70/30) SUV	−0.465	0.981

The concentration of the fluoroquinolones was 0.2 mM. In case of liposomal formulations the lipid concentration was 2 mg/ml. The applied UV-dose range was from 0 to 3 kJ/m^2.

On the basis of our results the order of photodegradation determined in aqueous media for LMFX, OFLX and CPFX seems to coincide with the order of phototoxicity reported by Horio et al. [4] (LMFX > OFLX > CPFX). The degradation constants for LMFX in liposomal suspensions are slightly different from that determined for the aqueous solution of LMFX. Because of the close proximity of degradation constant values it can be concluded, that the presence of lipids does not significantly accelerate or inhibit the UVB induced photodegradation of LMFX in comparison to that in aqueous medium.

For liposomal suspensions of LMFX the degradation rates do not seem to differ remarkably from each other. 1–25% alteration can be found in the degradation rate values among the liposomal LMFX samples. On the basis of the photodegradation rates determined for LMFX in DPPC SUVs, in DPPC MLVs and in DPPC/DOPC SUVs, no difference can be taken between the liposomal media with various lipid composition or lamellarity.

However, the ways of degradation and the photoproducts strikingly differ in various media. MS spectra showed evidences for degradation of the LMFX molecules even in the absence of UV irradiation. This can be due to hydrolytic degradation of the molecule which can result in two products identified by m/z as 316 and 288, from which the 316-fragment has been found in DPPC SUV samples only. Table 2 shows that on the bases of photoproduct m/z values there are differences between LMFX containing DPPC and DPPC/DOPC liposomes (Fig. 2). The presence of unsaturated fatty acid containing lipid in DPPC/DOPC liposomes leads to altered pathways of LMFX degradation in comparison to that of pure DPPC liposomes (Figs. 4–6). Comparing the LMFX degradation products

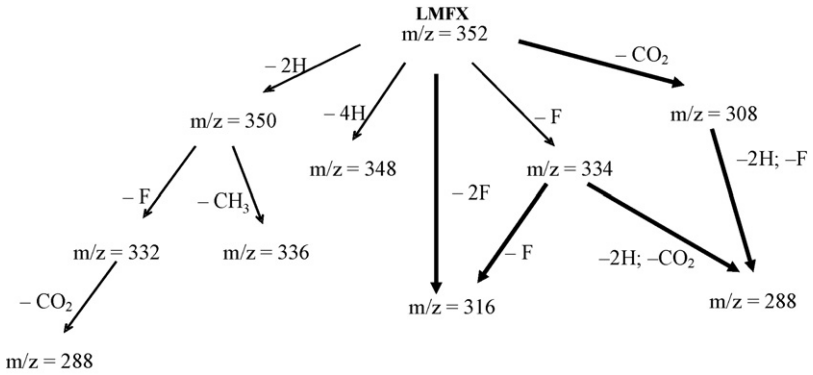

Fig. 2. Photodegradation ways of LMFX in aqueous and liposomal media showing the *m*/*z* values of the photoproducts.

Table 2
Degradation products of CPFX, OFLX and LMFX in aqueous and liposomal media

Fluoroquinolone	Medium	Characteristic photoproducts (*m*/*z* values)
CPFX [MH^+ = 332]	Aqueous	330; 306; 288
OFLX [MH^+ = 362]	Aqueous	348; 288; 101
LMFX [MH^+ = 352]	Aqueous	350; 348; 336; 332; 316; 308; 288
	DPPC SUV or DPPC MLV	350; 336; 332; 316; 308; 288
	DPPC/DOPC (70/30) SUV	350; 336; 332; 308; 288; 153; 69

The concentration of fluoroquinolones was 0.2 mM. In case of liposomal formulations the lipid concentration was 2 mg/ml.

in DPPC MLVs and DPPC SUVs, the lamellarity of liposomes does not seem to have an impact on the way of LMFX photodegradation (Figs. 4 and 5).

Evaluating the molecular changes as a result of UV-irradiation the formation of double bonds seems to be characteristic (Table 2 and Figs. 3–6). The MS–MS measurements – under artificial conditions – indicate the possibility of loss of 2, 4 or 6 hydrogens (*m*/*z* values are 350, 348 and 346) leading to the formation of 1, 2 or 3 double bonds, respectively, in the piperazine substitution of LMFX. However, under our experimental conditions, as a result of UV-irradiation, only *m*/*z* 350 and 348 can be detected in the samples

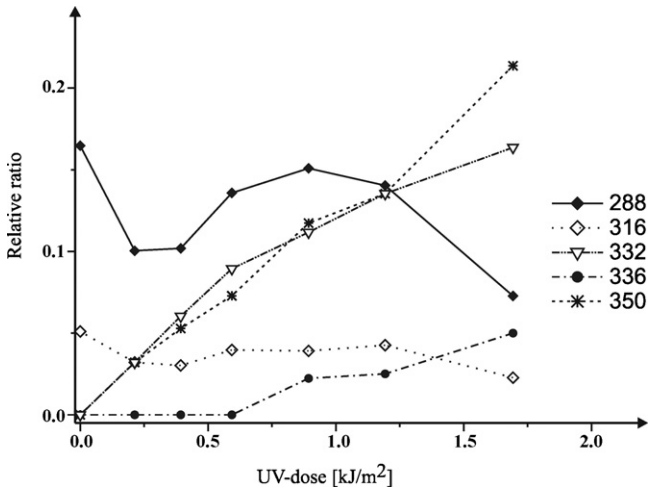

Fig. 4. Relative amounts of LMFX photoproducts *in DPPC SUVs* formed as a result of UVB irradiation. LMFX concentration was 0.2 mM. The lipid concentration was 2 mg/ml. All the peaks higher than 3% of the highest relative peak intensity of the MS spectra were analyzed. The sum of the analyzed peaks'relative intensity was taken as 1.

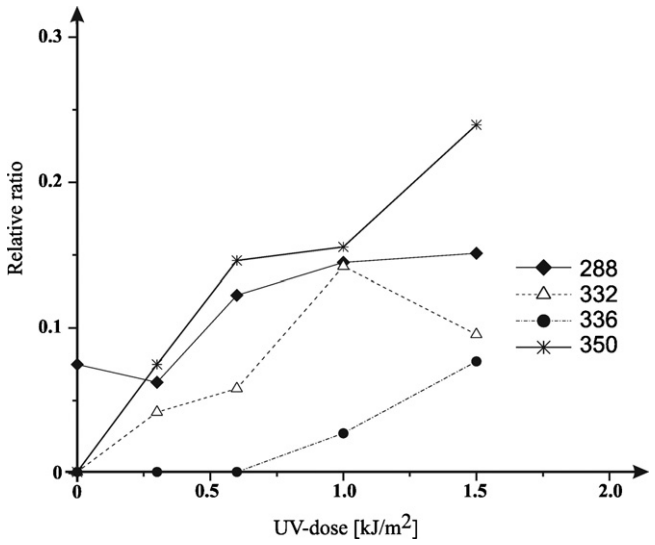

Fig. 3. Relative amounts of LMFX photoproducts *in water* formed as a result of UVB irradiation. LMFX concentration was 0.2 mM. All the peaks higher than 3% of the highest relative peak intensity of the MS spectra were analyzed. The sum of the analyzed peaks relative intensity was taken as 1.

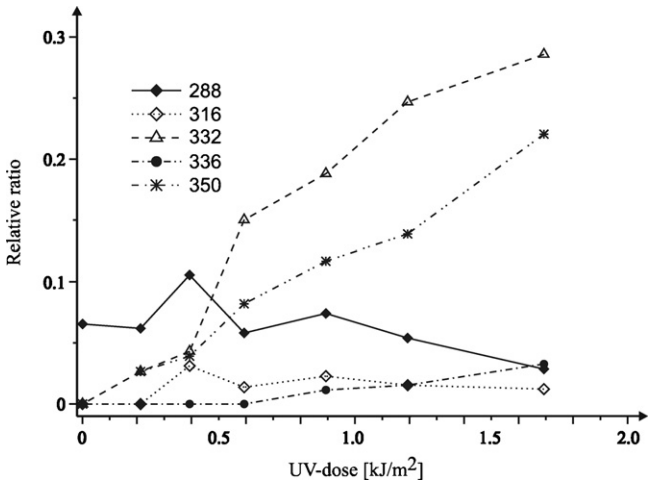

Fig. 5. Relative amounts of LMFX photoproducts *in DPPC MLVs* formed as a result of UVB irradiation. LMFX concentration was 0.2 mM. The lipid concentration was 2 mg/ml. All the peaks higher than 3% of the highest relative peak intensity of the MS spectra were analyzed. The sum of the analyzed peaks relative intensity was taken as 1.

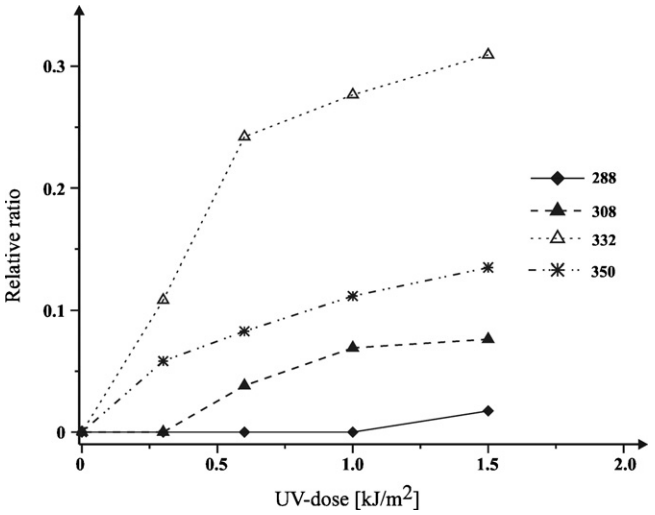

Fig. 6. Relative amounts of LMFX photoproducts *in DPPC/DOPC (70/30)* SUVs formed as a result of UVB irradiation. LMFX concentration was 0.2 mM. The lipid concentration was 2 mg/ml. All the peaks higher than 3% of the highest relative peak intensity of the MS spectra were analyzed. The sum of the analyzed peaks relative intensity was taken as 1.

(Fig. 2). The hydrogen loss seems to be more pronounced in the polar aqueous phase than in the liposomal LMFX samples.

The UV-light induced defluorination of LMFX can be observed not only in aqueous, but also in liposomal media. The loss of C6 and C8 fluorine atoms can be well detected. The loss of the first fluorine (resulting in *m*/*z* 334) is promptly followed by the loss of the other fluorine substituent (*m*/*z* 316) (Fig. 2). MS–MS measurement does not allow the position determination of the lost fluorine atoms. While in the MS–MS fragmentation spectra both fragments (*m*/*z* 334 and 316) can be well detected, under our experimental conditions the loss of both fluorine substituents seems to be preferred in comparison to the loss of only one of the fluorine substituents (it can explain the absence of *m*/*z* 334 peak). In pure DPPC vesicles the formation of *m*/*z* 316 is more common than in aqueous or unsaturated liposomal medium.

It is remarkable, that energetically the dehydrogenation followed defluorination leading to the formation of *m*/*z* 332 is favored in comparison to the loss of both fluorine substituents. The *m*/*z* 332 is the most typical degradation product in DOPC/DPPC liposomes, but in high amounts can be detected in pure DPPC vesicles and can be found in aqueous LMFX medium, too.

The characteristic feature of LMFX photodegradation is that either both fluorine substituents are lost – nearly at the same time – or following one double bound formation in the piperazine ring one of the fluorine substituents is lost. This latter way is more preferred in the liposomal systems containing double bonds in their fatty acid chains, while double defluorination mostly occurs in pure DPPC SUVs and MLVs (Figs. 4–6).

The *m*/*z* 336, as a fragmentation product of once dehydrogenated LMFX (*m*/*z* 350) can be detected in each LMFX sample. The *m*/*z* 336 photoproduct is very likely formed due to $-CH_3$ loss of the piperazine substitution (Figs. 2–6).

The CO_2 loss of LMFX resulting in the formation of *m*/*z* 308 does not play such a crucial role in the degradation process as –F or $-CH_3$ loss. Although CO_2 formation is present in all samples, the reaction most often occurs in unsaturated liposomal samples (Fig. 6). Probably, the feature of CO_2 loss – the reaction is accelerated due to free radicals – gives explanation, that the unsaturated lipid environment enhances the CO_2 formation. Degradation pathways has been studied for a great number of carboxylic acids, and was found that adjacent conjugated bonds and electron-withdrawing groups, which is in accord with the LMFX structure as well, promote loss of CO_2 groups [28]. According to our earlier results the presence of unsaturated bonds in the lipid molecules increases the free radical production due to UV-irradiation [17].

Providing the structural relationship of fluoroquinolones, the ground structure – *m*/*z* 288 – can be detected in all LMFX samples and is formed from all the three examined fluoroquinolones (LMFX, OFLX, and CPFX). Although samples were light protected before irradiation, hydrolytic degradation of LMFX can already be detected without UV-irradiation. Detailed degradation pathway for the above fragment in non-irradiated samples could not be deduced from the MS spectra, but one should account with dehydrogenation (–2H) and loss of F as well CO_2 groups. We detected differences in the amount of the 288-fragment in non-irradiated samples. It was the smallest one in aqueous solution and in DPPC-MLV containing samples, and the highest one in DPPC SUVs. On the contrary, no any degradation has been detected in DOPC/DPPC-samples without irradiation. We suppose that origin of the LMFX-degradation can be lipidperoxidation – in liposome containing samples – or presence of other type of free radicals (e.g. OH-radical), which in a subsequent step impacts the double bonds of LMFX [29]. The inverse reaction has already been described [30], where fluoroquinolones provoked peroxidation of unsaturated lipid squalene. This is inline with our observation that the presence of DOPC hinders the degradation of LMFX – without UV-irradiation – by reacting with the possible free radicals formed. (Interestingly when we used methanol/water 50/50 (w/w) mixture to dissolve LMFX for MS measurements, no signal was observed at *m*/*z* 288. This can be in agreement with the radical scavenging role of methanol.) During UV-irradiation the amount of photoproduct with *m*/*z* 288 increases as can be seen in Figs. 3–6.

In the preliminary phase of liposomal drug design one also has to take into consideration the impact of liposomal environment on the reaction ways of UV-induced LMFX photodegradation. It would be worth determining the in vivo toxicity of the LMFX photodegradation products, in order to be able to find the optimal liposomal carrier—however, it is beyond the scope of our present work.

4. Conclusions

Comparing the photostability of the examined fluoroquinolones (CPFX, OFLX and LMFX), LMFX was found to be the most photolabile. The presence of lipids does not accelerate the degradation process of LMFX, however, compared to aqueous phase it alters the ways of degradation leading to the formation of different photoproducts. In pure DPPC liposomes the double defluorination is a common and characteristic way of LMFX photodegradation in comparison to aqueous medium. While lamellarity of the DPPC vesicles does not influence the LMFX photodegradation, lipid composition has a significant impact on it: the presence of unsaturated fatty acid chains in the liposomal bilayer alters the LMFX-photodegradation-ways, making the CO_2 loss more common and increasing the frequency of dehydrogenation followed defluorination.

Our results are of high concern from the aspect of liposomal LMFX formulations: topical dosage forms can be exposed to natural or artificial UV-sources after administration and aseptic liposomal formulations may require UV-irradiation for their sterilization.

Acknowledgement

The authors are grateful to the Department of Mass Spectrometry of the Chemical Research Centre of the Hungarian Academy of Sciences for the MS–MS measurements.

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