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Review report on the PhD thesis of Marta Tikhomirov, DVM

„Intravenous lipid emulsions as a treatment in acute opioid poisoning – pharmacokinetic and pharmacodynamic evaluation in the rabbit model"

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The review report is organized in the following sections: project background, general description of the thesis, specific comments followed by a final evaluation statement.

Project background

The Ph.D. thesis aimed to evaluate the efficacy of lipid emulsions (ILE) in the treatment of acute opioid intoxication in rabbits. This work is based on an extensive set of *in vitro* and *in vivo* experiments. The *in vivo* results are interpreted based on pharmacokinetic-pharmacodynamic (PK/PD) models, a tool enabling quantitative description of the relationship between dose, concentration and effect of drugs. This way of data interpretation plays an important role in various phases of drug development. It is also ideally suited to answer various questions related to the interaction between ILE and opioids. The subject of the thesis is in my opinion interesting from the scientific and practical point of view.

General description of the thesis

The dissertation is a monograph. It covers 110 pages and consists of typical sections (Introduction, Methodology, Results, Discussion of Findings, and Conclusions). At the beginning the dissertation provides the source of funding for the work, cites publication with

part of the results presented in the dissertation, provides table of contents, abstract in Polish and English, and list of abbreviations. The dissertation ends with an extensive list of references.

Specific comments

The introduction is interesting, well-presented with all the necessary details allowing to understand the subject of research. It provides the basic facts about opioids. Highlights the known facts about the intravenous administration of lipid emulsion as a means of reducing toxicity after drug intoxication. In my opinion, more information on the PK/PD methodology and existing PK/PD models for fentanyl and buprenorphine would be of value to the readers. Such information would introduce the necessary context to understand results.

The purpose of the study is properly justified. However, the description presented on page 19, unnecessarily refers to the formula used in statistical hypothesis testing. To quote the author: "It was hypothesized that ILE can be useful in the treatment of acute opioid overdose". In my opinion, it is better to define a hypothesis as a statement, not as an assumption. It is rather difficult to test a hypothesis that aims to assess whether ILE can/might work because the answer to such a question is "yes". It is the magnitude and reproducibility of an effect in time and space that is important. In other words, to what extent does the observed effect generalize to other settings. The model development (e.g. proposing a theory that explain the phenomenon under study) and the estimation of parameters (their magnitude and uncertainty) would be a sufficient objective of the dissertation.

In the subsequent sections, author describes all the methods and experimental approaches used in the dissertation. Experiments are well designed, form a logical sequence, and are written with sufficient details to repeated them. They include the development of i) analytical methods for the quantification of fentanyl, buprenorphine and butorphanol in plasma and cell lysates, ii) a series of *in vitro* experiments (plasma protein binding, distribution coefficient in biological setting, cytotoxicity of ILE and disposition of studied drugs to the cell monolayer), iii) and a series of *in vivo* experiments used to determine the concentration-time profile of buprenorphine and fentanyl in ILE treated and no-treated animals. In addition to pharmacokinetics, various pharmacodynamic endpoints were measured (heart rate, blood pressure, end-tidal carbon dioxide, respiratory rate, etc.).

The methods section is followed by the description of results. In my opinion, the proposed analytical methods seem adequate and are properly validated to answer the questions posed in

the dissertation. Also, *in vitro* experiments serve their role by quantifying the basic physicochemical properties important to understand PK properties of opioids. However, the PK analysis has raised some concerns. The proposed model of FEN and BUP pharmacokinetics assumes stationarity, and this assumption is clearly violated given the proposed experimental design. ILE is administered after opioid administration and it introduces a time-varying effect of ILE on PK parameters of opioids. Under this setting PK parameters are biased and consequently are difficult to interpret. I will explain this using volume of distribution of the central compartment (Vc) as an example. Please note that for basic compartmental models, Vc is assumed to be the same for the whole range of times. It is also easy to check that various parts of PK profile (e.g. initial concentration, and terminal elimination rate) are influenced by Vc. Specifically, in the ILE-treated group of animals the initial part of the graph refers to the kinetics before ILE administration and this Vc should be the same as in NaCl-treated group. At the same time Vc should be different after ILE administration. And this difference should change over time due to ILE disposition. Assuming that Vc is constant introduces some bias in Vc, and consequently in all other PK parameters. To overcome the bias a more realistic model has to be proposed. What kind of model would allow to overcome the above-mentioned limitations?

In my opinion, it would be valuable to combine more theory into data analysis. Since FEN and BUP are high extraction drugs, they are expected to have clearance (for total concentrations) unchanged regardless of ILE administration. Simply, the excretion rate for these drugs should depend solely on liver blood flow. It has an interesting implication as the AUC (for total concentration) and clearance should be the same in the ILE-treated and NaCl-treated groups of animals. Please note the same clearance can be assumed *a priori*. What implication would such an assumption have on results? Also, the direct comparison of the "lipid sink" and "lipid shuttle" theory could be tested using models with structure based on these two theories.

It would also be beneficial to build PD model for all the endpoints in the same way PK models were built. It would ease the interpretation of results. Please note that ANOVA is usually not the best statistical approach for PK/PD designs. Usually a nonlinear mixed models are used. Such models would also allow for a joined analysis of the data from all the groups (control, NaCl, and ILE).

Two calibration curves (normal vs. lipemic) were used to quantitate fentanyl. They introduce another level of variation that is difficult to control. In my opinion, it would be beneficial to

analyze the concentrations obtained based on these two calibration curves jointly? The change of methods 5 min after ILE administration seems subjective. What about later times, when the concentration of ILE in blood is low?

The editorial side of the work is carefully done. I found only few aspects requiring correction/rethinking. These are listed below:

1. Page 15 "increasing the lipophilicity of the blood" and Page 16. "The concept of changing the blood lipophilicity using ILE". Partitioning is defined for two liquids. It is hard to understand what author means by "lipophilicity of the blood"
2. Page 15 "pethidine (1.7), methadone (3.9), (...) " and page. 17. „partition coefficient of 3.4 and 2.9 (...)" and Page 77. The numbers refer to the logarithm of partition coefficient, not to the partition coefficient.
3. Page. 19. „have a lipophilic molecule" should be "are lipophilic".
4. Page 20 "Can ILE be successfully utilized during acute opioid overdose". It should be emphasized that the conclusion concern rabbits. It is not necessarily true for humans.
5. Str. 33. "The power of the test was presumed to be 0.8 and the significant level of 0.05". Based on this description, it is impossible to deduce all the input data to calculate the power of the test (or tests since there is a large number of comparisons in the dissertation). I have an impression that this aspect is given here based on the presumption that it should be part of the dissertation. However, for the purposes of inference (or exploratory research), evaluating the power of the test is not necessary. It is more adequate for confirmatory research if done properly (which, honestly is quite rare).
6. Page 39 „The parameters after confirmation of normal distribution, where subject to the Student's t-test". It is not an optimal approach. The properties of a statistical test change in unknown ways with such a combined procedure.
7. Page 40. " The data was first evaluated to verify the normal distribution (...)". It is the normality of the residuals that matters, not the normality of the data points themselves.
8. Page 39. "η – sh" . It is not the best abbreviation (it suggests differences between variables).
9. Page 39. It is not a good practice to determine secondary parameters by non-compartmental analysis. It is better to derive them based on model parameters.

The non-compartmental analysis should be treated as an exploratory at best. Please also note that non-compartmental analysis assumes stationarity for all PK parameters!

10. Page 52. the "so called dynamite plot" conveys little information relative to the amount of ink it uses. It is much better to use a boxplot with the observations superimposed.
11. Page 53 and few other places. „tremendous" should be "large", "more than satisfactory" should be "satisfactory", "were deeply affected" should be "were affected" "excellent", "extremely" such "marketing words" should be avoided. It is better to write how large the effect actually is and how it compares to related effects.
12. Table 2. Should be "butorphanol"
13. Page. 55. "Several correlations were found". Why they were not presented. What such correlations mean.
14. Page 56. Shrinkage for Q3 is 100%.
15. "suggesting that no measurable interaction between NAL and ILE was identified", "ILE was found not to interfere with the efficacy of NAL-mediated reversion of FEN toxicity", "no beneficial effects associated with ILE were identified", "no interaction between NAL and ILE was detected". The above mentioned statements are risky. Please note that the "lack of evidence is not an evidence of lack". It is better to write: "We were unable to show the effect …"
16. Page 79. "The small free fraction available for lipid sequestration may be responsible for the relative low values of disposition coefficient" This sentence is hard to understand. The free fraction (due to protein biding) and disposition coefficient (due to intralipid binding) are independent parameters, e.g. the change in free fraction does not affect the disposition coefficient. But bound and unbound concentrations depend on free fraction, disposition coefficient, and concentrations of proteins and intralipids, etc. Please clarify.
17. Why author decided to correlate percent decrease and octanol/water Kd. The natural scale for such comparison is log of concentration ratios, log P, log Kd, etc.
18. "Nevertheless, as the ILE addition can sequester only free fraction of the drug". It is a false statement. If it was true it would be possible to extract free fraction. The addition of another partitioning agent shifts the equilibrium.

19. "higher overall plasma concentrations of the drug shortly after the administration (significant impact on AUC)" – it is not necessarily true. The initial increase in concertation can be counterbalanced by a decrease in concertation in later phases. AUC reflects the area from zero to infinity.
20. Page 85. "Despite the fact the linear elimination was not evident in every animal". How was the linearity of PK assessed? It is hard to believe that there are evidence of nonlinearity based on single-dose study.
21. What is the confidence interval for AUC_{NaCl}/AUC_{ILE} ratio? Does it contain 1. Does the data provide sufficient evidence about AUC difference between the ILE and NaCl-treated groups?
22. "All the parameters [k_{on} and k_{off}] are different for BUP and FEN, and can be influenced to the different degree by the presence of ILE in the blood" The ILE does not affect k_{on} and k_{off}. Those parameters are independent of ILE concentration. The rate of dissociation is affect, not the rate constant.
23. Page 88 The half-life for biophase distribution of 28.6 min seems unlikely. Please check if it is correct.
24. Page 89. "if the clinical settings requires this, concurrent use of both forms of therapy seems safe and ILE is not expected to diminish the effects of NAL". This is a very strong statement based on study performed in rabbits.
25. Page 89. "It is concluded that no potential risk associated with" – this study was not design to assess the risk. It is underpowered to answer such question.

The summary and general conclusions

The research topic undertaken by Marta Tikhomirov required the development of skills necessary to conduct *in vitro* and *in vivo* experiments, to develop and validate analytical methods, to understand various methods of data analysis, to perform PK modeling utilizing nonlinear mixed effect models, to understand complex biological processes, and to plan and execute complex and long-term experiments. The Ph.D. thesis proves that the student mastered the tasks. The experiments are valuable, thoughtful, and well-executed. The mentioned remarks and questions do not lower the positive evaluation of the doctoral dissertation written by Marta Tikhomirov. In my opinion, the dissertation, despite some shortcomings, is valuable from the scientific and practical point of view.

Final evaluation statement

This thesis is ready to be defended orally and meets the requirements laid down for the degree of Ph.D. by the statutes in the Journal of Laws of the Republic of Poland (Dz.U. 2003.65.595, March 14, 2003, and its subsequent amendments)

Gdańsk, July 6, 2021