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Approaches to improve the stability of the antiviral agent UC781 in aqueous solutions

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ABSTRACT

In this work, we evaluated the chemical stability profiles of UC781 based solutions to identify excipients that stabilize the microbicidal agent UC781. When different antioxidants were added to UC781 in sulfobutylether-β-cyclodextrin (SBE-β-CD) solutions and subjected to a 50 °C stability study, it was observed that EDTA was a better stabilizing agent than sodium metabisulfite, glutathione or ascorbic acid. Some antioxidants accelerated the degradation of UC781, suggesting metal-catalyzed degradation of UC781. Furthermore, we observed substantial degradation of UC781 when stored in 1% Tween 80 and 1% DMSO solutions alone or in those with 10 mM EDTA. On the other hand, improved stability of UC781 in the presence of 100 and 200 mM of EDTA was observed in these solutions. The addition of both EDTA and citric acid in the stock solutions resulted in recovery of more than 60% of UC781 after 12 weeks. Generally, 10% SBE-β-CD in the presence of EDTA and citric acid stabilized UC781 solutions: the amount of UC781 recovered approaching 95% after 12 weeks of storage at 40 °C. We also showed that the desulfuration reaction of the UC781 thioamide involves oxygen by running solution stability studies in deoxygenated media. Improved stability of UC781 in the present study indicates that the incorporation of EDTA, citric acid and SBE-β-CD and the removal of oxygen in formulations of this drug will aid in increasing the stability of UC781 where solutions of the drug are required.

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1. Introduction

It is estimated that at the end of 2007 about 33 million people worldwide were living with HIV (UNAIDS, 2008), the majority of HIV cases being reported in developing countries. In sub-Saharan Africa the preponderance of HIV transmission occurs through heterosexual contact. As women are twice as likely to acquire HIV from an infected partner during sexual intercourse than men (Glynn et al., 2001), women are disproportionately infected in this region. The high prevalence of HIV in women is partly due to the fact that women are biologically and socially more vulnerable to HIV infection (D'cruz and Uckun, 2006). Furthermore, in developing countries young women frequently remain in high-risk relationships due to economic dependence on their partners (Krishnan et al., 2008). Condoms are an effective barrier against HIV transmission, if used properly and regularly, and they are the only option available to women to protect themselves against sexually transmitted infections. Unfortunately, condom use is often outside a woman's control; hence, there is a great need to develop women-controlled prophylactic methods to slow or prevent transmission of HIV.

One strategy currently under development for controlling HIV transmission is the use of microbicides (Stone, 2002; D'cruz and Uckun, 2004, 2006). Microbicides are topical antiviral agents in drug delivery vehicles that can be used to protect against sexually transmitted diseases during rectal or vaginal intercourse. Used in combination with condoms, or instead of condoms, they are intended to reduce the risk of HIV transmission. Microbicides are designed to address the biological factors that make women more vulnerable to infection, in that they are applied topically to the vagina or rectum in order to prevent sexual transmission of HIV. Unlike condoms, microbicides represent a method that women could control themselves. Microbicides can be applied in advance of sexual activity, and without the active involvement or even knowledge of the male partner. Microbicides might be delivered in many forms: e.g. gels, films, sponges or as intravaginal rings that release the active ingredient over an extended duration (Woolfson et al., 2006; Gupta et al., 2008). Several microbicide products are being tested in clinical trials, although none is yet approved or available for widespread use (Microbicide pipeline, 2009). If proven effec-

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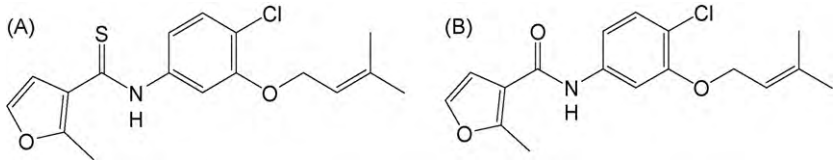

Fig. 1. Chemical structure of (A) UC781 and (B) UC22 which is the main degradation product of UC781.

tive, microbicides could be an inexpensive and readily available approach to HIV prevention for many women, especially those in developing countries. While the efficacy of a topically applied microbicide is likely to depend on the timely and correct application of the product relative to potential exposure, the availability of such products still represents an important strategy that could help stem the spread of HIV-1.

Among the compounds in microbicides development is the antiretroviral thiocarboxanilide UC781 (Fig. 1A). It is a non-nucleoside reverse transcriptase inhibitor (NNRTI), and a potent inhibitor of HIV-1 replication in cell culture systems (Balzarini et al., 1996; Barnard et al., 1997). It has been shown to inhibit HIV-1 strains which are resistant to nucleoside RT inhibitors with a potency similar to that used for inhibition of wild-type virus (Borkow et al., 1999). Furthermore, resistance to UC781 is only obtained when more than one mutation occurs in the NNRTI binding pocket (Pelemans et al., 2000). UC781 has been tested in animal models and in Phase I studies in humans (Patton et al., 2007; Sassi et al., 2007; Schwartz et al., 2008; ClinicalTrials.gov, 2009). Therefore, this compound shows potential for the treatment and prevention of HIV-1 infection. The main challenges facing UC781 in pharmaceutical development have been its poor aqueous solubility and its susceptibility to degradation in aqueous solutions. However, a gel form of UC781 is now in phase 1 clinical trials. The drug is a micronized suspension of UC781 crystals and has shown excellent stability on storage in the GMP mandated accelerated stability studies. However, the solution stability of UC781 remains poorly understood. The main oxidative degradation product of UC781 is UC22 (Fig. 1B). This product has some antiviral activity; however, it has been shown to be less potent compared to UC781 (Fletcher et al., 2005).

Several strategies to improve the stability and solubility of poorly soluble drugs have been described in literature (Chiou and Riegelman, 1971; Serajuddin, 1999; Damian et al., 2001; Goddeeris et al., 2008). Goddeeris et al. (2008) prepared fast disintegrating tablets of UC781 with vitamin E-TPGS 1000 and HPMC or PVPVA 64. Their findings showed that the dissolution properties of UC781 were significantly improved compared to physical mixtures formulated using the same excipients. Formation of inclusion complexes with hydrophilic carriers like cyclodextrins with a low association constant is believed to improve the solubility and stability of unstable drugs (Trapani et al., 2004; Brewster and Loftsson, 2007). Cyclodextrin molecules are cyclic oligosaccharides consisting of 6, 7, or 8 glucopyranose units per molecule which are α-1,4-linked to each other. Included in this category are hydroxypropyl-β-cyclodextrin (HP-β-CD), hydroxyethyl-β-cyclodextrin (HE-β-CD) sulfobutyl-β-cyclodextrin (SB-β-CD) and sulfobutylether-β-cyclodextrin (SBE-β-CD or Captisol®). The cyclodextrin cavity is relatively hydrophobic, whereas the outer part is hydrophilic in nature. Cyclodextrins are capable of forming a variety of complexes in which guest molecules are trapped, in part or entirely, by the hydrophobic cavity. Entrapment leads to changes in the physico-chemical properties of the hydrophobic guest molecules (Lemesle-Lamachea et al., 1996), improving solubility and stability properties. Recently, it has been shown that hydroxybutenyl-β-cyclodextrin can increase the stability of tamoxifen by 10–14-fold (Buchanan et al., 2007). Apart from using cyclodextrins, other research groups are focusing on using antioxidants and chelating agents to suppress the degradation of unstable products (Hovorka and Schöneich, 2001).

Previously, Yang et al. (2008) studied the physico-chemical and biological properties of several cyclodextrin complexes of UC781. They showed marked increases in the solubility of UC781 and report development of fast dissolving vaginal films of the inclusion complexes. However, chemical stability of UC781 in these inclusion complexes was not reported. Therefore, the objectives of this study were: (i) to develop inclusion complexes of UC781 with SBE-β-CD and HP-β-CD and assesses the stability of the drug complexes as a function of time, and (ii) evaluate the effect of several antioxidants on the degradation of UC781 in stock solutions and UC781/SBE-β-CD complexes. As part of these studies, we also evaluated the effect of deoxygenation of the stock solutions on the stability of UC781.

2. Materials and methods

2.1. Materials

UC781 (lot # 04-1803-02) and UC22 (lot # AC-1811-CMP-63) were obtained from CONRAD (Arlington, VA, USA). Sulfobutylether-β-cyclodextrin (SBE-β-CD or Captisol®) with a degree of sulfobutyl groups of 6.4 was obtained from CyDex, Inc. (Lenexa, KS, USA). Sodium dihydrogen phosphate monohydrate was purchased from Spectrum (New Brunswick, NJ, USA), disodium phosphate heptahydrate, ascorbic acid and hydroxypropyl-β-cyclodextrin were purchased from Sigma (St. Louis, MO, USA). Vitamin E-TPGS was purchased from Eastman chemical company (Kingsport, TN, USA), sodium metabisulfite and fumaric acid were purchased from Fluka (St. Louis, MO, USA). EDTA and glutathione were purchased from Acros Organics (Morris Plains, NJ, USA). Tween 80 was purchased from Fischer Scientific (Houston, TX, USA) and HPLC grade acetonitrile was purchased from EMD (Gibbstown, NJ, USA).

2.2. Methods

2.2.1. Preparation of UC781 stock solutions in 1% DMSO and 1% Tween 80

Thirty-four milligrams of UC781 was dissolved in 1 mL of DMSO in a 100 mL volumetric flask. 1 mL of Tween 80 was then added and the final volume made up to 100 mL using phosphate buffer (10 mM, pH 7). The final concentration of UC781 in the stock solution was 101.34 μM. 20 mL of the stock solution was filtered through 0.22 μm membrane filters (Chromafil AO-20/25). Aliquots of 500 μL of the filtrates were placed in 2 mL amber HPLC vials and sealed. Time 0 samples were immediately stored at −80 °C until all samples at other time points were ready for analysis. Days 3, 7, 14, 40, 56 and 84 samples were stored at 40 °C in the dark.

2.2.2. Preparation of UC781 stock solutions with EDTA or citric acid

EDTA was added to 20 mL of the stock solution from Section 2.2.1 at concentrations of 10, 100 and 200 mM EDTA. The pH of the resulting stock solutions after adding EDTA was adjusted to 7 using 10% NaOH. To make sure no particles were introduced into the HPLC system, all solutions were filtered through 0.22 μm membrane fil-

ters (Chromafil AO-20/25). Aliquots of 500 μL of the filtrates were placed in 2 mL amber HPLC vials and sealed. Time 0 samples were immediately stored at −80 °C until all samples at other time points were ready for analysis. Days 3, 7, 14, 40, 56 and 84 samples were stored at 40 °C in the dark. The number of vials for each condition was five ($n = 5$). In order to study the effect of combining EDTA and citric acid on the recovery of UC781 from the stock solutions, similar procedures as described above were repeated, but in this case 100 mM or 200 mM of citric acid were combined with respective equal amounts of EDTA in the stock solutions.

2.2.3. Preparation of 10% (w/w) SBE-β-CD solution with EDTA

Five grams of SBE-β-CD was added to a 50 mL volumetric flask and phosphate buffer (10 mM, pH 7) added to obtain final weight of 50 g. From this solution, two aliquots of 15 mL were taken and mixed with UC781, then sonicated for 10 min to disperse the drug. Suspensions of UC781 in SBE-β-CD solution were then autoclaved (Tuttnauer, Breda, The Netherlands) at 121.8 °C for 15 min. The suspensions were removed from the autoclave and cooled to room temperature. EDTA (100 or 200 mM) was added to one aliquot while the other was used as a control without EDTA. The pH of all suspensions was adjusted to 7.0 using 10% NaOH. The suspensions were filtered through 0.22 μm membrane filters (Chromafil AO-20/25). Aliquots of 500 μL of the filtrates were placed in 2 mL amber HPLC vials and sealed. Time 0 samples were immediately stored at −80 °C until samples at other time points were ready for analysis. Days 7, 14, 40, 56 and 84 samples were stored at 40 °C in the dark. This experiment was repeated using a combination of EDTA and citric acid for 4 weeks. The concentrations of both EDTA and citric acid used in this case were 10, 20, 50 and 100 mM. Before initiating the long-term stability of UC781/SBE-β-CD solutions with or without EDTA, we first carried out a 3-day stability study of UC781 in 10% (w/w) HP-β-CD and 10% (w/w) SBE-β-CD solutions. The preparation of these solutions was similar to what has been described above, except that 1 g of cyclodextrins was made up to 10 g using acetate buffer (10 mM, pH 7) into a 10 mL volumetric flask. The data from this mini-stability study were used to select a better excipient for future experiments.

2.2.4. Solubility studies

Solubility measurements of UC781 were performed according to the method of Higuchi and Connors (1965). Excess amounts of UC781 (20–40 mg) were put into 20 mL scintillation vials to which was added 15 mL of 10 mM phosphate buffer (pH 7) containing various concentrations of SBE-β-CD ($n = 3$). Concentrations of SBE-β-CD were 2.5, 5, 10, 25 and 50 mM. Suspensions of UC781 in SBE-β-CD were sonicated for 10 min at room temperature. The vials were then shaken using an orbital shaker (IKA® KS 260 Basic, NC, USA) at room temperature for 7 days (rotation speed was 300 rpm). Precautions to prevent the exposure of UC781 to light were taken by covering the vials with aluminum foil. The suspensions were filtered through 0.22 μm membrane filters and then analyzed for UC781 content by HPLC.

2.2.5. Effect of fumaric acid and vitamin E-TPGS on the stability of UC781 in SBE-β-CD/UC781 complex solutions

The effect of fumaric acid and vitamin E-TPGS on the stability of UC781 was evaluated using 10% SBE-β-CD in phosphate buffer (10 mM, pH 7). Fumaric acid and vitamin E-TPGS (0.15 g each) were added in separate 20 mL scintillation vials with 15 mL of 10% SBE-β-CD solution. The target concentration of fumaric acid and vitamin E-TPGS in the solutions was 1% (w/v). As a control, one solution was prepared using UC781 and 10%SBE-β-CD solution only. The pH of the solutions was adjusted to 7 using 10% NaOH. Excess UC781 (15–25 mg) was then added to the solutions and sonicated for 5 min followed by autoclaving at 121.8 °C for 15 min. The suspensions were removed from the autoclave and cooled to room temperature. They were then filtered through 0.22 μm membrane filters (Chromafil AO-20/25). Aliquots of 500 μL of the filtrates from each sample were placed either in 2 mL amber or 2 mL transparent HPLC vials and sealed. Time 0 samples were immediately stored at −80 °C until the samples at other time points were ready for analysis. Amber HPLC vials were stored at 40 °C in the dark for 4 weeks, while transparent vials were stored at 25 °C in the light conditions for 4 weeks. The number of vials for each condition was five ($n = 5$).

Table 1
Antioxidants used to stabilize UC781 in SBE-β-CD solutions at 50 °C in the dark[a].

Formulation	SBE-β-CD (%)	EDTA (mM)	GLT (mM)	AA (mM)	SMB (mM)
1	10	–	–	–	–
2	10 (NA)	–	–	–	–
3	10	100	–	–	–
4	10	–	100	–	–
5	10	100	100	–	–
6	10	–	100	100	–
7	10	–	100	–	100
8	10	100	–	–	100

[a] EDTA: ethylenediaminetetraacetic acid; GLT: glutathione; SMB: sodium metabisulfite; AA: ascorbic acid; NA: not autoclaved.

2.2.6. Effect of several antioxidants on the stability of UC781 in SBE-β-CD 10% at 50 °C

The effect of various antioxidants on stability of UC781 was evaluated using 10% SBE-β-CD in phosphate buffer (10 mM, pH 7). The composition of each formulation tested is shown in Table 1. Aliquots of SBE-β-CD solution (each 15 mL) were taken and mixed with UC781 (13–20 mg) and sonicated for 10 min. In total, 8 aliquots were prepared. Suspensions of UC781 from 7 aliquots were autoclaved at 121.8 °C for 30 min. The suspensions were removed from the autoclave and cooled to room temperature. Concentrations of antioxidants are shown in Table 1. All aliquots were adjusted to pH 7 using 10% NaOH and then filtered through 0.22 μm membrane filters (Chromafil AO-20/25). Samples of 500 μL of the filtrates from each aliquot were placed in 2 mL amber HPLC vials and sealed. Time 0 samples were immediately stored at −80 °C until samples stored at other time points were ready for analysis. Days 7, 14 and 28 samples were stored at 50 °C in dark. The number of vials for each condition was five ($n = 5$). As a control, one aliquot of UC781/SBE-β-CD was not autoclaved. The sample that was not autoclaved was left in an orbital shaker (IKA® KS 260 basic, NC, USA) for 15 h at room temperature with a rotation speed of 300 rpm and then filtered through membrane filters (0.2 μm). After filtration the filtrate was evaluated along with the autoclaved samples.

2.2.7. Quantification and calculation of concentration of UC781

HPLC was performed on an Agilent 1200 Series HPLC equipped with ChemStation32 software. All samples were run on an Agilent Zorbax 5 μm column (4.6 mm × 250 mm) at a flow rate of 1.1 mL/min under isocratic conditions: 80% acetonitrile/20% water with a run time of 10 min. At these conditions the retention time of UC781 was approximately 6.5 min. The retention time of the major oxidative degradation product (i.e. UC22) was observed at 5.6 min. The run of the samples was always preceded and ended by a set of UC781 standards. Standards were prepared by serial dilutions of stock solution of UC781 in mobile phase. A calibration curve with a correlation of 1 was established and used to quantify UC781. After every 20 samples one of the standard solutions was injected to ensure that the instrument did not drift during the run. UV signals were monitored at $\lambda = 300$ nm and peaks were integrated using ChemStation32 software. All chromatographic conditions were performed at 23.6 °C. The initial amount of UC781 was determined from time 0 samples stored at −80 °C. The recovery was

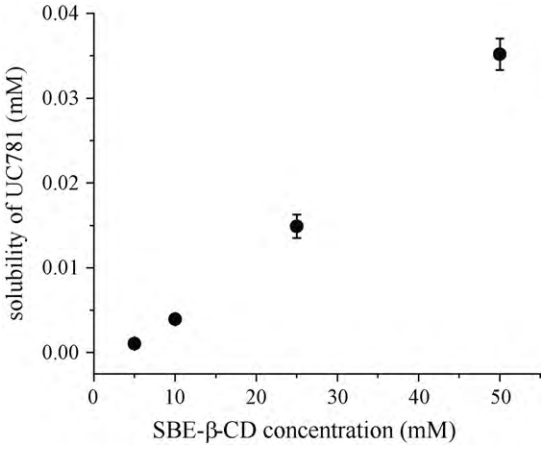

Fig. 2. Influence of SBE-β-CD on the solubility of UC781 at 25 °C. 50 mM is approximately a 10% (w/v) solution of SBE-β-CD. Mean ± SD ($n = 3$).

determined by using equation 1:

$$R = \frac{C_i}{C_o} \times 100 \quad (1)$$

where, C_i is the amount of UC781 at time t, while C_o is the amount of the drug at time 0.

2.2.8. Statistical analysis

Statistical analysis was performed by using the single factor ANOVA function of Microsoft Excel while kinetics of degradation was evaluated using Excel Solver software (Microsoft Excel 2007).

3. Results and discussion

3.1. Solubility results

Knowledge of the solubility of a drug substance is important for the drug development process, since the drug must dissolve in a fluid medium in order to elicit its intended action (Ikeda et al., 2005). If the solubility of a given drug is too low, its therapeutic efficacy can be compromised. The phase-solubility diagram of UC781 in the presence of SBE-β-CD was obtained by plotting the equilibrium concentration of UC781 against various molar concentration of SBE-β-CD (Fig. 2). The equilibrium solubility of UC781 increased linearly as a function of SBE-β-CD concentration over a range of 5–50 mM of SBE-β-CD. We choose this range because we desired to limit the concentration of the cyclodextrin studied due to the potential cytotoxicity of high concentrations of cyclodextrins reported by Yang (2008). The solubility curve started to deviate from linearity below 5 mM, yielding a negative y-intercept. Specifically, this curve is classified as an A_L^--type. At 50 mM of SBE-β-CD the increase in solubility of UC781 was approximately 88-fold compared to 5 mM. The solubility of UC781 at 50 mM was estimated to be 0.035 mM (11.8 μg/mL). Lockwood et al. (2003) estimated the solubility of astaxanthin, which is also a practically insoluble drug using SBE-β-CD in the range of 0–60% (w/v) and found that at a concentration of 10% (w/v) the concentration of astaxanthin dissolved was 0.03 μg/mL. Comparison of the solubility values of these insoluble drugs clearly shows that solubility of UC781 was favorably improved compared to astaxanthin. Examining the solubility of UC781 at the lowest concentration of SBE-β-CD used, it is evident the solubility of UC781 in pure water is less than <1 μg/mL. However, this value is still higher than EC50 for UC781 which was reported to be around 8 nM or 3 ng/mL by Balzarini et al. (Balzarini et al., 1996, 1998). The solubility of UC781 in phosphate buffer alone at these conditions could not be determined since the drug was undetectable by HPLC. The increase in solubility in the presence of SBE-β-CD suggests complex formation between these two components and this relationship may be used to provide information about the complexation efficiency. According to Higuchi and Connors' theory (Higuchi and Connors, 1965) this may be related to the formation of a soluble 1:1 (UC781: SBE-β-CD) inclusion complex. Normally, for a linear type A_L plot, the complexation constant is calculated by using Eq. (2) (Higuchi and Connors, 1965):

$$K_{1:1} = \frac{slope}{S_0(1 - slope)} \quad (2)$$

where $K_{1:1}$ represents the stability constant, and S_0 represent the intrinsic solubility of the drug in the absence of the complexing agent. The slope is determined from the phase solubility profile. Since our phase-solubility diagram showed a negative intercept, we cannot use Eq. (2) to calculate the stability constant because it results in overestimation of the complexation. This phenomenon has been described as being due to self-association of a poorly soluble drug and an excipient, and it is common in poorly soluble drugs with aqueous solubility less than 0.1 mM (Loftsson et al., 2002). In this case, the intrinsic solubility (S_0) of the drug will be much larger than the intercept of the phase-solubility diagram (S_{int}) resulting in a non-linear portion of an otherwise linear (A_L-type) phase-solubility diagram. Therefore, using this intercept can lead to incorrect $K_{1:1}$-values. A more accurate method for determination of the solubilizing efficiency of cyclodextrins has been proposed to be based on determining their complexation efficiency (CE), i.e. the concentration ratio between cyclodextrin in a complex and free cyclodextrin. CE is calculated from the slope of the phase-solubility diagrams, and it is independent of both S_0 and S_{int}. According to Loftsson et al. (2005), the complexation efficiency can be determined by using either the slope of the phase solubility profile or the complex to free cyclodextrin concentration ratio as shown in Eq. (3):

$$CE = S_0 K_{1:1} = \frac{[D/CD]}{[CD]} = \frac{slope}{1 - slope} \quad (3)$$

where S_0 is the intrinsic solubility of the drug, $[D]$ is the concentration of free drug, $[CD]$ is the concentration of free cyclodextrin, and $[D/CD]$ is the concentration of the complex. By using Eq. (3) the complexation efficiency for UC781 was found to be 0.0004; which means that only 4 cyclodextrin molecules out of 10,000 cyclodextrin molecules forms a complex with UC781. Loftsson et al. (2005) compared the CE of 28 drugs and found that the highest CE was 2.8 for diethylstilbestrol and the lowest was for ergotamine (i.e. 0.001). Comparing these data with our results shows that the CE of UC781 is 10-fold lower than the values of tamoxifen, omeprazole and cyclosporine A (Loftsson et al., 2005); all of which had a CE of 0.004. One of the main obstacles in pharmaceutical applications of cyclodextrins is their increase of the formulation bulk. In general, the complexation efficiency of most cyclodextrins is low, and consequently, the complex powder contains a significant amount of empty cyclodextrin molecules. The data obtained from the solubility study shows the amount of UC781 solubilized is minimal (i.e. for every 1 g of SBE-β-CD there is 0.26 mg of UC781). Several approaches to improve the complexation efficiency will be described in the subsequent sections.

3.2. Influence of SBE-β-CD and HP-β -CD on stability of UC781 after 3 days of storage

In the first set of these experiments, we evaluated the stability of UC781 in 10% (w/w) SBE-β-CD and 10% (w/w) HP-β-CD in 10 mM phosphate buffer (pH 7). Cyclodextrins did not interfere with the analysis of UC781. The complexation efficiency was increased by autoclaving the suspensions of UC781 in cyclodextrins at 121 °C.

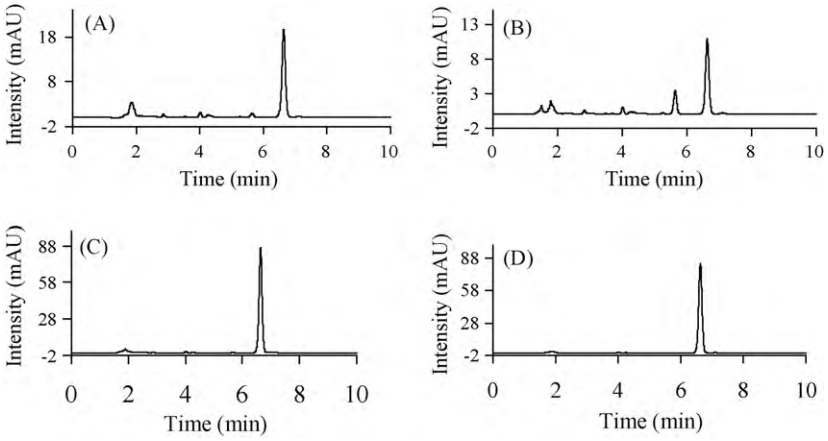

Fig. 3. Representative chromatograms of UC781 to show the effect of HP-β-CD and SBE-β-CD on the stability of UC781 after storage at 40 °C in the dark for 3 days. (A) UC781 with HP-β-CD at time 0; (B) UC781 with HP-β-CD after 3 days of storage, small peak with a retention time of 5.6 min is UC22 while larger peak with a retention time of 6.5 min is UC781; (C) UC781 with SBE-β-CD at time 0; and (D) UC781 with SBE-β-CD after 3 days, no UC22 formation in SBE-β-CD solution.

Fig. 3A shows a typical chromatogram of intact UC781 in HP-β-CD at time 0 with a retention time of 6.5 min. After 3 days of storage, UC781 partially degraded to UC22 (Fig. 3B). The chromatogram showed a good separation between UC781 and its main degradation product. On the other hand, the chromatogram of UC781 in SBE-β-CD clearly showed the absence of degradation of UC781 after storage for 3 days (Fig. 3D). Apart from increasing the stability of UC781, SBE-β-CD appears also to have a higher solubilizing effect compared HP-β-CD. The solubility of UC781 in the presence of HP-β-CD at 121 °C for 15 min was 0.005 mg/mL while for the case of SBE-β-CD it was 0.034 mg/mL (i.e. 7-fold increases in solubility).

Cyclodextrins have been reported to improve the stability of several labile drugs against hydrolysis, oxidation and photodecomposition, and thus increase their shelf life (Brewster et al., 1992; Ahn et al., 1997; Ma et al., 2000; Sortino et al., 2001). The interaction of cyclodextrins with unstable molecules can either suppress drug degradation, cause no effect, or accelerate degradation of drug molecules (Loftsson and Brewster, 1996). The data obtained in this set of experiments show that these two types of cyclodextrins are interacting with UC781 differently, and SBE-β-CD was selected for further experiments because it showed favorable solubilizing and stabilizing properties of UC781.

3.3. *Influence of adding vitamin E-TPGS and fumaric acid in 10% SBE-β-CD/UC781 complex in the stability of UC781*

Various strategies have been proposed to enhance the complexation efficiency of pharmaceutical compounds, including the use of polymers such as water soluble cellulose derivatives (Loftsson et al., 1994), addition of hydroxyl acids such as malic or tartaric acid (Agharkar et al., 1976; Varsha et al., 2009), or addition of surfactants to cyclodextrin complexes (Yang et al., 2004). These components enhance complexation efficiency by increasing the solubility of the drug. Increased solubility of the drug will shift the equilibrium toward complexation, resulting in improved complexation efficiency. Therefore, as part of these studies we evaluated the effect of adding fumaric acid and vitamin E-TPGS on the recovery of UC781 from SBE-β-CD solutions. The samples under investigation were stored in both light and dark conditions. The data presented in Fig. 4 (LEFT) show the stability of UC781 in various UC781/SBE-β-CD solutions after 28 days of storage at room temperature (exposed to light conditions). Vitamin E-TPGS and fumaric acid containing solutions stored at room temperature (exposed to light conditions) showed significant degradation of UC781, e.g. after 3 days of storage we observed a loss of 10% of UC781 in the presence of SBE-β-CD alone, 6.2% with vitamin E-TPGS and 9% with fumaric acid. Furthermore, after 14 days of storage at these conditions the loss of UC781 amounted to 33% in SBE-β-CD alone, 52% with vitamin E-TPGS and 32% with fumaric acid solutions. The loss of UC781 continued in this trend and after 28 days the drug loss reached 59%, 65% and 54% for SBE-β-CD alone, vitamin E-TPGS and fumaric acid solutions, respectively. Comparing the recoveries of UC781 in the presence of fumaric acid and vitamin E-TPGS showed that none of them are able to prevent UC781 degradation. In previous studies, we evaluated the stability of UC781 suspension in acetate buffer (pH 4) at room temperature (in the light conditions) and 40 °C/75% RH (in the dark). The data obtained showed that, unlike the aqueous solutions, the drug was stable in the suspensions at these conditions (Fig. 4). This supports the observation that chemical stability of UC781 may not be an issue when formulated in and aqueous semi-solid delivery systems as a crystalline suspension. Furthermore, Balzarini et al. (1998) showed that a UC781 gel (up to 5 wt% loading) was stable at 50 °C for 30 days.

The study on the effect of fumaric acid and vitamin E-TPGS was repeated but this time the samples were kept at 40 °C in the dark. Fig. 4 (RIGHT) shows the influence of fumaric acid and vitamin E-TPGS on the stability of UC781 in the UC781/SBE-β-CD complex at 40 °C (in the dark). The loss of UC781 after 3 days was less than 5%. At the end of 28 days losses were 8%, 7% and 6% for SBE-β-CD alone, fumaric acid and vitamin E-TPGS, respectively. If one compares degradation of UC781 at room temperature in the light conditions with that of dark conditions, it is clear that storing UC781 solutions in dark conditions significantly improves its stability by several orders of magnitude. Degradation of UC781 accelerated 2–2.6-fold when stored in transparent vials unprotected from light at room temperature in comparison with the drug solutions stored in dark conditions at 40 °C. Also, first order degradation rate constants of drug solutions in the light were about 10-fold higher compared to the samples stored in the dark. For example, UC781/SBE-β-CD complex with 1% fumaric acid had first order rate constants of $2.8 \times 10^{-8}\ s^{-1}$ and $3.2 \times 10^{-7}\ s^{-1}$ in the dark and light, respectively. These findings suggest that care must be taken when handling UC781 and it should be protected from photo-induced decomposition by using amber glass containers or covering with a protective material (e.g., aluminum foil). Addition of fumaric acid or vitamin E-TPGS did not improve the recovery of UC781 compared to SBE-β-CD alone. Significant degradation of UC781 at room temperature (exposed to light conditions) compared to dark conditions (40 °C) is an indication that the contribution of light to the degradation of UC781 outweighs the effect of temperature. Similar effects of

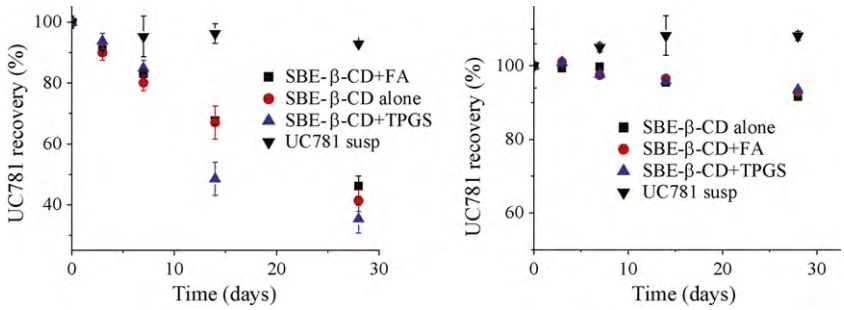

Fig. 4. Influence of vitamin E-TPGS and fumaric acid on the stability of UC781 in 10% SBE-β-CD solutions at 25 °C exposed to light conditions (LEFT) and at 40 °C in the dark (RIGHT). The concentration of fumaric acid was 1% (w/v) and vitamin E-TPGS 1% (w/v). The drug stored in the form of a suspension in acetate buffer (pH 4) was stable compared to the aqueous solutions of the drug in phosphate buffer with SBE-β-CD. Mean ± SD ($n = 5$).

light on the degradation of pharmaceutical compounds have been observed by other research groups (Won et al., 1995; Vermeire and Remon, 1999; Al Omari et al., 2007). Based on the results shown in Fig. 4, our goal of not losing more than 5% of UC781 after 28 days of storage was not achieved. Further experiments to improve the recovery of UC781 in aqueous solutions using several antioxidants and chelating agents were designed and the findings are presented in the subsequent sections.

3.4. Effect of several antioxidants on the recovery of UC781 at 50 °C (in the dark)

Evaluating the stability of the solid state and solubilized drug under accelerated and long-term conditions helps to identify conditions under which UC781 might be sensitive, and to identify degradation profiles under these conditions. The approach that is commonly used to minimize the oxidation of pharmaceutical compounds is addition of an antioxidant to the formulation. Antioxidants prevent processes that occur with some drugs or excipients on exposure to oxygen or in the presence of free radicals. These processes are often catalyzed by light, pH changes, and presence of trace metals or peroxides. In view of this knowledge, we evaluated the effect of multiple antioxidants on the stability of UC781 in SBE-β-CD complex at 50 °C and used the data to select an antioxidant providing favorable stability of UC781 for further experiments. All samples were stored at 50 °C (in the dark) and the data were collected after 0, 7, 14, and 28 days. The summary of results for this experiment is shown in Table 2. Stability of UC781 in the presence of antioxidants showed significant differences in degradation ($p < 0.05$) compared to UC781 in SBE-β-CD solution alone at all time points investigated. Some antioxidants showed recoveries of UC781 that were less compared to SBE-β-CD alone, suggesting the increased degradation of UC781. For example, solutions prepared with glutathione and ascorbic acid resulted in extensive degradation of UC781 compared to other combinations. After 28 days of storage the recoveries of UC781 from SBE-β-CD/ascorbic acid/glutathione, SBE-β-CD/glutathione/EDTA, and SBE-β-CD/glutathione were 3.4%, 5.2% and 29.50%, respectively. Glutathione is a peptide containing the amino acids glutamic acid, cysteine and glycine. Its serves as a co-enzyme in several reduction reactions and functions as an antioxidant mainly by reacting with potentially oxidizing agents and being oxidized itself (Maia et al., 2006).

Increased degradation of UC781 in the presence of these antioxidants suggests a metal-catalyzed degradation mechanism may be involved in the degradation of UC781 in aqueous solutions. Many antioxidants are good reducing agents for metal ion catalyzed oxidation. Antioxidants like ascorbic acid reportedly serve as pro-oxidants by reducing high oxidation state metal transition metal ions promoting formation of active oxygen species (Hong et al., 2004; Kramarenko et al., 2006). Underberg (1978) reported an increased degradation of promethazine in the presence of Cu^{+2}, Fe^{+3} and ascorbic acid. Likewise, Evans et al. (2004) observed an increased degradation of liquid formulations for adenovirus-based vaccines in the presence of ascorbic acid, while EDTA seemed to be an effective stabilizer of the formulations. Furthermore, our data have shown that the presence of sodium metabisulfite in the formulations accelerated the degradation of UC781. Similar results have been reported for furosemide (Shah et al., 1980) and amitriptyline (Enever et al., 1977).

Recovery of UC781 from non-autoclaved UC781/SBE-β-CD complex solution was very low compared to autoclaved UC781/SBE-β-CD solution. For example, recoveries of UC781 after 2 and 4 weeks were 92.2% and 85.2% for autoclaved solutions as opposed to 79.6% and 18.2%, respectively, for un-autoclaved solution. After 4 weeks of storage there was approximately a 4.5-fold loss in UC781 when the solution was prepared without autoclaving. These findings suggest autoclaving increases complexation efficiency thus stabilizing more drug compared with non-autoclaved UC781 consistent with a previously reported finding (Loftsson et al., 2007). This effect is

Table 2
Effect of several antioxidants on the stability of UC781 in UC781/SBE-β-CD complex solutions at 50 °C in the dark[a]. The data represent the percent of UC781 remaining compared to time 0 for each time point. Mean ± SD ($n = 5$).

Formulation	Time (days)				Rate constant (s^{-1})
	0	7	14	28	
1. SBE-β-CD alone	100.0 ± 0.2	96.3 ± 1.3	92.2 ± 0.8	85.2 ± 0.4	6.6×10^{-8}
2. SBE-β-CD alone (NA)	100.0 ± 1.3	90.5 ± 1.1	79.6 ± 0.9	18.2 ± 0.5	3.6×10^{-7}
3. SBE-β-CD + EDTA	100.0 ± 0.5	98.4 ± 0.9	98.4 ± 0.9	93.5 ± 0.4	ND*
4. SBE-β-CD + GLT	100.0 ± 1.2	93.9 ± 0.2	83.6 ± 1.7	29.5 ± 3.7	2.8×10^{-7}
5. SBE-β-CD + GLT + EDTA	100.0 ± 0.6	79.0 ± 1.0	53.3 ± 0.7	5.2 ± 0.8	6.3×10^{-7}
6. SBE-β-CD + AA + GLT	100.0 ± 1.0	85.1 ± 1.2	53.7 ± 1.4	3.4 ± 1.1	6.0×10^{-7}
7. SBE-β-CD + GLT + SMB	101.3 ± 1.2	95.2 ± 0.8	90.0 ± 1.4	81.5 ± 0.5	9.5×10^{-8}
8. SBE-β-CD + SMB + EDTA	100.0 ± 1.0	81.1 ± 1.0	65.1 ± 1.9	44.6 ± 2.1	3.4×10^{-7}

[a] EDTA: ethylenediaminetetraacetic acid; GLT: glutathione; SMB: sodium metabisulfite; AA: ascorbic acid; SBE-β-CD: sulfobutylether cyclodextrin; ND*: data did not fit the curves for rate constant calculation.

Table 3
Effect of EDTA on the stability of UC781 in stock solutions at 40 °C in the dark. The concentration of EDTA in the stock solutions ranged from 10 to 200 mM. The data represent the percent of UC781 remaining compared to time zero for each condition. Mean ± SD ($n = 5$).

Time (days)	UC781 stock solution alone	S[a] + EDTA[b] (10 mM)	S + EDTA (100 mM)	S + EDTA (200 mM)
0	100 ± 0.4	100 ± 0.3	100 ± 0.3	100 ± 0.1
3	44.5 ± 1.7	54.1 ± 3.1	67.4 ± 4.3	70.3 ± 3.2
7	8.0 ± 4.7	18.5 ± 1.3	ND	ND
14	2.1 ± 0.8	6.6 ± 0.8	50.6 ± 7.9	44.1 ± 0.7
40	0	0	35.2 ± 11.0	36.5 ± 0.9
56	0	0	14.3 ± 2.5	25.5 ± 1.6
84	0	0	3.0 ± 0.5	17.5 ± 2.1
Rate constant (s^{-1})	3.5×10^{-6}	2.5×10^{-6}	5.4×10^{-7}	4.5×10^{-7}

[a] S: Stock solution (1% DMSO and 1% Tween 80 in phosphate buffer, pH7).
[b] EDTA: ethylenediaminetetraacetic acid.

attributed to the increase in the amount of free drug in equilibrium with drug/cyclodextrin complexes. With the exception of formulation 3, the data from all other formulations fitted the first order kinetics. Formulations 5 and 6 showed higher rates of degradation, $6.3 \times 10^{-7}\ s^{-1}$ and $6.0 \times 10^{-7}\ s^{-1}$, respectively. These are the formulations that showed the least recovery of UC781 after 28 days of storage at 50 °C. Formulations 1 and 7 demonstrated a slower rate of degradation, $6.6 \times 10^{-8}\ s^{-1}$ and $9.5 \times 10^{-8}\ s^{-1}$, respectively. The recoveries of UC781 in formulation 3 at some time points were constant and this resulted in the data not being useful for rate constant calculations. However, this formulation had the best recovery of UC781 compared to other formulations in this set of experiment.

From data presented in Table 2, it is clear that favorable stability of UC781 was obtained with formulation 3 (i.e. UC781/SBE-β-CD with EDTA). At these extreme conditions (i.e. 50 °C) recovery of UC781 from UC781/SBE-β-CD complexes with EDTA after 4 weeks was 93.5% as opposed to 85.2% for SBE-β-CD alone. Retarding degradation of UC781 in the presence of EDTA suggests the presence of low concentrations of metal ions in the solutions which catalyze the formation of free radicals that can attack UC781. EDTA is a member of the polyamino-carboxylic acid family of ligands that usually binds metal cations through its two amines and four carboxylates. It forms especially strongly complexes with Mn^{+2}, Cu^{+2}, Fe^{+3}, and Pb^{+2}. Because metal ions are extensively enveloped by EDTA, their catalytic properties are often suppressed. Based on these results, EDTA was selected for further experiments.

3.5. Effect of EDTA and citric acid on the stability of UC781 from stock solutions

UC781 degradation in the presence of EDTA was compared with degradation without this additive. Table 3 shows the effect of using various concentrations of EDTA on the recovery of UC781 from the stock solution. We noticed a significant loss of the drug in the stock solution alone, for example, UC781 recovery after 2 weeks of storage at 40 °C (in the dark) was 2.1%, 6.6%, 50.6% and 44% for stock solution alone, 10 mM EDTA in stock, 100 mM EDTA in stock and 200 mM EDTA in stock, respectively. After 40 days of storage no UC781 was detected either in the stock solution alone or in stock solution with 10 mM EDTA. Complete degradation of UC781 resulted into the formation of five degradation products of which only one is known (i.e., UC22). Degradation products had retention times of 1.5, 1.8, 2.6, 3.7 and 5.5 min. The retention times of the degradants were shorter compared to the parent compound indicating that they are more polar compared to UC781. Fig. 5 shows the time course of degradation of UC781 in stock solution into sev-

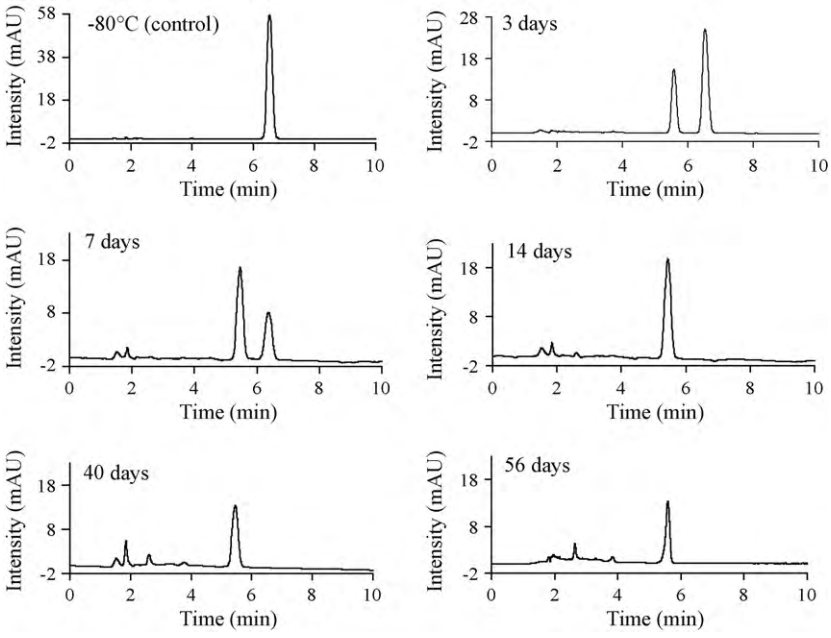

Fig. 5. Representative chromatograms showing the time course of degradation of UC781 in stock solutions at 40 °C in the dark. The solution stored at −80 °C showed no extra peaks indicating the absence of degradation of UC781 at a very low temperature. After 3 days of storage more than 50% of UC781 had already been transformed into UC22 and other three unknown degradative products. A small amount of UC781 remained after 7 days, and peak areas of the degradation products increased. The amount of UC781 in the stock solution after 14 days was negligible and peak areas of the degradation products increased. UC781 completely degraded into UC22 and four other unknown products after 40 days. After 56 days the peak areas of the unknown products started to decline.

Table 4
Effect of EDTA/citric acid on the stability of UC781 in stock solutions at 40 °C in the dark[a]. The concentrations of EDTA and citric acid in the stock solutions were either 100 mM or 200 mM each. The data represent the percent of UC781 remaining compared to time 0 for each condition. Mean ± SD (n = 5).

Time (days)	UC781 stock alone	S + EDTA + CA (100 mM)	S + EDTA + CA (200 mM)
0	100 ± 0.4	100 ± 0.1	100 ± 0.2
3	44.5 ± 1.7	79.3 ± 0.5	82.4 ± 0.7
7	8.0 ± 4.7	74.8 ± 0.8	77.9 ± 0.8
14	2.1 ± 0.8	73.9 ± 0.6	76.6 ± 0.7
40	0	70.0 ± 2.4	73.5 ± 1.2
56	0	66.4 ± 1.4	72.6 ± 11.8
84	0	61.1 ± 1.2	66.6 ± 2.2
Rate constant (s^{-1})	3.5×10^{-6}	1.7×10^{-7}	1.3×10^{-7}

[a] S: stock solution (1% DMSO and 1% Tween 80 in phosphate buffer, pH7); EDTA: ethylenediaminetetraacetic acid; CA: citric acid.

eral degradation products. After 12 weeks of storage the peak areas of the degradation products declined significantly. This observation indicates that UC22 is also degrading into other components. One of the degradation product, UC22 (Fig. 1B), is a desulfurated product of UC781. The chemical identity of other products could not be ascertained. Looking at the data collected after 2 weeks, recovery of UC781 was increased by 3 and 25 times compared to stock solution alone when 10 and 100 mM EDTA were added, respectively. Stock solutions with 100 and 200 mM EDTA showed some improvement in the recovery of the drug; however, a large amount of the drug was still susceptible to degradation (e.g. after 12 weeks of storage there was 97% loss of UC781 in the presence of 100 mM EDTA compared to 82% in 200 mM of EDTA). Degradation followed first order kinetics, and as shown in Table 3, the stock solution alone generally had a higher first order rate constant (e.g. 3.5×10^{-6} s^{-1} and 5.4×10^{-7} s^{-1}, for stock solution alone and 100 mM EDTA in stock solution, respectively). Based on these data, doubling the amount of EDTA in stock solutions from 100 to 200 mM EDTA had a marginal influence of UC781 stability. The recommended concentration of EDTA in pharmaceutical formulations is about 0.01–0.05% (Allen, 2008). The concentration of EDTA used in this case was several fold higher. Higher concentrations of EDTA improve stability of UC781, but in excess of the recommended limit.

Having seen the improvement in recovery of UC781 from stock solutions with EDTA, the next step was to combine EDTA and citric acid. Here, the aim was to augment stability of UC781 with two chelating agents. Table 4 shows the effect of combining EDTA and citric acid in the stock solution of UC781 in Tween 80/DMSO on the stability of UC781 for up to 12 weeks of storage. The data presented in Table 4 demonstrate the stabilizing effect of these two chelating agents and the recovery was better than when EDTA was used alone (see Tables 3 and 4). The recovery of UC781 after 12 weeks of storage was more than 60%, while in the case of stock solutions with EDTA alone we observed complete degradation of UC781 after 12 weeks. Also, first order degradation rate constants for EDTA and citric acid combinations were lower compared to stock solutions with or without EDTA (Table 4). Doubling the amount of EDTA and citric acid from 100 to 200 mM showed a marginal increase in recovery of UC781.

3.6. Effect of EDTA and citric acid on the stability of UC781 in UC781/SBE-β-CD complex solution

The next aspect of these studies was to evaluate the effect of adding EDTA or EDTA/citric acid in SBE-β-CD solution on the stability of UC781. Fig. 6(LEFT) shows that incorporation of EDTA resulted in an improvement in the stability of UC781 compared to SBE-β-CD alone. The concentration of EDTA was then increased from 100 to 200 mM so as to assess if degradation of UC781 was EDTA concentration dependent. However, doubling the amount of EDTA showed a slight improvement in the stability of UC781 at each time point. The data collected after 12 weeks of storage at 40 °C showed the recovery of UC781 in the presence of 100 and 200 mM to be 94.2% and 95.2%, respectively. In order to improve the recovery of UC781 in the presence of EDTA in SBE-β-CD solution, we used a combination of EDTA and citric acid at concentrations ranging from 10 to 100 mM. Since there was not much improvement in stability of UC781 by increasing the concentration of EDTA from 100 to 200 mM, we felt that there was no need to use 200 mM of EDTA in a citric acid combination. The representative data showing the effects of combining EDTA and citric acid in SBE-β-CD solution are presented in Fig. 6(RIGHT). After 4 weeks of storage at 40 °C the recovery of UC781 in the presence of 100 mM EDTA/citric acid in SBE-β-CD solution was 96%, while 20 mM EDTA/citric acid had a recovery of a 93.4%. Generally, lower concentrations of EDTA/citric acid did not improve the recovery of UC781. Furthermore, unlike with the stock solutions of UC781 in DMSO/Tween 80 (Tables 3 and 4) where there was a synergetic effect of EDTA and citric, there were no observed benefits of combining EDTA and citric acid in SBE-β-CD solution. Likewise, the first order rate constants of 100 mM EDTA with citric acid was not better compared to EDTA alone. However, the recoveries of UC781 from EDTA alone or with EDTA/citric acid in SBE-β-CD solutions were better than all combinations that we investigated in this study. Recovery of 95% of UC781 in aqueous solutions after 3 months of

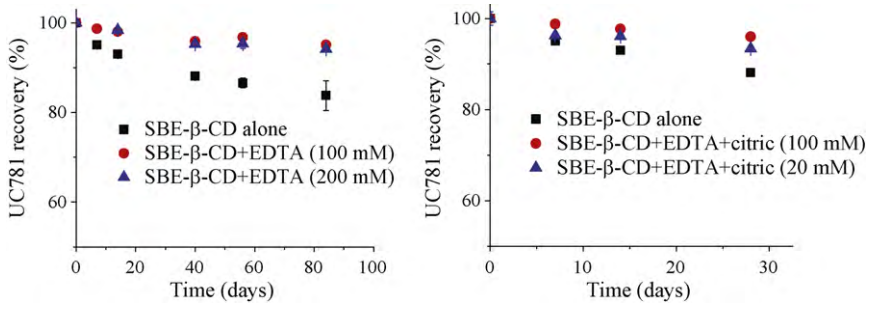

Fig. 6. Effect of adding 100 or 200 mM of EDTA (LEFT) and 20 or 100 mM of each EDTA + citric acid (RIGHT) to UC781/10%SBE-β-CD complex solutions on the stability of UC781 at 40 °C. Adding EDTA in UC781/SBE-β-CD complex resulted in an improvement in the stability of UC781 compared to SBE-β-CD alone.

storage at 40 °C can be regarded as a success in stabilizing this compound. The formulation of UC781 using this combination might result in a stable product in the semi-solid or solid formulation.

Furthermore, to elucidate the role of oxygen in the degradation of UC781, stock solutions of UC781 in DMSO and Tween 80 were deoxygenated. Solutions were freeze–thawed three times under nitrogen and vacuum, and then sealed in ampoules (under vacuum). One set of ampoules was stored at −80 °C (time 0) and the other one at 40 °C (in the dark) for 28 days. It was interesting to observe the absence of degradation of UC781 after 28 days of storage at 40 °C (in the dark) with a recovery of 100%. This finding shows that oxygen is involved in the degradation of aqueous solutions of UC781. Oxygen can induce degradation of pharmaceutical products via auto-oxidation. Several approaches to minimize the presence of oxygen include decreasing the headspace by filling the container as full as possible or replacing the headspace with nitrogen (Kasraian et al., 1999). Another option is to add an antioxidant to the formulation, however, as we have already mentioned in the previous sections, this approach is ineffective if oxidation is metal catalyzed. Findings from this study indicate that care is required to minimize or eliminate exposure to oxygen during key processing steps of UC781.

4. Conclusions

In this study, the stability of UC781 in stock solutions of UC781 with Tween 80 and DMSO was improved by several orders of magnitude when EDTA was used alone or in combination with citric acid. Stability of UC781 was further improved when SBE-β-CD was combined with EDTA alone or EDTA/citric acid; suggesting metal-catalyzed degradation is involved in degradation of UC781. Furthermore, deoxygenation of the stock solutions of UC781 suppressed degradation of UC781. The findings from the present study show that the use of EDTA alone or EDTA/citric acid combination substantially reduces the degradation of UC781 in aqueous solutions, and which might enable the formulation of stable formulations of UC781 for microbicides delivery.

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