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

Comment [N1]: References should be thoroughly checked throughout the whole paper.

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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. It was 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 was partly confirmed in a previous work. By continuing this study, degradation products, not found in the photodegradation of uncomplexed midazolam are observed in significant quantities when it was 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, and a proposed degradation mechanism of midazolam are discussed.

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

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In answer to "**Comment [N1]:**" at the top of this page, the references throughout the manuscript have been thoroughly checked.

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.[1] 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 safe and inexpensive means to rapidly achieve seizure control.[1] 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 administration *via* nasal route directly and provides rapid onset of action. Since no nasal preparation is commercially available, midazolam solution for injection has been used.[2,3] Since commercially available formulation for intravenous application contain low concentration of MDZ, due to its low solubility, large application volumes exceeding the limited nasal capacity have to be used to achieve adequate dosing. In order to deliver therapeutic midazolam doses in smaller volumes, nasal preparations with MDZ concentration exceeding its solubility must be developed. MDZ is water-soluble at pH values lower than 4 and lipid-soluble at pH values above 5 and therefore lipid soluble at physiological pH.[4] The water solubility of MDZs increases at pH values less than 4.0 due to ring opening and ionization[3] whereby 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 the physiological pH of 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 effectively able to accommodate and complex larger molecules, such macrocycles and steroids. However, the smaller cavity diameter of β-CDs (seven glucopyranose units) is found to accommodate and effectively complex most drug compounds (aromatic and hetero-

cyclic molecules). Hence, *β*-CD is commonly used as a complexing agent in many CD complex drug formulations.[5]

Formulations of a 0.2 % (w/v) oral solution of MDZ containing *γ*-CD and citric acid were previously investigated.[6] More recently, however, it was demonstrated that the use of randomly methylated-*β*-CD (RM–*β*-CD) as a solubilizer significantly reduces the rate of degradation of MDZ.[7] 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[8] and to reduce the rate of hydrolysis[9] and photodegradation.[10] Substitution of the hydroxyl with methoxy groups imparts a slightly lipophilic character to the molecule, which also facilitates permeation through the mucosa.[11]

Comment [N2]: g/L or something else?

Although the photostability of MDZ is well documented,[4,12–14] the rate of photodegradation and the nature and distribution of photodegradants may change when MDZ forms an inclusion complex with CD.[15–17] CDs have the potential to control 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 accelerate drug degradation.[18] The rate of secondary reactions may also be influenced and the chemical evolution of reaction intermediates controlled.[18] Quantitative high performance liquid chromatography (HPLC) methods have been used to study the decomposition of MDZ in formulations[19] and to determine the amount of MDZ and its metabolites in blood plasma.[20]

Since the presence of RM–*β*-CD results in different degradation from those observed in the absence of RM–*β*-CD, the aim of present study was to investigate more deeply the obtained products. Based on results of the photostability, kinetic studies and MS characterization, a degradation mechanism of midazolam is proposed as well as a model of the RM–*β*-CD–MDZ inclusion complex.

Phase solubility studies conducted according to the method of Higuchi and Connors[21] confirmed the ability of RAMEB 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. HPLC grade methanol was obtained from Romil

In answer to "Comment [N2]:" given at the top of this page, regarding the changing of the highlighted text "(w/v)" on line 79; the highlighted text should remain as "(w/v)". This is the abbreviation for "weight for volume" and is used when expressing percentage concentrations for solids dissolved in liquids.

Limited (Cambridge). Water for chromatography was obtained from a Milli-Q® water purification system (Millpore, MA, 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 and 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 °C) and detection was performed spectrophotometrically at 240 nm. Methanol was used as the organic mobile phase component. The optimum organic/aqueous phase ration was found to be 70:30 and the mobile phase was modified by addition of ammonium acetate as recommended for LC–MS studies to improve the performance of an LC–MS screening method. For very weak basic compounds, such as amides, the ammonium ions in the mobile phase promoted proton adduct responses.[22]

The retention times obtained with a µBondapak C18 10 µm column were compared with those obtained using a Waters Spherisorb ODS2 5 µm column.

Phase solubility studies

The phase solubility studies were performed in triplicate. A 75 mg of MDZ was placed in a conical flask and suspended in 5 ml of phosphate buffer solution (pH 5.8) 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 °C. The samples were then analysed after 24 h using a UV method. The presence of photodegradation products was ruled out by covering the flasks with foil during the phase solubility studied. The procedure was repeated with phosphate buffer at pH 5.0.

Comment [N3]: ?

UV method development

Midazolam (50 mg) was dissolved in 0.1 M HCl and diluted to 100 mL with the same solvent. 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 (λ_{max}) 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 5.0) 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 12 h (1.2 million lux h) in order to degrade the drug to approximately 10 % of its original concentration.[24] An Atlas SUNTEST CPS+ (Atlas Material Testing Technology B.V, Germany), fitted with a Xenon lamp and Solar ID65 filter was used for the irradiation of the samples. The temperature in the Suntest cabinet was maintained at 40±2 °C.

Samples (2 mL) were removed at 1 h intervals over the 12 h period, diluted to a final volume of 10 mL 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 representative samples of the drug and photoproducts in the presence and absence of RM–*β*-CD were selected and analyzed by LC–MS to obtain the molecular masses of the

In answer to Comment [N3]: regarding the highlighted text on line 135, "(w/v)"; the text should remain as "(w/v)". This is the abbreviation for "weight for volume" and is used when expressing percentage concentrations for solids dissolved in liquids.

degradation products. LC–MS/MS was then used to identify the photodegradants. For the 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×4.6 mm).

Comment [L4]: Please check

Thermal stability studies

To investigate the influence of temperature 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 °C. The samples were removed after 12 h and analyzed.

Comment [N5]: ?

RESULTS AND DISCUSSION

A simple UV method with 0.1 M HCl as the solvent was developed for use in midazolam–RM–*β*-CD phase solubility studies. The method showed good linearity, with the linear regression equation $y = -0.000051 + 0.0275A$ (y is the midazolam concentration and A the absorbance) with a correlation coefficient of 0.99. It was accurate with 98.9–101.6 % percent recovery. Precision of the method was determined by calculating the relative standard deviation (*RSD*). The *RSD* for replicate measurement at three different concentration within the linear range was less than 1 %, indicating adequate precision.[22]

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

Comment [N6]: ?

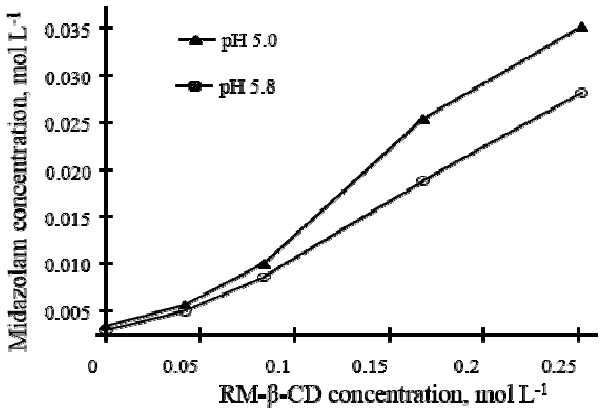


Fig. 1. Solubility of midazolam in phosphate buffers *versus* RM–*β*-CD concentration.

1. The word "FINIGAN" highlighted on line 164 (see Comment [L4]) should be changed to "FINNIGAN".
2. The text "w/v" highlighted on line 169 (Comment [N5]) should be changed to "(w/v)". As previously indicated, this is the abbreviation for "weight for volume" and is used when expressing percentage concentrations for solids dissolved in liquids.
3. The text "w/v" highlighted on line 183 (Comment [N6]) should be changed to "(w/v)". As previously indicated, this is the abbreviation for "weight for volume" and is used when expressing percentage concentrations for solids dissolved in liquids.

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 a 30 % w/v RM–*β*-CD solution with three known concentrations of MDZ (0.4, 0.5 and 0.6 mg mL^{-1}). Linearity was confirmed to be within the range 0.002 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.

Comment [N7]: ?

The limit of quantification (*LOQ*) was found to be 0.002 mg mL^{-1}. The robustness of the method was demonstrated during method development, when it was shown that the 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. The 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 due to 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 simultaneously that 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, until the end of the experiment. Analysis of the exposed samples containing RM–*β*-CD showed that degradation products begun to form during the first hour of exposure. No suspended particles were observed throughout the experiments, but a slight yellowish–brown color was observed.

The HPLC chromatograms of the degraded samples (*i.e.*, MDZ with and without CD) are shown in Fig. 2. The MDZ peak has a retention time of 10.5 min. Small quantities of the two degradation products at 5.5 and 6.5 min were detected in the MDZ solutions, but not in the MDZ RM–*β*-CD solutions. Two other degradation products, only observed in MDZ RM–*β*-CD solutions, had retention times of 8.3 and 8.7 min 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 and 17.2 min. Thus, all degradants, except the one that eluted at 17.2 min, were more polar molecules than MDZ as they had shorter retention times. Peaks of the

The text "w/v" highlighted on line 193 (Comment [N7]) should be changed to "(w/v)". As previously indicated, this is the abbreviation for "weight for volume" and is used when expressing percentage concentrations for solids dissolved in liquids.

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.

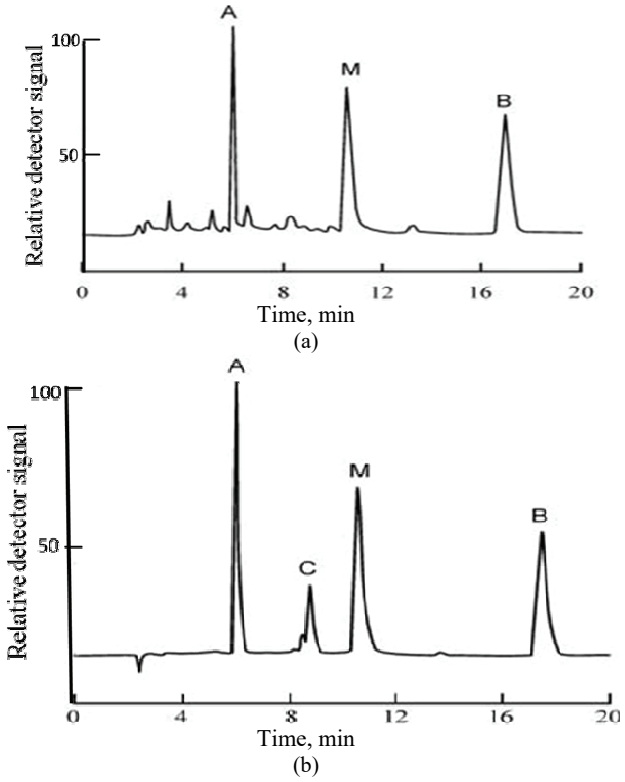


Fig. 2. HPLC chromatograms showing the peak of midazolam, M, after 8 h photodegradation (a) in the absence and (b) in the presence of RM–*β*-CD and the peaks of degradants **A**, **B** and **C**.

Results of the thermal stability studies showed 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 degradation that was observed in the photostability 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 was similar, with slightly decreased photostability in the presence of RM–*β*-CD. Two degradants, labeled **A** and **B**. with retention times 6.0 and 17.2 min, respectively, were observed, while in the presence of RM–*β*-CD, two

249 further degradants were evident at 8–9 min. For the purpose of this study, only
250 identification of the major component, marked as a degradant **C**, was considered.
251 Production of degradant **A** follows degradation of the drug. The maximum
252 concentration of photodegradant **C** was reached after 6 h, Production of the third
253 degradant, degradant **C**, reached a maximum amount after about 7 h, when
254 approximately 90 % of the drug had been degraded. The amount of **C** then dec-
255 reased over the next 3 h to about 15 % of its maximum value. At this total time of
256 10 h, the amount of degradant **B** reached a maximum. A similar trend was seen in
257 the forced degradation studies. These results indicate that photodegradant **C** may
258 be an intermediate that decomposes into degradant **B**.[1]
259 The results of LC–MS analysis including the M+1 peaks for the degradants,
260 their retention times and proposed structures are listed in Table I. The degrad-
261 ation products observed only in solutions of MDZ, at retention times 5.5 and 6.5
262 min, exhibited peaks at *m*/*z* 342 (14 %, relative abundance – RA) and 343 (27 %
263 RA), respectively. The structures proposed for the photoproduct with *m*/*z* 342 are
264 the hydrolysis products 1-hydroxymethylmidazolam (**1**) or 4-hydroxymidazolam
265 (**2**), which are also the primary metabolites of MDZ. The photoproduct with *m*/*z*
266 343 has proposed structure (**4**).

267 TABLE I. Retention times, M + 1 peak values and proposed structures of photodegradants

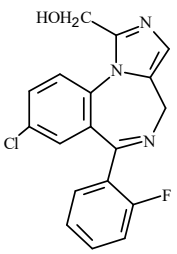
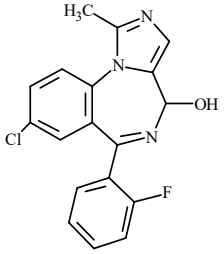
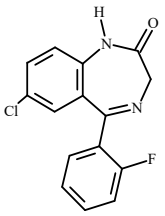
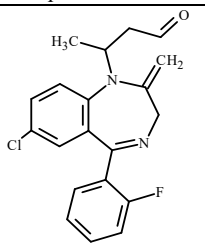
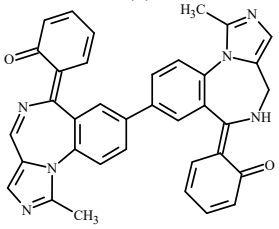
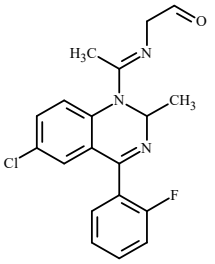
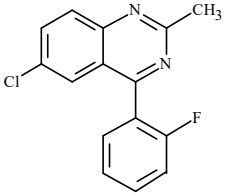
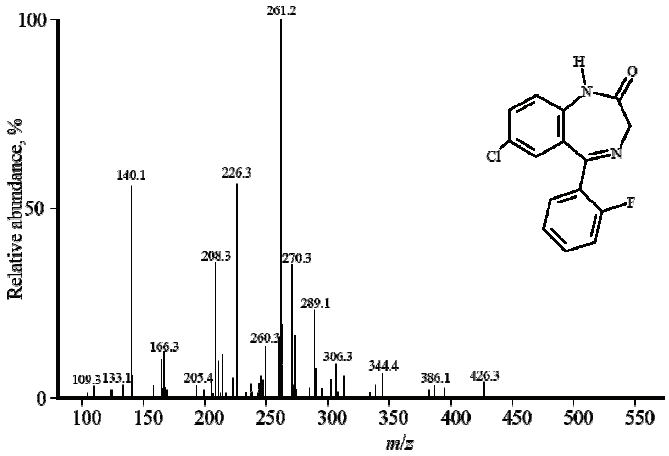
Retention time, min	M + 1	Proposed structure
5.5	342	 (**1**) or  (**2**)
6.0	289	 (**3**)

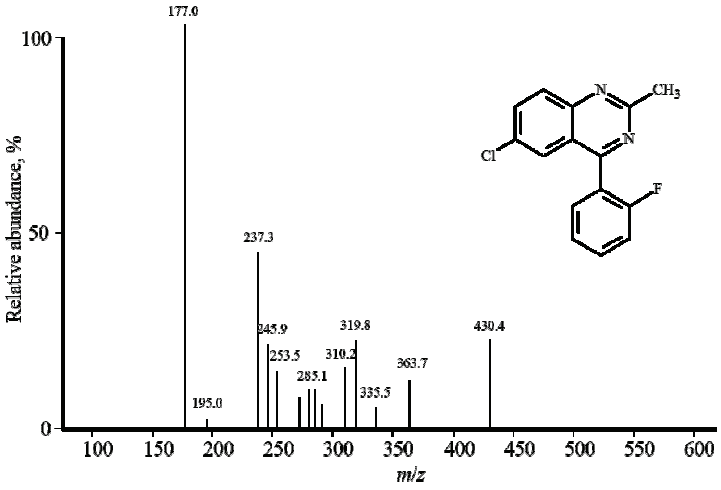
TABLE I. Continued

Retention time, min	M + 1	Proposed structure
6.5	343	 (**4**)
8.3	575	 (**7**)
8.7	358	 (**6**)
17.2		 (**5**)

Mass spectra of degradation products, with *m/z* values of 289, 273, 358 and 575 obtained by MS-MS analysis, are shown in Figs. 3a–d and 4a–d, respectively. In Fig. 3a, the molecular-ion peak (*m/z* 289, 23 % RA) is still visible, with an NH_4 adduct (*m/z* 306, 11 % RA) also observed. The molecular mass of this com-



(a)



(b)

Fig. 3. Mass spectra of: a) degradant **A** with the M + 1 peak at *m/z* 289 and b) degradant **B** with the M+1 peak at *m/z* 273.

pound is the same as one reported by Andersin *et al.*[12] and, on this basis, degradant **A** is proposed to be *N*-desalkylflurazepam, or 7-chloro-5-(2-fluorophenyl)-1,3-dihydro-2*H*-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 observed at 270 (35 %

RA, M–H_2O) and 260 (16 % RA, M–CO) were previously reported.[23] Other peaks observed were at 226 (57 % RA), 208 (36 % RA) and 140 (56 % RA). Photodegradation of MDZ to degradant **A** follows the opening of the imidazole ring with subsequent substitution to form benzodiazepinone.

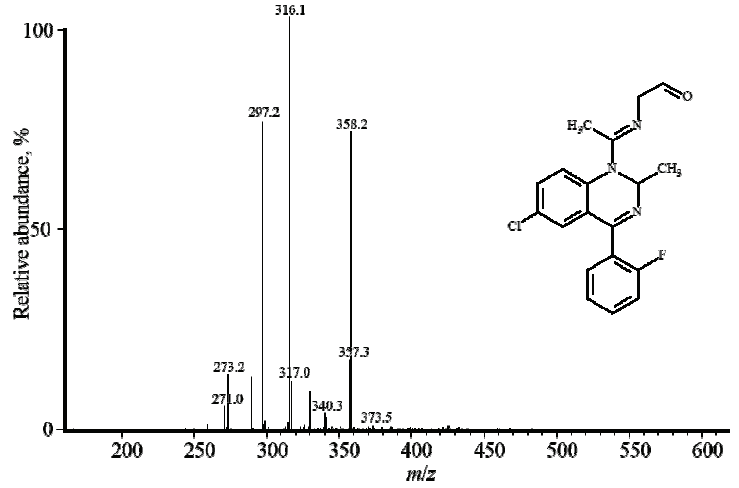


Fig. 4. Mass spectra of: a) degradant **C** with the M + 1 peak at *m/z* 358 and b) degradant M + 1 peak at *m/z* 574.9.

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–C_6H_4F) 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 the formation of a precipitate in solutions of MDZ 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 h 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 that 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, could be explained by the ability of RM–*β*-CD to improve the solubility of the hydrophobic degradant compounds present.

The experimentally determined UV λ_{max} values of degradants **A** and **B** were consistent with literature values (Table II) for their proposed structures.

TABLE II. UV λ_{max} values of degradants **A, B** and **C** (nm)

Degradant	Experimental	Literature values[13]
A	229	230
	318	318
B	230	206
	327	230
		326
C	215	–
	333	

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. 4a) shows an 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 CH_3, followed by subsequent loss of CH_3CH_2O. A fragment equivalent to the M+1 peak of degradant **B**, *m/z* 273 was 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.[14] The degraded solutions were covered in foil, stored in a refrigerator (5±3 °C) and analyzed by

HPLC after a few weeks. After this time, the peak due to the drug had slightly decreased and the peak for degradant **C** had almost disappeared, while the peak for **B** had significantly increased, which is 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. 4b) shows a base peak at *m/z* 556 (100 % RA, M–H_2O), 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-3*H*-quinazolin-4-ylidene)-cyclohexa-2,4-dienone) of MDZ without CD in the aqueous solution.[14] 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,[24] 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, it is suggest that an important part of the degradation 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 Andersin and coworkers[12] was not observed in the present work. Even the degradant that 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 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,[25] and thus the reaction could be directed along one of the competing pathways.[26]

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 attack of other reactive molecules and thus increase its chemical stability.[9] CDs are known to accelerate or to retard 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 mole-

cular 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 MDZ RM–*β*-CD complex formation by the shift observed in the peaks for both MDZ and RM–*β*-CD.[27] ^{1}H- and ^{13}C-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 an inclusion complex. The structure of the MDZ RM–*β*-CD complex was established using two dimensional NMR (ROESY) spectral data.[28] The signals for the protons belonging to the aromatic ring containing fluorine exhibit strong cross correlation peaks with the CD cavity protons. Taking into account the 1:1 stoichiometry of the complex, it was concluded that the fluorine-containing ring penetrates the *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. 5). Thus, the possibility of another 1:1 complex involving penetration of the chlorine-containing ring was ruled out because interaction of the protons from the ring containing chlorine with the cavity protons was not evidenced. Therefore, it could be concluded 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.

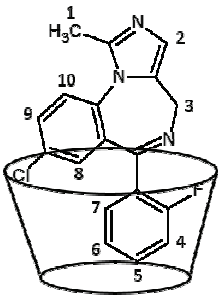


Fig. 5. Proposed model of the RM–*β*-CD–MDZ inclusion complex.

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 the CD cavity due to more rapid diffusion.[29]

CONCLUSIONS

It has been shown that complexation with RM–*β*-CD 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(2*H*)-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-dienone[33] 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 midazolam, it also alters its photostability. Introduction of 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 might or might 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 to understand whether the site of degradation is within the cyclodextrin cavity or not. Further studies should be undertaken to determine the phototoxicity of the degradants. Generally, photosensitivity reactions occur in patients to whom light sensitive drugs have been administered, and have been related to 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.

ИЗВОД

ХЕМИЈСКА КАРАКТЕРИЗАЦИЈА ПРОИЗВОДА ФОТОРАЗГРАДЊЕ КОМПЛЕКСА МИДАЗОЛАМА СА НАСУМИЧНО МЕТИЛОВАНИМ *β*-ЦИКЛОДЕКСТРИНОМ МЕТОДАМА HPLC И LC–MS/MS

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Мидазолам, потенцијални анксиолитички лек са седативним својствима, подложан је фоторазградњи и хидролизи у воденим растворима. Установљено је да су интраназалне формулације ефикасне у контроли епилептичних напада. Како би се постигла адекватна терапијска доза, назалне водене формулације би морале да буду са већом концентрацијом мидазолама него што је његова растворљивост. Један од начина да се постигне терапијска доза јесте комплексирање мидазолама насумично метилованим *β*-циклодекстрином. Стога је важно да се утврде брзина разграње мидазолама и одговарајући настали производи уколико је комплексиран. Установљено је да је комплексирани мидазолам фотонестабилинији од некомплексираног. Оно што је више значајно јесте да се код комплексираног мидазолама јављају нови производи разградње у значајним концентрацијама. Смањена фотостабилност је праћена појавом два нова производа разградње, једног са прелазном структуром и једног димера. Настанак фотодеградационих производа одиграва се по истом механизму који је установљен испитивањем принудне разградње, што потврђује присуство прелазних структура. Дата је дискусија настанка фотодеградационих производа, који су окарактерисани MS подацима, и предложен је одговарајући механизам разградње мидазолама.

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