**Chemical characterization of photodegradation products of midazolam complexes with randomly methylated-β-cyclodextrin by HPLC and LC-MS/MS**

Snezana Agatonovic-Kustrin1[[1]](#footnote-1), Mosimotsana Lebete 3, Michael E. Brown3, David W. Morton2 and Beverley D. Glass4

*1Facultyof Pharmacy, Universiti Teknologi MARA (UiTM), Selangor, Malaysia*

*2The School of Pharmacy and Applied Science, La Trobe Institute of Molecular Sciences, La Trobe University, Bendigo, Victoria, Australia.*

*3Faculty of Pharmacy, Rhodes University, Grahamstown, South Africa.*

*4Pharmacy, College of Medicine and Dentistry, James Cook University, Townsville, Queensland, Australia.*

*Abstract:* Midazolam, a potent anxiolytic drug with sedative properties, is susceptible to degradation by both light and hydrolysis in aqueous solution. When formulated as an intranasal product it was found to be effective in achieving seizure control in epileptic patients. In order to deliver an adequate therapeutic dose to a patient, a nasal formulation requires the concentration of midazolam to be higher than its’ aqueous solubility. One way to increase midazolam solubility to a therapeutic concentration, is complexation with randomly methylated-β-cyclodextrin. Thus, it is important to determine how complexation with cyclodextrin affects the rate of degradation and type of midazolam degradants that are formed. We have found that complexation with cyclodextrin decreases its photostability. More importantly, the degradation profile for midazolam is significantly altered when it is complexed with randomly methylated-β-cyclodextrin, which we partly confirmed in our previous work.[1](#_ENREF_1)

By continuing our study we have found that degradation products, not observed on the photodegradation of uncomplexed midazolam are observed in significant quantities when it is complexed with randomly methylated-β-cyclodextrin. The decreased photostability was accompanied by the appearance of two new degradation products, an intermediate structure and a dimer. Photoproduct formation followed the same pattern as in the forced degradation studies, further confirming the presence of an intermediate. The production of these new photodegradants, characterized with their MS spectra, as well as a proposed degradation mechanism of midazolam is discussed.

*Keywords:*benzodiazepine photostability; high performance liquid chromatography; liquid chromatography-tandem mass spectrometry

RUNNING TITLE: ANALYSIS OF Degradation ofmidazolam complexes

INTRODUCTION

 The benzodiazepine, midazolam (MDZ), is a potent drug with anxiolytic, hypnotic, amnestic, anticonvulsant, skeletal muscle relaxant, and sedative properties, and is commonly used as a preoperative anesthetic agent, especially for pediatric patients.2 A growing interest in alternative forms of drug administration has led to the development of nasal formulations of MDZ for sedation before surgical, dental or diagnostic procedures, and for treatment of seizures in children and adult patients as a safe and inexpensive means to rapidly achieve seizure control[2](#_ENREF_2).  The nasal route has been explored widely for delivery of a large number of drug molecules, due to its rich vasculature and thin epithelial lining that enables drug to reach systemic circulation after administered via nasal route directly and provides rapid onset of action. As no nasal MDZ preparation is commercially available, MDZ solution for injection has been used for this purpose.[3-4](#_ENREF_3) However, due to its low solubility, large application volumes of MDZ solution for injection exceeding limited nasal capacity are required in order to achieve adequate dosing. In order to deliver therapeutic MDZ doses in smaller volumes, nasal preparations with MDZ concentration, exceeding its solubility must be developed. MDZ is water-soluble at pH less than 4 and lipid-soluble at pH above 5 and therefore lipid soluble at physiological pH.[5](#_ENREF_5) MDZ’s water solubility increases at pH values less than 4.0 due to ring opening and ionization4 where MDZ is reversibly converted to the corresponding benzophenone, an open ring form with a highly ionizable primary amino group. Thus, at pH > 5 the drug is largely present in the lipid soluble, closed ring form. Although, solubility of MDZ increases at lower pH, the use of an intranasal acidic MDZ solution would result in severe irritation and swelling in the nasal cavity. In order to deliver an adequate therapeutic dose to a patient, a nasal formulation requires the concentration of MDZ to be higher than its water solubility (i.e. <0.1 mg L-1 at physiological pH 7.4).

Cyclodextrins (CDs) are commonly used to improve the solubility of poorly water-soluble drugs, by a process of inclusion complexation of the drug into the CD cavity. The cavity size is the major factor in determining which type of CD should be used for complexation with a particular drug. α-CDs contain six glucopyranose units, resulting in a small cavity, which can only incorporate low molecular weight compounds or compounds with aliphatic side chains. γ-CDs, on the other hand have eight-glucopyranose units, resulting in a substantially larger cavity so that the CD hydrophobic groups cannot effectively interact with many molecules to facilitate complexation. γ-CDs are able to effectively accommodate and complex larger molecules such macrocycles and steroids. However, the smaller cavity diameter of ß-CDs (seven glucopyranose units) is found to accommodate and effectively complex most drug compounds (aromatic and heterocyclic molecules). Hence, ß-CD is commonly used as a complexing agent in many CD complex drug formulations.[6](#_ENREF_6)

 Formulations of a 0.2 % (w/v) oral solution of MDZ containing γ-CD and citric acid have previously been investigated[7](#_ENREF_7). More recently however, it has been demonstrated that the use of randomly methylated-β-CD (RM-β-CD) as a solubilizer significantly reduces the rate of degradation MDZ[8](#_ENREF_8). RM-β-CDs have replaced γ-CD with partially methylated CDs in order to improve the solubility and stability of MDZ oral formulations, with the aim of developing a palatable buccal-nasal formulation. Due to their high aqueous solubility (> 500 mg mL-1) and their ability to form inclusion complexes, methylated CDs are widely used in drug formulations to improve drug solubility[9](#_ENREF_9) and to reduce the rate of hydrolysis[10](#_ENREF_10) and photodegradation.[11](#_ENREF_11) Substitution of the hydroxyl with methoxy groups imparts a slightly lipophilic character to the molecule, which also facilitates its permeation through the mucosa.[12](#_ENREF_12)

 Although the photostability of MDZ is well documented[5](#_ENREF_5), [13-14](#_ENREF_13) the rate of photodegradation and the nature and distribution of photodegradants may change when MDZ forms an inclusion complex with CD.[15-17](#_ENREF_15) The CDs have the potential to control both chemical and photochemical reactions, due to the microplanarity of the host cavity and limited molecular mobility of the guest molecule, due to steric constraints. The nature of the lowest excited states of the guest molecule, deactivation pathways and the fate of the reaction, may be modulated by the CD microenvironment. Thus, structural changes in drug molecules that occur when they form a complex with CD may have the potential to accelerate drug degradation.[18](#_ENREF_18) Quantitative high performance liquid chromatography (HPLC) methods have been used to study the decomposition of MDZ in formulations[19](#_ENREF_19) and to determine the amount of MDZ and its metabolites in blood plasma.[20](#_ENREF_20)

 Since the presence of RM-β-CD results in a different degradation profile to that observed in the absence of RM-β-CD, the aim of present study was to further investigate the degradants observed and how they may affect the safety/stability of nasal formulations of complexed MDZ. Based on results of the photostability kinetic studies, MS characterization, a degradation mechanism of MDZ is proposed as well as the model of the RM-β-CD-MDZ inclusion complex. Phase solubility studies were conducted according to the method of Higuchi and Connors,[21](#_ENREF_21) confirmed the ability of RM-β-CD to solubilize the target concentration of 10 mg mL-1 of MDZ.

EXPERIMENTAL

Chemicals

MDZ was kindly donated by Aspen-Pharmacare, South Africa and was used without further purification. RM-β-CD was purchased from Cyclolab (Hungary). All other chemicals used were of Analytical Reagent grade. Sodium hydroxide and ammonium acetate were obtained from Saarchem-Holpro (RSA). HPLC grade methanol was obtained from Romil Limited (UK). Water was in this work was from a Milli-Q® water purification system (Millipore, USA).

HPLC Instrumentation

Analysis of MDZ and its degradants was performed using HPLC (Spectra series P100 pump, Thermo separation products, Virginia, USA) in an isocratic mode using a UV 100 variable wavelength ultraviolet (UV) detector. The flow rate was 1 mL min-1; the injection volume was 20 μL; the column was used at room temperature (25 ± 2 ºC); and detection was carried out spectrophotometrically at 240 nm. The mobile phase was a mixture of methanol: 30 mM ammonium acetate aqueous solution (70:30 v/v) that was adjusted to pH 7 ± 1 using 0.2 M NaOH solution. The flow rate was 1 mL min-1; the injection volume was 20μL; the column was at room temperature (25 ± 2 ºC); and detection was carried out spectrophotometrically at 240 nm. Separation was performed on a Waters Spherisorb ODS2 5 μm (250 mm x 4.6 mm) column.

*Phase Solubility Studies*

The phase solubility studies were performed in triplicate. 75 mg of MDZ was placed in a conical flask and suspended in 5 mL of phosphate buffer solution (pH 5.0) containing 0, 5, 10, 20, 30% (w/v) RM-β-CD (i.e. 0.042, 0.084, 0.168, 0.252 mol L-1). The conical flaks were then stoppered, covered in foil, and shaken in a water bath at 25 ± 1 ºC. They were covered in foil so that no photodegradation products would be produced during the phase solubility studies. The samples were then analysed after 24 hrs using a UV method (see below).

*UV Method Development*

50 mg of MDZ was dissolved and diluted to 100 mL using 0.1 M HCl. The resultant solution was diluted to achieve concentrations of 0.005, 0.01, 0.015, 0.02 and 0.025 mg mL-1. The absorbances of these solutions were measured at 258 nm (λmax) and a standard calibration curve was constructed by plotting absorbance versus concentration.

Photostability kinetic studies

MDZ solutions (0.5 mg mL-1) in phosphate buffer (pH 5), were prepared in the presence and absence of RM-β-CD, in 2 mL clear glass (USP standard type 1 glass) ampoules (including dark controls) and irradiated at 550 W m-2 for a period of 12 hours (1.2 million lux hours) in order to degrade the drug to approximately 10 % of its original concentration.[22](#_ENREF_22) An Atlas SUNTEST CPS+ (Atlas Material Testing Technology B.V, Germany), fitted with a Xenon lamp and Solar ID65 filter was used for irradiation of samples. The temperature in the Suntest cabinet was maintained at 40 ± 2 ºC.

2 mL samples were removed at 1 h intervals over the 12 h period, diluted to a final volume of 10 mL with phosphate buffer and then analyzed. Control samples were covered in foil and treated in the same way as the exposed samples. Degradation was calculated as a percentage of the height of the drug peak, with respect to the peak height obtained from analysis of the original solution. The appearance of the major degradation products was also monitored and calculated as a percentage of the highest concentration achieved.

LC-MS/MS

The HPLC chromatograms obtained from the photostability kinetic studies were examined and a representative sample of drug and photoproducts in the presence and absence of RM-β-CD was selected and analyzed by LC-MS to obtain molecular masses of the degradation products. LC-MS/MS was then used to identify the photodegradants. For MS studies a Finnigan LCQ ion trap mass spectrometer (ITMS) (FINIGAN MAT, USA) equipped with an atmospheric pressure chemical ionization (APCI) ion source was used. Separation was performed on a Waters Spherisorb 5 μm ODS2 column (250 mm x 4.6 mm).

*Thermal stability studies*

To investigate temperature dependence on the degradation of MDZ, 2 mL aliquots of MDZ solution (0.5 mg mL-1, with and without 30 % w/v RM-β-CD) were sealed in ampoules, covered in foil and placed in ovens at temperatures of 25, 30, 40, 50, 60 and 70 ºC. The samples were removed after 12 h and analyzed.

RESULTS AND DISCUSSION

A simple UV method using 0.1 M HCl as a solvent was developed for the MDZ-RM-β-CD phase solubility studies. The method has shown a good linearity, with a linear regression equation *y* = -0.000051 + 0.0275*A* (*y* – MDZ concentration; *A* – absorbance) and correlation coefficient of 0.99. It was accurate with percent recovery of 98.9 - 101.6%. Precision of the method was determined by calculating the relative standard deviation. RSD for replicate measurement at three different concentration within the linear range was less than 1% indicating adequate precision.[23](#_ENREF_23)

The highest concentration of MDZ of 8.4 mg mL-1 (below the target concentration), was reached with phosphate buffer pH 5.8, was while at pH 5.0 the concentration of 10.6 mg mL-1 was achieved, with 30 % w/v RM-β-CD. In both cases the phase solubility curves obtained were of the Ap-type, suggesting the formation of a higher order complexes (Fig. 1). The results of the phase solubility studies indicated that the desired solubility of 10 mg mL-1 was achieved using 30% w/v RM-β-CD at pH 5.0.

**Figure 1.**

 The developed HPLC method was found to have sufficient accuracy with an average recovery of 100 ± 2 % for replicate measurements at low, mid and high concentrations of MDZ within the working concentration range. The accuracy of the method was investigated by spiking the 30 % w/v RM-β-CD solution with three known concentrations of MDZ (0.008 mg mL-1, 0.01 mg mL-1 and 0.012 mg mL-1). Linearity was confirmed to be within the range of 0.004 to 0.02 mg mL-1, with a high correlation coefficient of 1.00. The precision of the method, in terms of repeatability, was satisfactory with relative standard deviations of 0.62 % and 0.40 % (*n* = 6) at low and mid concentrations within the working range. The limit of quantitation (LOQ) was found to be 0.002 mg mL-1.

Robustness of the method was demonstrated during the method development, where it was shown that best peak resolution was reached, when the pH of the mobile phase was between 6 and 8, the concentration of ammonium acetate in the mobile phase was between 20 and 40 mM, with the organic/aqueous phase ratio maintained at 70:30 methanol/water. Ruggedness of the method was confirmed by the robustness and intermediate precision results, which had a % RSD of 0.9 and 0.5 % respectively, thus ensuring that the method is precise and suitable for use for the analysis of MDZ. The LC-MS method was reliable in evaluating the specificity of the assay in the absence of RM-β-CD, but for the solutions containing RM-β-CD, a photodiode array detector was required in order to obtain a peak for the presence of MDZ alone.

In the samples without RM-β-CD, degradation products started appearing after 2 h of irradiation. A slight yellowish-brown color developed at the same time which intensified on continued irradiation. After 6 h, a precipitate formed which was readily soluble in the dilution solvent used for analysis (methanol/water). The control samples, with and without RM-β-CD, were clear and colorless, throughout the experiment. Analysis of the exposed samples containing RM-β-CD showed that degradation products had begun to form during the first hour of exposure. No suspended particles were observed throughout the experiment, but a slight yellowish-brown color was observed.

Fig. 2 shows the HPLC chromatograms of the degraded samples (i.e. MDZ with and without RM-β-CD). The MDZ peak has a retention time of 10.5 min. Small quantities of a two degradation products at 5.5 and 6.5 min were detected in MDZ solutions, but not in MDZ RM-β-CD solutions. Two other degradation products, only observed in MDZ RM-β-CD solutions, have retention times of 8.3 min and 8.7 min respectively with the degradation product with the higher retention time being more prominent. The major degradation products, that were observed in both MDZ and MDZ RM-β-CD solutions, had retention times of 6.0 min and 17.2 min respectively. Thus, all degradants, except the one which eluted at 17.2 min, were more polar than MDZ as they had shorter retention times. Peaks of major degradants are marked as **A**, **B** and **C** (degradation product **C** being only present in the MDZ-RM-β-CD solution) on the HPLC chromatograms.

**Figure 2.**

Results of the thermal stability studies show that up to 1.5 % degradation of the drug occurred in all samples subjected to heat for 24 h, both in the presence and in the absence of RM-β-CD, suggesting that most of the degradation that was observed in the photostabibilty studies samples was due to light and not heat, as previously suggested.7 Degradation of MDZ in the presence and in the absence of RM-β-CD is similar, with slightly decreased photostability in the presence of RM-β-CD. Two degradants, labelled **A** and **B** with retention times 6.0 and 17.2 min, respectively, were observed, while in the presence of RM-β-CD, two further degradants were evident at 8-9 min. For the purpose of this study, only identification of the major component, marked as a degradant **C** was considered.

Production of the degradant **A** follows degradation of the drug. The maximum concentration of photodegradant **C** is reached after 6 hours, Production of the third degradant, degradant **C**, reaches a maximum amount after about 7 hrs, when approximately 90% of the drug has been degraded. The amount of **C** then decreases over the next 3 hrs to about 15% of its maximum value. At this total time of 10 hrs, the amount of degradant B reaches a maximum. The similar trend has been seen in the forced degradation studies. These results indicate that photodegradant **C** may be an intermediate that decomposes into degradant **B**.

The results of LC-MS analysis including the M+1 peaks for the degradants, their retention times and proposed structures are listed in Table I. The degradation products observed only in solutions of MDZ, at retention times 5.5 and 6.5 min, exhibited peaks at *m/z* 342 (14 %, relative abundance (RA)) and 343 (27 %, RA), respectively. Structures proposed for the photoproduct with *m/z* 342 are the hydrolysis products 1-hydroxymethylmidazolam **(1)** or 4-hydroxymidazolam **(2)**, which are also the primary metabolites of MDZ. The photoproduct with *m/z* 343, has proposed stucture **(4)** which could form from the contraction of the diazepine ring, followed by contraction of the imidazole ring and subsequent substitution of the alkylamino group by a carbonyl in solution.

**Table I.**

Mass spectra of degradation products, with *m/z* 289, 273, 358 and 575 obtained by MS-MS analysis, are shown in Fig. 3 (a-d), respectively. In Fig. 3a, the molecular-ion peak (*m/z* 289, 23 % RA) is still visible, with an NH4 adduct (*m/z* 306, 11 % RA) also observed. The molecular mass of this compound is the same as one reported by Andersin *et al*[13](#_ENREF_13) and, on this basis, degradant **A** is proposed to be N-desalkylflurazepam, or 7-chloro-5-(2-fluorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one (structure **(3)**)and is one of the starting compounds in the synthesis of MDZ. The base peak at *m/z* 261 (100 % RA, M - 27) could be due to loss of HCN, and the peaks abserved at 270 (35 % RA, M - H2O) and 260 (16 % RA, M - CO) have been previously reported[24](#_ENREF_24). Other peaks observed were at 226 (57 % RA), 208 (36 % RA) and 140 (56 % RA). Photodegradation of MDZ to degradant **A** follows opening of the imidazole ring with subsequent sustitution to form the benzodiazepinone.

The M + 1 peak *m/z* 273, which corresponds to the loss of Cl,21 for degradant **B** was not detected (Fig. 3b). The base peak is at *m/z* 177 (100 % RA, M - C6H4F) and other peaks were *m/z* 237 (44 % RA, M - Cl), 245 (21 % RA) and 253 (14 % RA, M - F). The molar mass of degradant **B,** suggests that it is 6-chloro-2-methyl-4-(2-fluorophenyl)-quinazoline.12 Andersin *et al*. also reported a formation of precipitate in solutions of MDZ that were exposed to daylight. Their analysis of the purified precipitate revealed that the precipitate was 6-chloro-2-methyl-4-(2-fluorophenyl)-quinazoline. In this study, the precipitate appeared after 6 hrs of exposure and the amount of **B** was also approaching a maximum value. The proposed identification of **B** as structure **(5)**, is consistent in that the compound is more hydrophobic than degradant **A** (structure **(3)**). The retention time of **B** was 17.2 min in contrast to the other degradants which had shorter retention times than MDZ (*R*t = 10.5 min). Due to its non-polar nature, it is not surprising that degradant **B** precipitated out of the aqueous solution as its concentration increased. The absence of a precipitate in the RM-β-CD solutions, in spite of the evidence of the presence of **B** as a major degradation product, can be explained by the ability of RM-β-CD to improve the solubility of hydrophobic degradant compounds present.

**Fig. 3.**

Experimentaly determined UV λmax values of degradants **A** and **B** were consistent with literature values (Table II) for their proposed structures.

**Table II.**

The retention time (8.7 min) of degradant **C** suggests that it is more polar than MDZ. It possibly contains oxygen, resulting in a higher molecular mass. The mass spectrum of degradant **C** (Fig. 4c) shows a M + 1 peak at *m/z* 358 (72 % RA). It is important to stress that a compound with this molecular mass has not been previously reported in photodegradation studies of MDZ. The base peak at *m/z* 316 (100 % RA), and the peak at *m/z* 297 (75 % RA) could be due to loss of CH3, followed by subsequent loss of CH3CH2O. A fragment equivalent to the M + 1 peak of degradant **B**, *m/z* 273 is observed. From the kinetic studies it was postulated that degradant **C** is an intermediate in the formation of **B.** This means that **C** may have the structure of **B** as part of its structure.[1](#_ENREF_1) The degraded solutions were covered in foil, stored in a refrigerator (5 ± 3ºC) and analyzed by HPLC after a few weeks. After this time the peak due to the drug had slightly decreased, the peak for degradant **C** had almost disappeared, while the peak for **B** had singificantly increased, further indication that **C** may be an intermediate of **B.** The structure of **C** was therefore proposed to be structure **(6)**. Degradation of MDZ through **C** to **B** is an additional reaction pathway that was observed in the presence of RM-β-CD, which explains the slight increase in the degradation rate.

Another degradant caused by the presence of RM-β-CD may be a dimer of one of the degradation products.The mass spectrum (Fig. 3d) shows a base peak at *m/z* 556 (100 % RA, M - H2O), and other peaks at 381 (52 % RA) and 367 (95 % RA). The proposed structure (structure **(7)**) is a dimer of a major degradation product (6-(6-chloro-2-methyl-3H-quinazolin-4-ylidene)-cyclohexa-2,4-dienone) of MDZ without CD in aqeous solution.12 The MS data suggests the dimer is formed through loss of a Cl atom from each molecule, resulting in free radicals, which then combine with each other. Formation of this dimer and intermediate degradant **C** (structure **(6)**) is unique to the CD environment. The reactions are made possible by the ability of CD to stabilize free radicals,[25](#_ENREF_25) and by conformational control which results in stabilization of certain reaction intermediates. Since hydroxylation is attributed to the escape of degradants from the CD cage, we suggest that an important part of the deradation reaction pathway results from the recombination of radicals which are not in the solvent but are trapped in the CD cavity.

The major degradation product (molar mass 323.1) in solutions exposed to a high pressure mercury lamp by Anderson and coworkers[13](#_ENREF_13) was not observed in our work. Even the degradent which formed the dimer (*m/z* 575) was only present in small quantities. Degradant **B,** one of the major degradation products in this study, occurred in the solutions exposed to daylight, where **A** was only present in small amounts.

The results of this study indicate that MDZ undergoes a highly sequence-selective photoreaction pathway following inclusion complexation with RM-β-CD. RM-β-CD can thus alter the photobehavior of a MDZ molecule, by changing the ground state distribution of reactive and non-reactive conformers (“conformational control”) resulting in selectivity,[26](#_ENREF_26) and thus the reaction can be made to proceed along one of the competing pathways.[27](#_ENREF_27)

 A variety of non-covalent intermolecular bonds are involved in the formation of a stable complex with RM-β-CD that should protect the MDZ molecule against the attack of other reactive molecules and thus increase its chemical stability.[10](#_ENREF_10) CDs are known to accelerate or to slow down various reactions. For example, when an ester group of a guest molecule is fixed close to the catalytic site of CD (hydroxyl group of the sugar), the ester will be hydrolyzed faster. However, the rate of hydrolysis will decrease when the ester is inside the CD cavity. The molecular size of MDZ, calculated using Molecular Modeling pro 5.10 is estimated to be 14.67 Å, in molecular length (*x*), 11.59 Å width (*y*), and 4.27 Å depth (*z*). However, the RM-β-CD diameter inside cavity is 6.0-6.5 Å, while its outside diameter is 15.4 Å and height is 7.9 Å which corresponds approximately to the size of an aromatic ring (calculated size 6.18 Å in length, 6.82 Å in width, and 3.54 Å in depth). Therefore, RM-β-CD is only able to accommodate slightly more than one aromatic ring within its cavity.

 NMR studies have confirmed the MDZ RM-β-CD complex formation by the shift observed in the peaks for both MDZ and RM-β-CD.[28](#_ENREF_28) Proton and 13C-NMR have provided an idea how the guest substrate is positioned in the CD cavity. The shielding of the CD cavity protons and associated shift changes in the signal for the MDZ protons in the mixtures of CD and MDZ are attributed to the aromatic ring penetrating into the CD cavity, thereby confirming the formation of the inclusion complex. The structure of the MDZ RM-β-CD complex was established using two dimensional NMR (ROESY) spectral data.[29](#_ENREF_29) The signals for the protons belonging to the aromatic ring containing fluorine, exhibit strong cross correlation peaks with CD cavity protons. Taking into account the 1:1 stoichiometry of the complex it was concluded that the fluorine containing ring penetrates the RM-β-CD cavity resulting in the formation of a 1:1 complex. The signals for the H-8 of the chlorine containing aromatic ring (next to the chlorine substituent) also exhibited a strong interaction with the protons inside the CD cavity. However H-9 and H-10, did not show any cross peaks with the CD cavity protons (Fig. 4). Thus, the possibility of another 1:1 complex involving penetration of the chlorine containing ring was ruled out because the interaction of the protons from the ring containing chlorine with the cavity protons was not evident. Therefore, we can conclude that the fluorine containing aromatic ring and the chlorine substituent and H-8 from the other aromatic ring are likely to be inside the RM-β-CD cavity.

**Figure 4.**

The “cage effect” of inclusion of molecules in the CD cavity cause radical pairs to undergo reaction before being able to diffuse into the surrounding medium. This occurs to a lesser extent in the solution outside of the CD cavity due to more rapid diffusion.[30](#_ENREF_30)

CONCLUSION

It has been shown that complexation with RM-β-CD has slightly decreased the photostability of MDZ. The decreased stability was accompanied by the appearance of two new degradation products: (i) an intermediate [(E)-{1-[6-chloro-4-(2-fluorophenyl)-2-methylquinazolin-1(2H)-yl]ethylidene}amino]acetaldehyde (structure **(6)**/degradant **C**) that degrades to 6-chloro-2-methyl-4-(2-fluorophenyl)-quinazoline (structure **(5)**/degradant **B**); and (ii) a dimer (structure **(8)**) formed from free radicals (structure **(7)**) derived from previously reported photodegradant 6-(8-chloro-1-methyl-4,5-dihydro-2,5,10*b*-triaza-benzo[*e*]azulen-6-ylidene)-cyclohexa-2,4-dienone12  as a result of the loss of a Cl atom. Photodegradation product formation followed the same pattern as in the forced degradation studies, further confirming the presence of an intermediate. The photodegradation chemistry of a drug molecule is different when encapsulated because the interior of the cyclodextrin cavity constitutes an isolated environment where the included species is usually present as a single molecule. The photochemistry is therefore generally restricted to intramolecular events, except in cases of multiple occupancy.

While the presence of the RM-β-CD improves the aqueous solubility of MDZ it also alters its photostability. The formation and presence of these new photo degradants must be taken into account when using RM-β-CD to improve MDZ solubility and develop a feasible nasal formulation. Although photoinduced reactions may or may not be identical *in vitro* and *in vivo*, basic knowledge on the reaction mechanisms and products is important to ensure safe handling, packaging and labeling of the formulation and reduced potential for adverse effects. The differences in the photostability upon complexation with cyclodextrin are affected by the way the drug is encapsulated into cyclodextrin cavity, which helps us to understand whether the site of degradation is within the cyclodextrin cavity or not. Therefore, further studies should be undertaken to determine the toxicity of these photodegradants. Generally, photosensitivity reactions have occurred in patients to whom light sensitive drugs have been administered, and have been related with the formation of phototoxic degradants. Such drugs decompose to form radical intermediates and highly reactive products, which react with the tissue cells resulting in adverse effects, making the detection and identification of photodegradants important. Thus, there is a need to determine the effect of the inclusion complexation with cyclodextrins on the photodegradation profile of drugs.

REFERENCES

1. B. D. Glass; M. E. Brown; S. Daya; M. S. Worthington; P. Drummond; E. Antunes; M. Lebete; S. Anoopkumar-Dukie; D. Maharaj, Influence of cyclodextrins on the photostability of selected drug molecules in solution and the solid-state. *Int. J. Photoenergy* **3** (2001) 205.

2. S. Björkman; G. Rigemar; J. Idvall, Pharmacokinetics of midazolam given as an intranasal spray to adult surgical patients. *Br. J. Anaesth.* **79** (1997) 575.

3. T. Mahmoudian; M. M. Zadeh, Comparison of intranasal midazolam with intravenous diazepam for treating acute seizures in children. *Epilepsy Behav.* **5** (2004) 253.

4. E. Lahat; M. Goldman; J. Barr; T. Bistritzer; M. Berkovitch, Comparison of intranasal midazolam with intravenous diazepam for treating febrile seizures in children: prospective randomised study. *BMJ (Clinical Research Ed.)* **321** (2000) 83.

5. R. Andersin, Solubility and acid-base behaviour of midazolam in media of different pH, studied by ultraviolet spectrophotometry with multicomponent software. *J. Pharm. Biomed. Anal.* **9** (1991) 451.

6. G. Tiwari; R. Tiwari; A. K. Rai, Cyclodextrins in delivery systems: Applications. *Pharm. Bioallied Sci.* **2** (2010) 72.

7. F. Marçon; D. Mathiron; S. Pilard; A.-S. Lemaire-Hurtel; J.-M. Dubaele; F. Djedaini-Pilard, Development and formulation of a 0.2% oral solution of midazolam containing γ-cyclodextrin. *Int. J. Pharm.* **379** (2009) 244.

8. D. Mathiron; F. Marcon; J. M. Dubaele; D. Cailleu; S. Pilard; F. Djedaini-Pilard, Benefits of methylated cyclodextrins in the development of midazolam pharmaceutical formulations. *J. Pharm. Sci.* **102** (2013) 2102.

9. J. Mannila; T. Jarvinen; K. Jarvinen; M. Tarvainen; P. Jarho, Effects of RM-beta-CD on sublingual bioavailability of Delta9-tetrahydrocannabinol in rabbits. *Eur. J. Pharm. Sci.* **26** (2005) 71.

10. T. Loftsson; M. E. Brewster, Pharmaceutical applications of cyclodextrins. 1. Drug solubilization and stabilization. *J. Pharm. Sci.* **85** (1996) 1017.

11. S. Scalia; R. Tursilli; V. Iannuccelli, Complexation of the sunscreen agent, 4-methylbenzylidene camphor with cyclodextrins: effect on photostability and human stratum corneum penetration. *J. Pharm. Biomed. Anal.* **44** (2007) 29.

12. T. Loftsson; S. B. Vogensen; M. E. Brewster; F. Konradsdottir, Effects of cyclodextrins on drug delivery through biological membranes. *J. Pharm. Sci.* **96** (2007) 2532.

13. R. Andersin; J. Ovaskainen; S. Kaltia, Photochemical decomposition of midazolam. III. Isolation and identification of products in aqueous solutions. *J. Pharm. Biomed. Anal.* **12** (1994) 165.

14. R. Andersin; S. Tammilehto, Photochemical Decomposition of Midazolam. II. Kinetics in Ethanol. *Int. J. Pharm.* **56** (1989) 175.

15. P. Bortolus; G. Grabner; G. Kohler; S. Monti, Photochemistry of Cyclodextrin Host-Guest Complexes. *Coord. Chem. Rev.* **125** (1993) 261.

16. J. Mielcarek, Photochemical stability of the inclusion complexes formed by modified 1,4-dihydropyridine derivatives with beta-cyclodextrin. *J. Pharm. Biomed. Anal.* **15** (1997) 681.

17. U. Tadanobu; I. Keiko; H. Fumitoshi; U. Kaneto, Stoichiometry-dependent changes of solubility and photoreactivity of an antiulcer agent, 2′-carboxymethoxy-4,4′-bis(3-methyl-2-butenyloxy) chalcone, in cyclodextrin inclusion complexes. *Eur. J. Pharm. Sci.* **1** (1993) 81.

18. S. Sortino; S. Giuffrida; G. De Guldi; R. Chillemi; S. Petralia; G. Marconi; G. Condorelli; S. Sciuto, The photochemistry of flutamide and its inclusion complex with beta-cyclodextrin. Dramatic effect of the microenvironment on the nature and on the efficiency of the photodegradation pathways. *Photochem. Photobiol.* **73** (2001) 6.

19. R. Andersin; S. Tammilehto, Photochemical decomposition of midazolam. IV. Study of pH-dependent stability by high-performance liquid chromatography. *Int. J. Pharm.* **123** (1995) 229.

20. J. A. Carrillo; S. I. Ramos; J. A. Agundez; C. Martinez; J. Benitez, Analysis of midazolam and metabolites in plasma by high-performance liquid chromatography: probe of CYP3A. *Ther. Drug Monit.* **20** (1998) 319.

21. T. Higuchi; K. A. Connors, Chapter 4. Phase Solubility Studies. In *Advances in Analytical Chemistry and Instrumentation*, Reilley, C. N.; McLafferty, F. W., Eds. Interscience: New York, 1965; p 117.

22. ICH Topic Q1B, Photostability testing of new drug substances and medicinal products, Step 5, Note for guidance on the photostability testing of new active substances and medicinal products (CPMP/ICH/279/95). European Medicines Agency: London, 1998.

23. H. G. Brittain, Validation of nonchromatographic analytical methodology. *Pharm. Technol.* **22** (1998) 82.

24. R. Selkämaa; S. Tammilehto, Photochemical decomposition of midazolam. I. Isolation and identification of products. *Int. J. Pharm.* **49** (1989) 83.

25. A. V. Veglia; A. M. Sanchez; R. H. Derossi, Change of Selectivity in the Photo-Fries Rearrangement of Phenyl Acetate Induced by Beta-Cyclodextrin. *J. Org. Chem.* **55** (1990) 4083.

26. B. N. Rao; N. J. Turro; V. Ramamurthy, Modification of chemical reactivity via inclusion complex formation: photochemistry of dibenzyl ketones and benzyl phenylacetates. *J. Org. Chem.* **51** (1986) 460.

27. G. D. Reddy; V. Ramamurthy, Modification of photochemical reactivity by cyclodextrin complexation: alteration of photochemical behavior via restriction of translational and rotational motions. Alkyldeoxybenzoins. *J. Org. Chem.* **52** (1987) 5521.

28. A. R. Hedges, Industrial Applications of Cyclodextrins. *Chem. Rev.* **98** (1998) 2035.

29. S. M. Ali; S. K. Upadhyay, Complexation study of midazolam hydrochloride with β-cyclodextrin: NMR spectroscopic study in solution. *Magn. Reson. Chem.* **46** (2008) 676.

30. G. D. Reddy; G. Usha; K. V. Ramanathan; V. Ramamurthy, Modification of photochemistry by cyclodextrin complexation. Competitive Norrish type I and type II reactions of benzoin alkyl ethers. *J. Org. Chem.* **51** (1986) 3085.

1. Corresponding author. E-mail: snezana@puncakalam.uitm.edu.my [↑](#footnote-ref-1)