**Response to reviewer’s comments on the manuscript "Chemical characterization of photodegradation products of midazolam complexes with randomly methylated-β-cyclodextrin by HPLC and LC-MS/MS"**

The manuscript has been revised in accordance with the two reviewer’s suggestions/comments. The responses/changes made to the manuscript to reviewer’s suggestions/comments are given below. The Figures and Tables have also been corrected/revised in accordance with the requirements of the Journal and reviewer’s suggestions. The revised figures and tables are in the file entitled “Corrected figures and tables”.

**Reviewer B**

Does the manuscript contain enough significant original material?: yes

Is the manuscript clearly and concisely written?: yes

Are the conclusions adequately supported by the data?: no

Does the manuscript give appropriate credit to related recent publications?: yes

Are the references appropriate and free of important omissions?: no

Response: the references have been updated in accordance with the reviewers’ comments.

Is the length of the manuscript appropriate?: no

Does the manuscript need condensation or extension?: yes

Is the quality of the figures (including legends and axes labelling) satisfactory?: no

Response: the Figures have been updated in accordance with the reviewers’ comments.

Are the nomenclature and units in accordance with SI?: yes

Are the English grammar and syntax satisfactory?: yes

**Reviewer B additional comments**

1.      Please consider the manuscript title. In the title “chemical characterization of photodegradation products of midazolam...” there is no mentioning of the degradation at high temperature condition which is also a subject of this work.

Response: 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 degradation that was observed in the photostability studies samples was due to light and not heat, as previously suggested. Therefore, as temperature is not important, we believe it should not be included in the title.

2.      L17-19 You mentioned in the abstract that is very important to claim the complex influence on miadazolam dissolution, but you didn’t research that at all. A significant part of the abstract is dedicated to the miadazolam solubility/dissolution rate from cyclodextrin complex (L11-17) and this
wasn’t your preocupation in this study. Only a small part of the abstract is about midazolam photodegradation (L20-25). In my opinion, the abstract needs complete correction with a focus on the degradation products and the importance of this research and minimization of the part regarding
miadazolam dissolution from cyclodextrin complex.

Response: Midazolam solubility and dissolution are well documented in the literature. The abstract has been rewritten with the focus on the degradation products.

3.      L36-9 The dissolution of midazolam is shown at pH 7.4 and the temperature of 24°C. This data doesn’t show a realistic case study, so it isn’t relevant from the biopharmaceutical aspect.

Response: We do not show the dissolution of midazolam at pH 7.4 so we are not sure what the reviewer is referring to here.

4.      L73-83 There is no clear aim - why is it important to investigate the degradation products of the complex? What is the difference compared to the author’s previous work (ref. 1) and other referenced works?

Response: A statement has been added to the conclusion to the address this issue. “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.”

5.      L89-91 For some substances you stated only the country, and for others both the county and the city. Please be uniform.

Response: In order to be uniform, the name of the city has been removed from the details of the substances in the manuscript.

6.      There is no data about sample preparation (preparation of miadazolam and RM-β-CD complex ) in the Experimental part of the manuscript.

Response: This was described in the experimental part of the manuscript within “photostability kinetic studies” and “thermal stability studies”.

7.      L92-100 Which is the pH value of the mobile phase? During method validation, the pH value of the mobile phase is the crucial parameter for the method’s robustness. Why is it not noted?

Response: The sentence; “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.” has been added to the section entitled “HPTLC instrumentation” (page 4, revised manuscript).

8.      L105-107 It is necessary to cite the original reference (ICH directive Q1B Stability testing: photostability testing of new drug substances and products) instead of reference 20.

Response: Reference 20 has been replaced “with the original reference” (i.e. reference 23 in the revised manuscript).

9.      L118 Why was the pH 5 buffer used? It is known that the pH value of nasal liquid is between 5.5 and 6.5. Additionally, you described midazolam stability at pH values higher than 5.

Response: The buffer of pH = 5 was used in in order to achieve targeted concentration of midazolam. It is not soluble at physiological pH as indicated in the introduction.

10.     L129-131 What was the solvent of midazolam and RM-β-CD complex? Is it connected with the Photostability kinetic studies? Why were these temperatures chosen? On which device was the examination performed? Did you control moisture, according to the ICH directive?

Response: It was clearly indicated in the “photostability kinetic studies” section that “MDZ solutions (0.5 mg mL-1) in phosphate buffer (pH 5), were prepared in the presence and...”.

Forced degradation is carried out to produce representative samples for developing stability-indicating methods for drug substances and drug products. Some scientists have found it practical to begin with extreme conditions such as 80 °C or even higher temperatures and testing at shorter time points, so that the rate of degradation can be evaluated. Regarding the effects of moisture, we have evaluated solutions so we are not sure how moisture should affect stability.

11.     In my opinion, it would be very good to confirm the interaction between midazolam and RM-β-CD by using FT-IR.

Response: Interaction of MDZ with CD with studied with NMR as indicated in the reference provided

12.     L161 Based on what have you chosen the time intervals for presentation of degradation products? There are results after 6 and 8 hours. Please note that the presented results are after 8 hours.

Response: The ICH guideline states that stress testing is intended to identify the likely degradation products which further helps in determination of the intrinsic stability of the molecule and establishing degradation pathways, and to validate the stability indicating procedures used [[ ICH guidelines, Q1A (R2): Stability Testing of New Drug Substances and Products (revision 2), International Conference on Harmonization. Available from: 〈http://www.fda.gov/downloads/RegulatoryInformation/Guidances/ucm128204.pdf〉, 2003.]](file://E:\Research%20papers\Midazolam%20paper\J%20Serb%20Chem%20Soc%20submission\Revised%20manuscript\%5b%20ICH%20guidelines,%20Q1A%20(R2):%20Stability%20Testing%20of%20New%20Drug%20Substances%20and%20Products%20(revision%202),%20International%20Conference%20on%20Harmonization.%20Available%20from:%20〈http:\www.fda.gov\downloads\RegulatoryInformation\Guidances\ucm128204.pdf〉,%202003.%5d). But these guidelines are very general in conduct of forced degradation and do not provide details about the practical approach towards stress testing.

13.     L179-187 Please improve the discussion regarding the degradation of midazolam and midazolam/RM-β-CD complex. On which temperatures were the degradation products noticed? Is the degradation the same at 20°C and at 80°C?

Response: The discussion and conclusion have been revised to provide a more detailed discussion of the degradation results observed.

14.     L188-194 this paragraph is completely the same as in the previous work (reference 1). Make it shorter and referenced.

Response: The paragraph has been rewritten. It has been referenced already to previous work.

15.     The legends, x axis and y axis on Fig 2 are not clear. On Fig 1 different segments are marked with Roman numbers (I, II), and on Fig 2 with letters (a). Please be uniform according to the instructions.

Response: The marking on Fig. 1 has been corrected and Fig. 2 has also been corrected according to Journal formatting requirements.

16.     Please check the lines in Tab 2.

Response: The lines in Table 2 have been checked. They are correct.

17.     Fig 3: Structures 3 and 5 are described in reference 1. My suggestion is to add a new sentence which contains the information about that and additional structures 1,2 and 4. Please explain why the structure 17 from reference 1 wasn’t detected. Structures 1,2 and 4 are noted in Table 1, so
there is no need for double presentation of data (Figures and Tables).

Response: See response to additional comment 19.

18.     Fig 4: Midazolam structures from Fig 3 are repeated. Structures 3 and 5 are repeated. The structure 6 was presented in the previous work (reference 1). Please clarify. The structure with the origin from intermediary 2 was shown in Table 1, therefore there is no need for repeating, simply describe it in the text.

Response: See response to additional comment 19.

19.     Based on the previous two comments, I suggest you delete figures 3 and 4. If you think that the presented data is necessary, you can show them in a supplement (please see the instructions for authors)

Response to additional comments 17-19: Figures 3 and 4 have been deleted as suggested by the reviewer.

20.     In the conclusion add more details about the significance of the presented results according to formulations and ICH directives for photostability tracking.

Response: The conclusion has been rewritten with the more emphasis on photo stability and photo degradants.

21.     Please check the references based on the instructions (for example ref 8)

Response: All of the references have been checked to ensure they are formatted in accordance with the Journal with corrections/changes made to the manuscript where necessary.

22.     Please check the typing errors (e.g. ® should be in the superscript -®).

Response: “®” has been superscripted in the text as requested. The manuscript has also been checked for typing errors and changes have been made where appropriate.

REPORT:

In my opinion, this manuscript should: be published after minor revision without additional review

If manuscript is suitable for publishing, referees recommendation : Original scientific paper

**Reviewer C:**

Does the manuscript contain enough significant original material?: yes

Is the manuscript clearly and concisely written?: no

Response: the manuscript has been changed in accordance with the reviewers’ suggestions.

Are the conclusions adequately supported by the data?: yes

Does the manuscript give appropriate credit to related recent publications?: yes

Are the references appropriate and free of important omissions?: yes

Is the length of the manuscript appropriate?: yes

Does the manuscript need condensation or extension?: yes

Response: the manuscript has been changed in accordance with the reviewers’ suggestions.

Is the quality of the figures (including legends and axes labelling) satisfactory?: yes

Are the nomenclature and units in accordance with SI?: yes

Are the English grammar and syntax satisfactory?: yes

**Reviewer C additional comments**

Some minor corrections are recommended:

Line 11: susceptible to degradation by both light and... (instead of to both degradation).

Response: The correction has been made.

Line 19:  ...but also in slightly (in is missing).

Response: The abstract has been rewritten.

Line 23:  significant (incorrect spelling, missing c)

Response: The spelling has been corrected.

Line 24: photodegradants (incorrect spelling, missing o)
Response: The spelling has been corrected.

Lines 45-56: Can we shortened. Too long introduction about cyclodextrins.

Response: The introduction has been shortened.

Line 60: They replaced...What does "they" stand for?
Response: The phrase; “They replaced…” has been rewritten as “RM-β-CDs have replaced….”.

Line 66: "permeation of the mucosa". Shouldn't it be through or into the mucosa? Or is it the permeability of the mucosa?
Response: The phrase; “…permeation of the mucosa” has been replaced with “…permeation through the mucosa”.

Line 83: Period is missing at the end of the sentence.
Response: A period has been added to the end of the sentence.

Experimental part:  There is overlapping between "Irradiation of samples" and "Photostability kinetic studies", i.e. both sections are describing the preparation of the samples. Please combine them into one section.

Response: The sections "Irradiation of samples" and "Photostability kinetic studies" have been combined into a single section.

Line 122: What was the diluent used to obtain the final volume of 10 mL?
Response: The phrase; “…diluted to a final volume of 10 mL…” has been replaced with “…diluted to a volume of 10 mL with phosphate buffer…” (page 5 of the revised manuscript).

RESULTS AND DISCUSSION

The accuracy was investigated for the concentrations of 0.4 mg/mL, 0.5 mg/mL, and 0.6 mg/mL. However, the linearity was confirmed for the range of 0.002 to 0.02 mg/mL. Shouldn't the accuracy be tested within the linearity range? Please explain.

Response: The sentences referred to here; “The accuracy of the method was investigated by spiking the 30 % w/v RM-β-CD solution with three known concentrations of MDZ (0.4 mg mL-1, 0.5 mg mL-1 and 0.6 mg mL-1). Linearity was confirmed to be within the range of 0.002 to 0.02 mg mL-1, with a high correlation coefficient of 1.00.” has a number of numerical errors.

It has been checked and has been rewritten with the correct concentrations. It now reads; “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 revised text is on page 6 of the revised manuscript just below the Fig. 1 caption).