**Response to Reviewers of the manuscript Manuscript No: 189 entitled A STEPWISE PROTOCOL FOR DRUG PERMEATION ASSESSMENT THAT COMBINES HEAT SEPARATED PORCINE EAR EPIDERMIS AND VERTICAL DIFFUSION CELLS**

**Reviewer 1 comments:**

1. The caption of Figure 1 could be extended to include more details on separate photographs shown.

The authors thank you for this suggestion, and supply additional information to Figure 1 caption:

*„Figure 1. Schematic representation of the protocol for the preparation of heat-separated porcine ear epidermis that precedes the actual drug permeation assessment:*

*a) the photograph of a porcine ear, as received after slaughtering, b) the photograph of the outer side of porcine auricle skin when separated from cartilage, c) the use of the spherical cutter ensures uniform membrane dimensions, d) the photograph of the isolated epidermis placed in a Petri dish versus a full thickness skin membrane, and e) the final setup of the modified Franz diffusion cells.”*

Additionally, the file Corrected\_Fig1 has also been uploaded, with precise labelling (a-e) of each photograph.

2. permeation parameters Jss, Q30h and Kp are discussed in the text but actual values are not shown. Please rewrite this section to include at least some of the values to demonstrate the utility of the protocol.

Both reviewers presented similar concerns for the section of the manuscript that initially spanned from line 235 to 247. For this reason, please find that the entire section has been rewritten in order to include some of the actual values of the calculated parameters, as well as the statistical significance, where applicable.

*„Aceclofenac permeation profiles shown in Figure 2, as well as the values of steady-state flux, permeation coefficient and the total amount of aceclofenac that permeated through the skin at the end of the experiment imply that the presented protocol is successful in discerning differences in aceclofenac availability from the investigated hydrophilic creams. As expected, the protocol was able to point towards the differences in aceclofenac permeation parameters from APG vs. R samples. In fact, the difference in the two samples’ microstructure has resulted in a nearly two-fold higher Jss, Q30h and Kp values (p < 0.05) in favour of the aceclofenac sample stabilized with the natural-origin emulsifier (calculated values for sample APG were 1.22±0.19 µg/cm2h, 21.04±2.04 µg/cm2 and 0.12±0.02 mg/cm2h, respectively; while the corresponding values for sample R were 0.57±0.09 µg/cm2h, 12.62±1.88 µg/cm2 and 0.06±0.01 mg/cm2h). Admittedly, such substantial formulation variations (i.e. „large“ variations) could also be revealed via in vitro release tests equipped with synthetic membranes, such as polycarbonate ones our group has used in reference [30]. Naturally, the differences of the aforementioned permeation parameters among the samples that endured smaller composition variations (i.e. co-solvent addition) were not as prominent but were still notable (Jss was found to be 1.6-fold; Q30h 1.8-fold and Kp 1.5-fold higher for the sample APG+Ipa as compared to the sample APG+Gly). In fact, the sample APG+Ipa provided a significant increase in aceclofenac permeation rate, Q30h and permeation coefficient (1.50±0.08 µg/cm2h, 34.36±2.97 µg/cm2 and 0.15±0.01 mg/cm2h, respectively), all p < 0.05 when compared to other three tested samples. Contrary to these findings, when synthetic membranes are used instead of heat-separated porcine ear epidermis, the impact of isopropyl alcohol as a permeation enhancer with multifaceted mechanism of skin interactions [32], could not be detected. In fact, aceclofenac release testing through polycarbonate membranes favoured the basic APG sample, and not APG+Ipa sample (release rate and extent attributed to the sample APG were significantly higher when compared to all other tested samples; p < 0.05) [30]. This is another obvious contribution of bio-derived membranes application in elucidation of slight differences in the assessed topical semisolids.”*

3. There are some suggestions for minor corrections in English language and style. Please see the attached pdf file with highlights.

Please check the uploaded revised manuscript to see that all of these suggestions were welcomed and suitable corrections made accordingly.

**Reviewer 2 comments:**

1. Abstract

line 18 and 19: please edit sentence like: …testing equipment; however, all these provide data on drug release using inert synthetic membranes.

Thank you for this suggestion. Please find that this part of the abstract is now corrected.

2. line 20: not just morphologically but structurally in general (thickness, hair follicle content, pigmentation, collagen and lipid composition). Please add that in sentence line 20. Just to note that, recent studies even confirmed immunological similarity of pig and human skin, where subsets of cells responsible for immune response in porcine skin are classified corresponding to the subsets described in the human skin (Summerfield et al. 2015, The immunology of the porcine skin and its value as a model for human skin, Molecular Immunology, Volume 66, Issue 1, July 2015, Pages 14-21)

We acknowledge that there are other similar aspects between human and porcine skin apart from their morphological similarities. For that reason, the word *morphologically* was replaced with *structurally* in this sentence of the abstract, and all the following parts of the text. Additionally, please find that line 76-78 of the manuscript now mentions immunological similarities as well, along with the reference you have suggested (now reference 19). Please find that the numeration of all the references is also revised.

3. line 28: the last sentence is too long. It should be separated in two parts like: The developed protocol is a straightforward and reliable in vitro option for the evaluation of rate and extent of drug delivery into/through the skin. Please change word “option” with “test” in this sentence. The second sentence should be edited like: Moreover, this protocol may be routinely applied even in averagely equipped laboratories during formulation development or preliminary bioequivalence assessment of generic topical semisolids.

Please find that you suggestion has been entirely accepted.

4. Introduction

line 47.change “performed with” with “performed by”

The sentence has been rewritten to „performed using”, as suggested by Reviewer 1.

5. line 55. I suggest to add “time-consuming” beside “expensive in vivo studies”

Acknowledged and corrected.

6. line 63. I suggest to change term “reveal” with simulate

Acknowledged and corrected.

7. line 67. the same comment like for “morphologically” in line 20 in abstract.

As previously mentioned, the term *morphologically* was changed to *structurally* throughout the manuscript.

8. line 70. please add “available” in front of “literature”. Also, I suggest citation of comprehensive review “Pig and guinea pig skin as surrogates for human in vitro penetration studies: A quantitative review” Toxicology in Vitro Volume 23, Issue 1, February 2009, Pages 1-13 by Ana M.Barbero and H. Frederick Frasch, among provided literature (13-15).

We are familiar with the mentioned paper by Barbero and Frash (reference number 11), and agree that it should be cited again in this section.

9. line 75. please add “structural” beside “morphological and permeability similarities”

Please find that the section is corrected in the following way:

*„For that reason, Organisation for Economic Co-operation and Development (OECD) published a guideline stating the suitability of porcine ear skin for percutaneous absorption studies, due to the demonstrated structural, morphological and permeability similarities to the human skin [16-18]. Apart from that, recent findings even confirmed certain immunological similarities of porcine and human skin [19].”*

10. Experimental

Line 93. In sentence “Existing publications….”, please cite some of the publications related to this statement.

Thank you for this comment. We absolutely agree that the sentence that mentioned the existing publications indeed needs to provide at least some actual references. For that reason, please find that the following new references were added to the reference list:

22. Abd E, Yousef SA, Pastore MN, Telaprolu K, Mohammed YH, Namjoshi S, Grice JE, Roberts MS. Skin models for the testing of transdermal drugs. Clin Pharmacol. 2016; 8: 163-176.

23. Mitra A, Leyes A, Manser K, Roadcap B, Mestre C, Tatosian D, Jin L, Uemura N. Use of minipig skin biopsy model as an innovative tool to design topical formulation to achieve desired pharmacokinetics in humans. J Pharm Sci. 2015; 104: 1701-1708.

24. Tay SL, Heng PW, Chan LW. An investigation of the chick chorioallantoic membrane as an alternative model to various biological tissues for permeation studies. J Pharm Pharmacol. 2011; 63: 1283-1289.

25. Zhang H, Zhu X, Shen J, Xu H, Ma M, Gu W, Jiang Q, Chen J, Duan J. Characterization of a liposome-based artificial skin membrane for in vitro permeation studies using Franz diffusion cell device. J Liposome Res. 2016; 28: 1-10.

11. Line 99. Please replace “obtain with” with “obtain by”

Acknowledged and corrected.

12. Results and discussion

Line 164 I would rather say “provides uniform size”?

Acknowledged and corrected.

13. Line 239-246 please add statistical significance

Line 246-247 please add few sentences more in order to compare results obtained from test using bio-derived membrane vs. pharmacopoeial testing using synthetic membrane, since you have already performed all these in your work-Ref No 25. This would point out the real contribution of bio-derived membrane application in elucidation of slight differences in tested topical semi-solid formulation and its potential in BE assessment.

Both reviewers presented similar concerns for the section of the manuscript that initially spanned from line 235 to 247. For this reason, please find that the entire section has been rewritten in order to include some of the actual values of the calculated parameters, as well as their statistical significance, where applicable. Additionally, the findings of the test that relies on synthetic membranes were also commented.

*„Aceclofenac permeation profiles shown in Figure 2, as well as the values of steady-state flux, permeation coefficient and the total amount of aceclofenac that permeated through the skin at the end of the experiment imply that the presented protocol is successful in discerning differences in aceclofenac availability from the investigated hydrophilic creams. As expected, the protocol was able to point towards the differences in aceclofenac permeation parameters from APG vs. R samples. In fact, the difference in the two samples’ microstructure has resulted in a nearly two-fold higher Jss, Q30h and Kp values (p < 0.05) in favour of the aceclofenac sample stabilized with the natural-origin emulsifier (calculated values for sample APG were 1.22±0.19 µg/cm2h, 21.04±2.04 µg/cm2 and 0.12±0.02 mg/cm2h, respectively; while the corresponding values for sample R were 0.57±0.09 µg/cm2h, 12.62±1.88 µg/cm2 and 0.06±0.01 mg/cm2h). Admittedly, such substantial formulation variations (i.e. „large“ variations) could also be revealed via in vitro release tests equipped with synthetic membranes, such as polycarbonate ones our group has used in reference [30]. Naturally, the differences of the aforementioned permeation parameters among the samples that endured smaller composition variations (i.e. co-solvent addition) were not as prominent but were still notable (Jss was found to be 1.6-fold; Q30h 1.8-fold and Kp 1.5-fold higher for the sample APG+Ipa as compared to the sample APG+Gly). In fact, the sample APG+Ipa provided a significant increase in aceclofenac permeation rate, Q30h and permeation coefficient (1.50±0.08 µg/cm2h, 34.36±2.97 µg/cm2 and 0.15±0.01 mg/cm2h, respectively), all p < 0.05 when compared to other three tested samples. Contrary to these findings, when synthetic membranes are used instead of heat-separated porcine ear epidermis, the impact of isopropyl alcohol as a permeation enhancer with multifaceted mechanism of skin interactions [32], could not be detected. In fact, aceclofenac release testing through polycarbonate membranes favoured the basic APG sample, and not APG+Ipa sample (release rate and extent attributed to the sample APG were significantly higher when compared to all other tested samples; p < 0.05) [30]. This is another obvious contribution of bio-derived membranes application in elucidation of slight differences in the assessed topical semisolids.”*

14. Conclusion

Line 265 the same comment like for “morphologically” in line 20 in abstract.

Acknowledged and corrected.