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ORIGINAL ARTICLE

Photostabilization of oxolinic acid in hydroxypropyl-β-cyclodextrins; implications for the effect of molecular self-assembly phenomena

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Abstract Oxolinic acid (OXA) is a first-generation quinolone antibacterial agent, known to cause drug induced photosensitivity. In the present work its photoinduced degradation was monitored under simulated solar irradiation. The effect of photoprotecting agents on OXA stability was also assessed by drug complexation with hydroxypropyl-β-cyclodextrin (HPβCD). The complex was studied by UV-Vis and ^{1}H (2D) NMR Spectroscopy. A photostability indicating chromatographic method was developed and validated. Because OXA is insoluble in acidic solutions, and because an acidic solvent is necessary for successful chromatographic separation, a procedure was developed to pre-treat the sample. This method is suitable for the separation of degradation products from OXA and from each other. The method was also evaluated in the presence of HPβCD, in order to ensure that inclusion complexation did not generate inaccuracies. Investigation of OXA photodegradation profiles confirms first order kinetics and acceleration at higher initial sample concentrations. A 94% photostabilization upon complexation with HPβCD was achieved. Furthermore, molecular self association phenomena were determined by self titration experiments, using ^{1}H NMR Spectroscopy and suggestions were made for the photostabilization mechanism of cyclodextrins.

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Introduction

Oxolinic acid (OXA) is a first-generation quinolone antibacterial agent (Fig. 1). It is commonly prescribed for the treatment of Gram-negative urinary tract infections and is generally used in veterinary applications [1]. Quinolones inhibit DNA gyrase (topoisomerase II), a bacterial enzyme involved in DNA replication, recombination and repair. Quinolones attack only the bacterial gyrase-DNA complex, binding neither the gyrase nor DNA. The gyrase-DNA complex is responsible for the supercoiling of bacterial DNA and thus necessary for bacterial replication. The gyrase, an enzyme consisting of two subunits of gyrA and two subunits of gyrB, binds and then cuts the double-stranded DNA. Both strands are then moved so that supercoils are inserted. Quinolones disrupt this process thus resulting to bactericidal activity [2, 3].

Quinolone drugs in general must be protected from light, during preparation procedure, packaging, storage and administration as they are known to be photolabile molecules and cause drug-induced photosensitivity [4]. OXA has proven to be a potent, safe and well-tolerated drug; however during its exposure to light it undergoes photodecomposition, leading to possible side effects [5, 6]. Therefore, it is important to study the rate of its photodecomposition, and further investigate the effect of photostabilizing additives, in an attempt to improve the drug stability.

Cyclodextrins (CDs) are often used as photostabilizing excipients [7–11]. They are shallow truncated cone-shaped cyclic oligosaccharides. They are formed from six, seven,

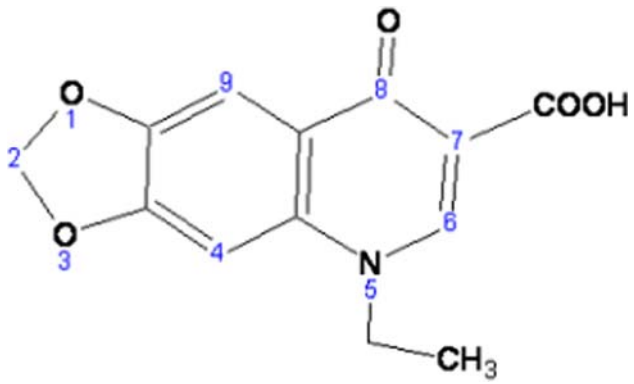

Fig. 1 Chemical structure and numbering of oxolinic acid

eight or more D-glucopyranose units, linked with α-(1 → 4) glucosidic bonds [7]. CDs contain a central hydrophobic cavity into which guest molecules can be inserted, forming noncovalent host–guest inclusion complexes. Complexation with these biodegradable excipients can significantly improve solubility, chemical stability, palatability and bioavailability of the guest molecules [8, 9], making CDs very useful for drug formulations, foods and cosmetics. In this work, the effect of photoprotecting agents on OXA stability was assessed by drug complexation with hydroxypropyl-β-cyclodextrin (HPβCD), a highly soluble synthetic derivative of βCD.

Although OXA photostability has been previously investigated [12, 13] any specific photostability indicating method could not be found in the literature. In the present work, an HPLC method, reported for OXA determination was firstly applied [14], however it failed to provide an acceptable resolution of degradation products from oxolinic acid and from each other. Therefore, a photostability indicating chromatographic method was developed and validated. Because OXA is insoluble in acidic solutions of mobile phase (non-ionized form), and because an acidic solvent is necessary for successful chromatographic separation, a procedure was developed to pre-treat the sample. This method is suitable for the separation of degradation products from OXA and from each other.

Analytical methods based on the spectrophotometric data, very often present accuracy problems when applied to cyclodextrin complexes [15–17] since inclusion in the CD cavity affects the UV absorptivity of the guest molecules, leading to Beer's law deviations. Consequently, the new HPLC method was also evaluated in the presence of HPβCD, in order to ensure that inclusion complexation did not generate inaccuracies.

The photodegradation profiles of OXA were studied in the presence and absence of HPβCD using the previously prepared OXA:HPβCD complexes, characterized by ^{1}H NMR Spectroscopy [18].

Materials and methods

Materials

OXA was obtained from Sigma-Aldrich Co. (St Louis, USA), stored in a cool place under light protection, and used without further purification. Trifluoroacetic acid and other reagents were of analytical grade and were purchased from Merck (Darmstad, Germany). Tetrabutylammonium bromide was purchased from Fluka (Buchs, Switzerland). All solvents used were of HPLC grade and were purchased from Carlo Erba Reactifs SDS (Val de Reuil CEDEX, France). Water used was deionised and filtered through a Milli-Q Plus water purifying system from Millipore (Bedford USA). Deuterated solvents for NMR were purchased from Euriso-top (Gif-Sur-Yvette, France).

Equipments

Liquid chromatography experiments were carried out using a high-performance liquid chromatograph consisting of a Waters 1515 Isocratic HPLC Pump (Waters Co, Milford, MA, USA), equipped with a constant temperature oven, a Waters 996 Photodiode Array Detector, and a Waters 717 Plus auto sampler. The software package Empower from Waters was used for acquisition and analysis.

The photodegradation experiments were performed with a Suntest CPS, Accelerated Exposure Xenon Burner Machine, from Atlas Material Testing Technology LLC (Chicago, Illinois, USA), which accurately simulates the wavelength distribution of solar radiation and meets guidelines established by ICH, the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use for light source characteristics [19].

^{1}H-NMR spectra were recorded on a 400 MHz Bruker DRX-Avance spectrometer, operating at 400.13 MHz. The 2D-ROESY spectra (two-dimensional Rotating Frame Nuclear Overhauser Effect spectroscopy) were acquired with 2,048 points in the t_2 dimension and 256 increments in t_1 with a spin lock time of 250 ms. The HDO signal was suppressed by low power presaturation (60 dB) during the relaxation delay and the mixing period. For the elucidation of the association pattern, the probe temperature was held constant at 298 K.

UV-Vis spectra were recorded on a Perkin-Elmer Lambda 7 spectrometer, in wavelength ranging from 200 to 300 nm. For the determination of the formation constant of the complexes (K_f) the absorption changes for OXA were monitored and they were measured at $\lambda_{max} = 268$ nm, in the presence and absence of HPβCD.

Liquid chromatography method

The new developed method

Separation of photodegradation products from OXA and from each other was performed on a 3.5 μm XBridge™ C_{18} 3.0 × 150 mm column (Waters Co., Milford, MA, USA). A mixture of 20% acetonitrile/80% aqueous solution of 0.005% tetrabutylammonium bromide (TBA) adjusted to pH 3.5 with trifluoroacetic acid (v/v) was used as mobile phase. Flow rate was adjusted to 0.5 mL min^{-1}. The detector wavelength was set at $\lambda_{max} = 268$ nm and the injection volume was 20 μL.

Samples used for method development were irradiated until degradation of 20–30% of the OXA concentration was achieved.

Method development

Column selection

A C_8 XTerra, 3.5 μm (4.6 × 150 mm) chromatographic column was first tested. The results were not satisfying, since coelution of degradation products was observed. The following columns with a C_{18} stationary phase were then tried: C_{18} XTerra, 3.5 μm (4.6 × 150 mm), C_{18} Atlantis, 3 μm (4.6 × 150 mm), C_{18} XBridge, 3.5 μm (3 × 150 mm) (all from Waters Co), and polar embedded C_{18} Synergi 4u Fusion-RP 80A column, 4 μm (2.00 × 150 mm) (Phenomenex, USA). The Atlantis column gave good results, probably because the remaining free silanol groups of the stationary phase can interact with retained OXA and its photoproducts. Satisfactory separation was also achieved with the polar embedded C_{18} column. However, for both Atlantis and Phenomenex columns, the pH of the mobile phase that gave the best results was outside of the manufacturer's specifications. The C_{18} XBridge column gave the best results. Moreover, the XBridge stationary phase shows the necessary stability at the selected pH (3.5).

Optimization of mobile phase pH

In a series of experiments, the pH of the aqueous part of the mobile phase was adjusted to levels ranging from 2.5 to 9.5. The results showed that in alkaline region, where OXA is fully ionized ($pK_a = 6.9$), no retention was observed and the drug substance eluted almost simultaneously with all of its photoproducts. The adjustment of pH with phosphoric acid buffer or acetic acid resulted in limited resolution and detection of fewer degradation products. Addition of trifluoroacetic acid gave good separation. Optimal resolution and selectivity were observed at pH 3.5. Moreover, the addition of TBA at a concentration of 0.005% further improved the resolution, enabling the separation of peaks that had previously co-eluted. The effect of higher concentrations of TBA was also examined, but no further improvement was achieved.

Organic modifier effect

The effect of methanol or acetonitrile was studied in a series of experiments ranging from 10 to 65% (v/v). At higher concentrations of organic solvents, no significant improvement in the separation of the degradation products was observed. Addition of a secondary organic solvent, such as tetrahydrofuran and methanol (with acetonitrile as primary organic modifier) or acetonitrile (with methanol as primary organic modifier) in a 5 to 10% ratio provided no advantage. Specifically, limited retention of some photoproducts resulted in coelution with the front of the solvent. The optimum mobile phase composition was 20% acetonitrile/80% aqueous solution of 0.005% TBA adjusted to pH 3.5 with trifluoroacetic acid (v/v) .

Temperature effect

Column temperature was maintained at 30 °C since coelution occurred at several higher temperatures ranging from 35 to 50 °C.

Overcoming accuracy problems generated from oxolinic acid insolubility

Over the course of the successive injections required for the HPLC analysis, microcrystals appeared in the sample vials during their maintenance in the instrument's autosampler. To address this difficulty, a sample pre-treatment procedure was developed.

In the selected acidic pH of the mobile phase, a condition essential to achieve adequate resolution, OXA exists in its non-ionized form. In this form, it has very limited aqueous solubility. The inaccurate quantifications obtained were therefore attributed to partial precipitation of OXA upon interaction with the strong acidic media of the mobile phase. The interaction of mobile phase with OXA occurs firstly, during sample dilution at the sample vials, in autosampler and possibly, in the column during chromatographic analysis.

When working with nonpolar analytes in aqueous solutions, microcrystal formation is a physicochemical process that often occurs in the cases, the solute concentration is higher than its solubility in the particular media being used. Sample pre-treatment procedure has often been proposed as the best solution for this problem challenge.

Several sample pre-treatments were assayed in order to address the possible formation of OXA microcrystals. Both the dilution of samples with the mobile phase and the addition

of small amounts of organic solvents as carriers proved inadequate. Other techniques were also applied for dissolving OXA in the samples. None of them resulted in satisfactory relative standard deviations (RSDs). Addition of 0.1 to 1% HPβCD in the mobile phase was another technique that was tested. Unfortunately the presence of CDs in the mobile phase led to coelution of the analytes. Instead, the addition of HPβCD to each sample, in approximately equimolar quantity to the initial concentration of OXA, proved effective.

Sample preparation

In all cases, accurately measured quantities of OXA were dissolved with an equimolar quantity of NaOH (0.25 M) solution to prepare an OXA-sodium salt solution. This was necessary in order to overcome the pronounced insolubility of OXA in water.

Method validation

To study linearity, two stock solutions were prepared by dissolving 16.1 and 19.8 mg of OXA in 100 mL water. From these solutions, with an appropriate dilution scheme, a calibration curve with the following solution concentrations was created: 0.018, 0.020, 0.247, 0.740, 1.061, 3.452, 6.903, 9.862, 15.161 and 37.902 $\times$ 10^{-5} M.

To test method precision, a stock solution was prepared by diluting 50 mg of OXA in 50 mL water. From this stock solution, a solution of 1.91 $\times$ 10^{-4} M concentration was prepared. The final dilution was made by combining 5 mL from the described 1.91 $\times$ 10^{-4} M OXA solution and 1 mL from a fully photodegraded OXA sample (see below), and water to 25 mL final volume. To evaluate inter- and intra-day precision, the above process was repeated eight different times.

To verify accuracy, two stock solutions were prepared by diluting 19.5 and 22.6 mg of OXA in 100 mL water. These stock solutions were appropriately diluted to obtain three solutions with the following concentrations: $A = 10.383$, $B = 5.191$ and $C = 8.959 \times 10^{-5}$ M. Finally, eleven solutions were prepared by combining 5 mL from A, 1 mL of fully photodegraded OXA and increasing volumes from B or C. The total sample volume brought to 25 mL with water. OXA concentrations analyzed were as follows: 2.435, 2.492, 2.700, 2.793, 2.907, 3.115, 3.152, 3.510, 3.868, 4.227, 4.585 $\times$ 10^{-5} M. Some of the above samples were also spiked with increasing proportions of a 1.2 $\times$ 10^{-5} M HPβCD solution.

Photodegradation profiles

Experiments with different initial concentrations of the sodium salt of OXA were conducted. After appropriate dilutions, samples with concentrations of 3.0, 6.0 and 15.0 $\times$ 10^{-6} M were prepared.

Preparation of the oxolinic acid:hydroxypropyl-β-cyclodextrin complexes

Accurately weighed HPβCD was added to an aqueous OXA solution (1.26 $\times$ 10^{-5} M), in order to obtain a molar ratio of 1:1. The solution was shaken in a water bath at a constant temperature of 35 $\pm$ 1 °C and 50 rpm. Complex formation was followed by continuously monitoring the UV spectra (Fig. 2) since the absorptivity of OXA changes upon complexation [20]. Equilibrium between free OXA molecules and complexes [OXA + HPβCD $\leftrightarrow$ OXA:HPβCD] was achieved after 48 h, when the changes detected at λ_{max} and absorbance reached a plateau.

UV-VIS spectroscopy study and binding constant of the complexes

UV spectra of 3.94 $\times$ 10^{-5} M OXA solution in the presence of different HPβCD concentration were studied [21, 22]. The HPβCD concentration varied from 1.89 $\times$ 10^{-5} to 1.51 $\times$ 10^{-2} M. The complex formation constant can be defined as:

$$K_f = \frac{[\text{OXA} : \text{HP}\beta\text{CD}]}{[\text{OXA}] \cdot [\text{HP}\beta\text{CD}]}$$

The K_f, value for the inclusion complex OXA:HPβCD was determined according to Scott's equation (Eq. 1) [21, 22].

$$[\text{HP}\beta\text{CD}]/\Delta\text{A} = [\text{HP}\beta\text{CD}]/([\text{OXA}] \times \Delta\varepsilon) + 1/([\text{OXA}] \times \Delta\varepsilon \times K_f) \quad (1)$$

where: ΔA represents the change in OXA absorbance after addition of HPβCD in different proportions ($\Delta A = A_{HP\beta CD} - A_{OXA}$) and $\Delta\varepsilon$ is the difference of the molar absorptivities for free and complexed OXA. If the resulting plot of [HPβCD]/ΔA versus [HPβCD] yield a straight line, the K_f value can be calculated from the following equation (Eq. 2):

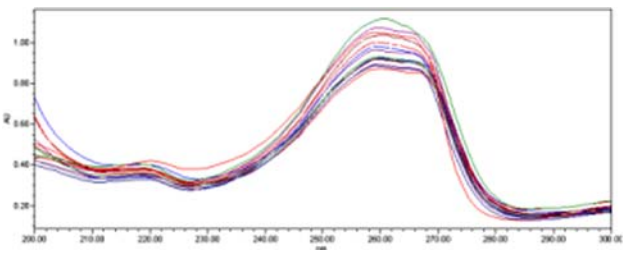

Fig. 2 Ultraviolet absorption spectra of oxolinic acid solutions at 268 nm, in the presence of various concentrations of hydroxypropyl-β-cyclodextrin (HPβCD)

$$K_f = \text{slope}/\text{intercept} \quad (2)$$

where: slope and intercept correspond to regression coefficients a and b of the regression equation:

$$[\text{HP}\beta\text{CD}]/\Delta\text{A} = \text{a}\ [\text{HP}\beta\text{CD}] + \text{b}$$

Photodegradation study

The photodegradation of OXA under xenon lamp irradiation was monitored. The rate constant of photodegradation was determined at 750 W/m^2, the maximum level of light intensity provided by the instrument. Initial sample volumes and other experimental parameters were kept constant in all cases. 4 mL aliquots of samples for photodegradation experiments were transferred into a 1 cm path length cuvette (quartz) with Teflon screw cap. According to ICH guidelines, samples were exposed to forced irradiation side-by-side with a validated quinine chemical actinometric system to ensure repeatability of the results. At predetermined time intervals, 200 μL aliquots of the irradiated solution were withdrawn from the cuvette. A volume of HPβCD aqueous solution with approximately the same molarity as the initial OXA concentration was added to amber glass vials and immediately diluted with the mobile phase to a final volume of 800 μL. The samples were then analyzed by the aforementioned LC method. The photodegradation study was performed by monitoring the remaining OXA concentration.

As a dark control experiment, samples were wrapped in aluminum foil and treated in the same way as above. The coexistence of thermal reactions was excluded by testing the thermal stability of samples stored at 30 °C for 24 h.

NMR spectroscopy study

Complex formation was evidenced by one- (1D) and two-dimensional (2D) ^{1}H NMR spectroscopy [18]. The complex stoichiometry was determined by the continuous variation method (Job plot), constructed from data acquired by 1D ^{1}H NMR [23]. Furthermore, OXA molecular self-association phenomena were determined by self-titration experiments [17], in an attempt to correlate the concentration effect with the modified electronic environment of drug assemblies, generated at high concentrations.

The stoichiometry of the OXA:HPβCD complexes was determined according to the Job's continuous variation method, by NMR spectroscopy as described by Djedaini and Perly [23]. According to this method equimolar 1.9×10^{-2} M solutions of (1) OXA and (2) HPβCD were prepared. The two solutions were combined in different proportions and treated as above to prepare the complexes. The stoichiometry was determined by taking into account the chemical shift displacement of selected protons of OXA. The displacements measured from NMR spectra were plotted versus molar ratio [OXA]/[OXA] + [CD].

In addition, a 1.9×10^{-2} M solution of the OXA:HPβCD complex, prepared as above was used for the confirmation of complexation with 2D ROESY experiments. For the self titration experiments, eight different OXA solutions were prepared over a concentration, ranging from solution A, 1.9×10^{-2} M to solution H, 0.16×10^{-2} M.

Results and discussion

Validation of the new HPLC method

The assay showed good linearity between 1.85×10^{-7} and 3.79×10^{-4} M. Linear regression yielded an r^2 value of 0.9993 (slope = 82,336.098 ± 791.199, intercept = 22,735.630 ± 106,884.199). The limit of detection was 0.59×10^{-7} M, while the limit of quantitation was 1.83×10^{-7} M. Precision of the method was examined in two different days at 11 new concentration levels each day (triplicate injection). The RSD values for within-day precision were 0.48 and 0.81% and for day-to-day precision was 0.67%. The experiment was also repeated in the presence of HPβCD with a determined RSD of 1.09%. Assaying the accuracy, the mean error across all samples in different concentration levels was −0.45%.

Binding constant of oxolinic acid:hydroxypropyl-β-cyclodextrin complexes

Increasing concentration of HPβCD in solutions the absorbance of OXA in UV-Vis increases. Evaluating the results, a linear correlation in Scott's plot at 268 nm was observed, which confirms the 1:1 stoichiometric ratio. The slope was 3.4479 ± 0.1047 and the intercept 0.0013 ± 0.0006. Using the Eq. 2 the K_f value can be calculated. $K_f = 2.65 \times 10^3$ M^{-1}.

Photodegradation profiles of oxolinic acid

Examination of the HPLC profiles revealed that the photodegradation of OXA is not catalysed by the presence of degradation products.

Kinetic order

Photodegradation of OXA follows first-order kinetics at the concentrations studied. When monitoring the remaining drug concentration versus time, an exponential decay was obtained. The kinetic data with their experimental errors are presented in Table 1. The k values were calculated

Table 1 Parameters of first order photodegradation of oxolinic acid (OXA) sodium salt in aqueous solutions

OXA ($\times 10^{-6}$ M)	k ($\times 10^{-3}$ h^{-1}) (Slope)	Intercept	r^2
3.0	−26.5 (±0.001)	11.572 (±0.018)	0.9907
6.0	−33.6 (±0.039)	12.101 (±0.048)	0.9611
15.0	−37.8 (±0.003)	0.8154 (±0.033)	0.9851
6.0 (with HPβCD 1:1)	−2.0 (±0.000)	4.9179 (±0.004).	0.9413

from the slopes of the straight lines obtained by plotting the logarithm of concentrations versus time.

Initial concentration effect

Several photochemistry articles conclude that, as initial sample concentration increases, the measured rate constant (k) decreases [24–26]. However, other studies contradict this conclusion [27–30]. In this study, OXA behaves like the latter group. Photodegradation experiments performed with OXA samples of different initial concentrations (C_0) revealed that any increase in C_0 lead to higher k values, until a plateau was reached.

Cyclodextrin effect

Aqueous solutions of OXA as free drug and as a complex with HPβCD were irradiated. The logarithm of the remaining OXA concentration under both forms was comparatively studied versus time. In both cases, a linear correlation was obtained (Table 1). The calculated k values showed that a 94% photostabilization upon complexation with HPβCD was achieved. The corresponding plots are depicted in Fig. 3. The remarkable photostabilization upon complexation led us to hypothesize that in this case, the separation of drug molecular assemblies, either by sample dilution or by inserting them in a CD cavity, has a protective effect.

Knowing that, in some cases, molecular assemblies that exist at high sample concentrations affect chromatographic behavior, and in light of the above results, a new approach was described. The observed acceleration of photodecomposition at higher concentrations and the enhanced stability in the presence of HPβCD led us to hypothesize that inter- and intramolecular interactions, occurring at higher initial concentrations, induce molecular modifications capable of altering molecular behavior under irradiation, such as relaxation time and pathways.

NMR study

Oxolinic acid self-assemblies

The planarity of the quinolone ring and the nature of the functional groups present in the OXA molecule suggest the possibility of several types of intra- and intermolecular interactions. Intermolecular interactions are known to weaken upon dilution and in most cases induce downfield shifts of nearby protons, as a result of monomer formation.

The self-association phenomena of OXA were studied to further investigate possible OXA molecular aggregation. The concentration-dependent chemical shift displacements of the OXA aromatic protons are listed in Table 2. The chemical shift changes of the most affected proton, H-4, are plotted versus sample concentration. A linear correlation was obtained: $\Delta\delta = [0.090\ (\pm 0.018)] \times C_{oxa} - [0.011\ (\pm 0.002)]$. The r^2 value was 0.998, showing a rather uniform effect upon dilution, due to separation of OXA molecules. The spectra from self-titration experiments are shown in Fig. 4. The above-mentioned results confirm the presence of OXA molecular assemblies in aqueous solutions.

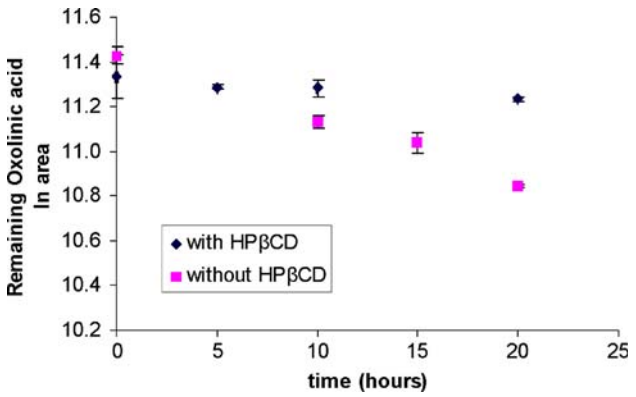

Fig. 3 Photodegradation plot in the presence and absence of cyclodextrins showing the stabilization effect of cyclodextrins

Table 2 Chemical shifts (ppm) of oxolinic acid (OXA) solution (A) 1.9×10^{-2} M and solution (H) 0.16×10^{-2} M

OXA protons	(A) 1.9×10^{-2} M (δ ppm)	(H) 0.16×10^{-2} M (δ ppm)	$\Delta\delta = \delta_H - \delta_A$ (ppm)
H-2	5.9213 (s, 2H)	5.9725	0.0512
H-4	6.9235 (s, 1H)	7.0792	0.1557
H-6	8.1043 (s, 1H)	8.1448	0.0405
H-9	7.3234 (s, 1H)	7.4444	0.1210
H-(CH_3)	1.2462 (t, 3H, J ≈ 7 Hz)	1.2910	0.0448
H-(CH_2)	4.0794 (q, 2H, J ≈ 13 Hz, J ≈ 7 Hz)	4.1790	0.0996

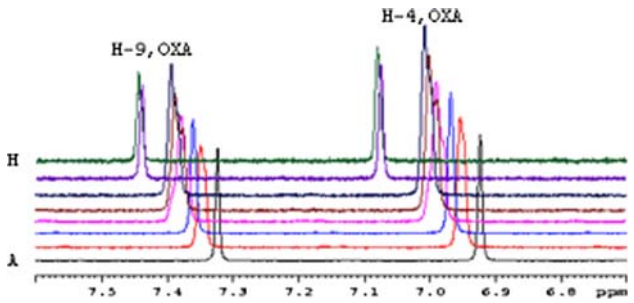

Fig. 4 Chemical shift displacement of H-4 and H-9 protons of oxolinic acid in eight different concentrations ranging from solution A = 1.9×10^{-2} M to solution H = 0.16×10^{-2} M

Oxolinic acid-hydroxypropyl-β-cyclodextrin inclusion complex formation and stoichiometry

Proton NMR spectroscopy is commonly used for the investigation of the nature of CD complexes in solutions [23]. In order to examine the inclusion of OXA in HPβCD, 2D ROESY and titration experiments were conducted.

Evaluation of the 1D NMR data revealed spectral modifications of the H-9 of OXA upon complexation in both shape and resonance frequency. The H-9 proton is affected not only by complexation with CD but also by changes in the neighboring C-8 carbonyl group occurring as a result of OXA insertion in CD cavity. In the free drug spectrum, a single peak at 7.32 ppm can be assigned to H-9; in the presence of HPβCD, an asymmetrical double peak from 7.46 to 7.51 ppm was observed. The strong downfield shift of H-9 could be attributed to the change in environment of this proton upon complexation. The two resonances of H-9 in the soluble complex probably represent pure OXA and the complexed form of OXA in a slow exchange regime on the NMR time scale at 298 K. The downfield shift of H-9 in the presence of HPβCD may be attributed to proton interactions of CDs and the approaching OXA carbonyl group, which weaken the carbonyl group's effect on the neighboring H-9. These chemical shift displacements, in addition to the observed interaction with CD, confirm that the H-9 of OXA is located inside the CD cavity.

Evidence of the drug-CD complex formation can be obtained by detection of intermolecular interactions between the protons of the CDs internal cavity and the protons of the drug. Such interactions of OXA aromatic protons and H-3 and H-5 of cyclodextrin internal cavity, were indeed detected (see below) and demonstrate that, inclusion does take place.

A partial 2D ROESY spectrum for the OXA:HPβCD complex is presented in Fig. 5. Cross peaks between H-9 of OXA and H-3, H-5 and H-6 of HPβCD were clearly seen. Intermolecular interactions of protons H-3 and H-5 of the CD cavity with the drug's protons are particularly important as they indicate that OXA is inserted into the CD cavity (inclusion complex formation). Crosspeaks between H-4 and H-6 of OXA with H-1 (anomeric protons) of HPβCD were also observed, which simply verify the molecular proximity. The possible structure of the complex is depicted in Fig. 6.

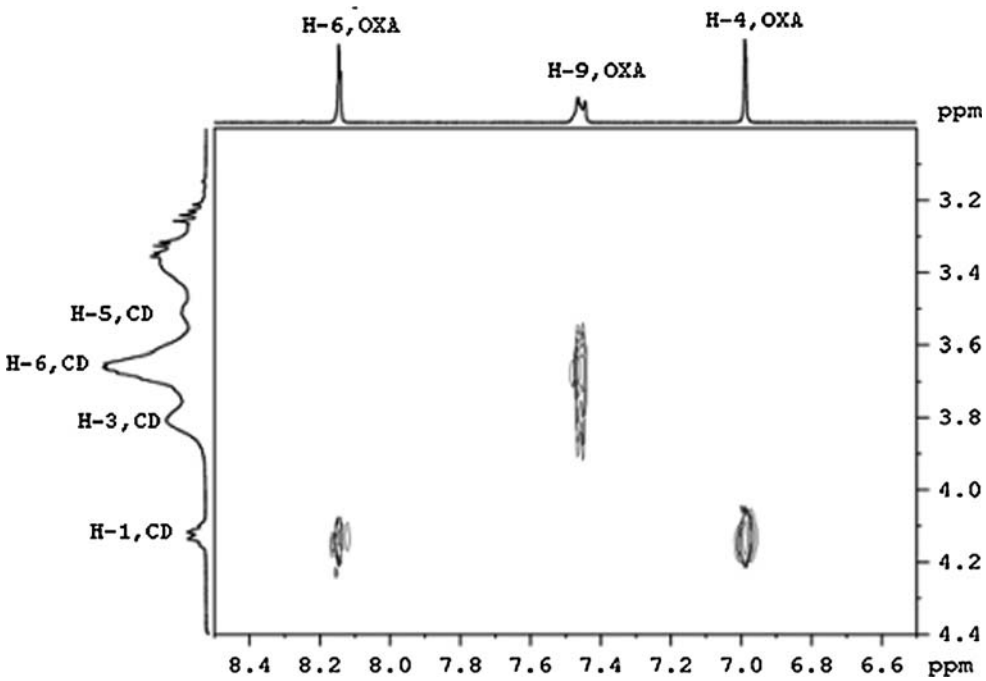

Fig. 5 Partial spectrum of 400 MHz ROESY (1.9×10^{-2} M oxolinic acid sodium salt solutions in D_2O) illustrating the intermolecular interactions between oxolinic acid and hydroxypropyl-β cyclodextrin protons

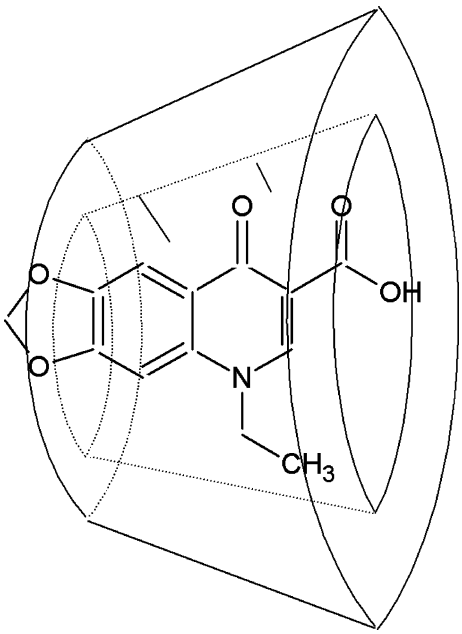

Fig. 6 The proposed structure of oxolinic acid:hydroxypropyl-β-cyclodextrin complex

Complex stoichiometry

Chemical shift displacement of drug protons in the presence of HPβCD complexes is a physical parameter directly related to the concentration of the complex and therefore can be measured for a set of samples with continuously varying molar fractions of the components. The maximum concentration of the complex will be present in the sample where the molar ratio corresponds to the complex stoichiometry [23]. A continuous variation plot (Job's method) showed the maximum value at a molar fraction of 0.5 and had a highly symmetrical shape, revealing the existence of an OXA:HPβCD complex with 1:1 stoichiometry (Fig. 7).

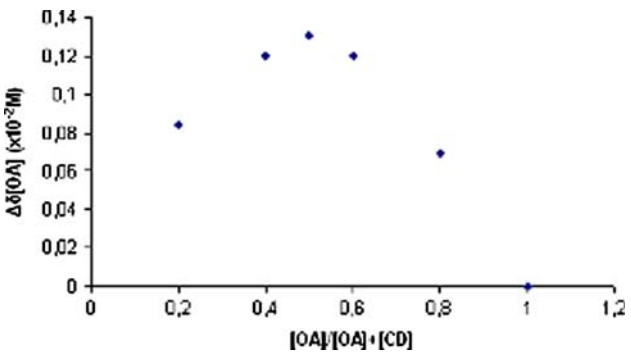

Fig. 7 The 1:1 stoichiometry of the oxolinic acid:hydroxypropyl-β-cyclodextrin complexes. Job plot obtained using chemical shift variations from the selected proton H-9 of oxolinic acid

Conclusions

A new HPLC method for measuring OXA photostability was developed and validated. The new method, enabling the separation of degradation products from OXA and from each other, was found to be specific and suitable for photostability studies of OXA, according to ICH guidelines. The addition of appropriate amounts of HPβCD as a sample pre-treatment procedure proved adequate to overcome OXA insolubility at the pH of the mobile phase. The guiding principle of the demonstrated method was that in order to solve the problem of OXA insolubility with the addition of CDs, it was essential to have an HPLC method compatible with CDs available.

Investigation of OXA photodegradation profiles confirms first order kinetics and acceleration at higher initial sample concentrations. A 94% photostabilization upon complexation with HPβCD was also achieved.

Our study of the complexation of OXA with HPβCD yielded two important observations. First, it is likely that OXA complexation with CDs competes with the molecular assembly phenomenon, reversing the effects seen at high concentrations of OXA in solution. This allows us to assume that the dissociation of the drug dimers or aggregates, by sample dilution or by inserting them in a CD cavity, has similar photoprotective effect.

Secondly, the crucial photostabilizing effect of CDs on OXA leads us to conclude that other techniques that enable the separation of molecular assemblies into monomers may reveal new and unique methods for photostabilization and will be important sources for investigation in the future.

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References

1. Greenwood, D.: Activity of flumequine against *Escherichia coli*: in vitro comparison with nalidixic and oxolinic acids. Antimicrob. Agents Chemother. **13**, 479–483 (1978)
2. Neugebauer, U., Szeghalmi, A., Schmitt, M., Kiefer, W., Popp, J., Holzgrabe, U.: Vibrational spectroscopic characterization of fluoroquinolones. Spectrochim. Acta Part A **61**, 1505–1517 (2005). doi:10.1016/j.saa.2004.11.014
3. Noble, C.G., Maxwell, A.: The role of GyrB in the DNA cleavage-religation reaction of DNA gyrase: a proposed two metal-ion mechanisms. J. Mol. Biol. **318**, 361–371 (2002). doi:10.1016/S0022-2836(02)00049-9
4. Tønnesen, H.H.: Formulation and stability testing of photolabile drugs. Int. J. Pharm. **225**, 1–14 (2001). doi:10.1016/S0378-5173(01)00746-3
5. Cárdenas, A.M., Vargas, F., Fernández, E., Hidalgo, M.E.: Phototoxic potential of quinolones. J. Photochem. Photobiol. B Biol. **10**, 249–255 (1991). doi:10.1016/1011-1344(91)85006-3

6. Cárdenas, A.M., Cortés, M.P., Fernández, E., Peña, W.: Lipid peroxidation and loss of potassium from red blood cells produced by phototoxic quinolones. Toxicology **72**, 145–151 (1992). doi:10.1016/0300-483X(92)90108-Q
7. Szejtli, J.: Cyclodextrin Technology. Kluwer Academic Publisher, Dordrecht (1998)
8. Tønnesen, H.H., Másson, M., Loftsson, T.: Studies of curcumin and curcuminoids. XXVII. Cyclodextrin complexation: solubility, chemical and photochemical stability. Int. J. Pharm. **244**, 127–135 (2002). doi:10.1016/S0378-5173(02)00323-X
9. Tønnesen, H.H., Duchêne, D.: Cyclodextrins and their pharmaceutical applications. Int. J. Pharm. **329**, 1–11 (2007). doi:10.1016/j.ijpharm.2006.10.044
10. Sapino, S., Cariotti, M.E., Cavalli, R., Trotta, M., Trotta, F., Vione, D.: Effect of alkyl-γ-cyclodextrins on the stability of retinol. J. Incl. Phenom. Macromol. Chem. **57**, 451–455 (2007). doi:10.1007/s10847-006-9233-8
11. Cariotti, M.E., Sapino, S., Marino, S., Ugazio, E., Trotta, F., Vione, D., Chirio, D., Cavalli, R.: Influence of hydroxypropyl-β-cyclodextrin on the photostability and antiradical activity of Trolox. J. Incl. Phenom. Macromol. Chem. **61**, 279–287 (2008). doi:10.1007/s10847-008-9420-x
12. Pouliquen, H., Delépée, R., Larhantec-Verdier, M., Morvan, M.-L., Le Bris, H.: Comparative hydrolysis and photolysis of four antibacterial agents (oxytetracycline, oxolinic acid, flumequine and florfenicol) in deionized water, freshwater and seawater under abiotic conditions. Aquaculture **262**, 23–28 (2007). doi:10.1016/j.aquaculture.2006.10.014
13. Hidalgo, M.E., Pessoa, C., Fernández, E., Cárdenas, A.M.: Comparative determination of photodegradation kinetics of quinolones. J. Photochem. Photobiol. B Biol. **73**, 135–138 (1993). doi:10.1016/1010-6030(93)80042-8
14. Delépée, R., Pouligen, H.: Determination of oxolinic acid in the bryophyte *Fontinalis antipyretica* by liquid chromatography with fluorescence detection. J. Chromatogr. B **775**, 89–95 (2002). doi:10.1016/S1570-0232(02)00279-9
15. Rozou, S., Antoniadou-Vyza, E.: An improved HPLC method overcoming Beer's law deviations arising from supramolecular interactions in tolfenamic acid and cyclodextrins complexes. J. Pharm. Biomed. Anal. **18**, 899–905 (1998). doi:10.1016/S0731-7085(98)00227-1
16. Rozou, S., Antoniadou-Vyza, E.: Chromatographic behaviour of naproxen–cyclodextrin complexes stationary phase C_8 alkyl chain as competitor for the drug release from cyclodextrin cavity. J. Chromatogr. A **1041**, 187–193 (2004). doi:10.1016/j.chroma.2004.05.002
17. Rozou, S., Voulgari, A., Antoniadou-Vyza, E.: The effect of pH dependent molecular conformation and dimerization phenomena of piroxicam on the drug:cyclodextrin complex stoichiometry and its chromatographic behaviour: a new specific HPLC method for piroxicam:cyclodextrin formulations. Eur. J. Pharm. Sci. **21**, 661–669 (2004). doi:10.1016/j.ejps.2004.01.007
18. Schneider, H.J., Hacket, F., Rodiger, V., Ikeda, H.: Cyclodextrins. Chem. Rev. **98**, 1755–1785 (1998). doi:10.1021/cr970019t
19. Moore, D.E.: Standardization of kinetics studies of photodegradation reactions. In: Tønnensen, H.H. (ed.) Photochemistry of Drug and Drug Formulations, 2nd edn, pp. 42–65. CRC Press, LLC, USA (2004)
20. Loukas, Y.L., Vyza, E.A., Valiraki, A.: Inclusion complexes and stability studies of an organophosphorous insecticide with cyclodextrins: spectrophotometric and kinetic determination of stability constant. Analyst **120**, 533–538 (1995). doi:10.1039/an9952000533
21. Letellier, S., Maupas, B., Gramond, J.P., Guyon, F., Gareil, P.: Determination of the formation constant for the inclusion complex between rutin and methyl-β-cyclodextrin. Anal. Chim. Acta **315**, 357–363 (1995). doi:10.1016/0003-2670(95)00263-Y
22. Zarzycki, P.K., Lamparczyk, H.: The equilibrium constant of β-cyclodextrin-phenolopthalein complex; influence of temperature and tetrahydrofuran addition. J. Pharm. Biomed. Anal. **18**, 165–170 (1998). doi:10.1016/S0731-7085(98)00150-2
23. Djedaini, F., Perly, B.: NMR of cyclodextrins derivatives and inclusion compounds. In: Duchêne, D. (ed.) New Trends in Cyclodextrins and Derivatives, pp. 227–231. Edition de Santé Paris, Paris (1991)
24. Das, D.P., Parida, K., De, B.R.: Photo-oxidation of phenol over titania pillared zirconium phosphate and titanium phosphate. J. Mol. Catal. A **240**, 1–6 (2005). doi:10.1016/j.molcata.2005.06.022
25. Emery, R.J., Papadaki, M., Freitas dos Santos, L.M., Mantzavinos, D.: Extent of sonochemical degradation and change of toxicity of a pharmaceutical precursor (triphenylphosphine oxide) in water as a function of treatment conditions. Environ. Int. **31**, 207–211 (2005). doi:10.1016/j.envint.2004.09.017
26. Shie, J.-L., Lee, C.-H., Chiou, C.-S., Chang, C.-T., Chang, C.-C., Chang, C.-Y.: Photodegradation kinetics of formaldehyde using light sources of UVA, UVC and UVLED in the presence of composed silver titanium oxide photocatalyst. J. Hazard. Mater. **155**, 164–172 (2008). doi:10.1016/j.jhazmat.2007.11.043
27. Blažková, A., Mezeiová, B., Brezová, V., Čeppan, M., Jančovičová, V.: Photochemical transformations of sodium anthracene-1-sulfonate in oxygen-saturated aqueous titanium dioxide suspensions. J. Mol. Catal. **153**, 129–137 (2000). doi:10.1016/S1381-1169(99)00312-X
28. Lin, Y.-J., Teng, L.-S., Lee, A., Chen, Y.-L.: Effect of photosensitizer diethylamine on the photodegradation of polychlorinated biphenyls. Chemosphere **55**, 879–884 (2004). doi:10.1016/j.chemosphere.2003.11.059
29. Kotzagiorgis, E.C., Michaleas, S., Antoniadou-Vyza, E.: Improved photostability indicating ion-pair chromatography method for pergolite analysis in tablets and in the presence of cyclodextrins. J. Pharm. Biomed. Anal. **43**, 1370–1375 (2007). doi:10.1016/j.jpba.2006.11.019
30. Voulgari, A., Benaki, D., Michaleas, S., Antoniadou-Vyza, E.: The effect of β-cyclodextrin on tenoxicam photostability, studied by a new liquid chromatography method; the dependence on drug dimerisation. J. Incl. Phenom. Macrocycl. Chem. **57**, 141–146 (2007). doi:10.1007/s10847-006-9206-y