Response to Reviewer 2 Comments

Thank you very much for taking the time to review this manuscript. We have carefully considered the comments and concerns made by the reviewer. Our point-by-point response are listed below. We hoped this revision improves the manuscript such that you and the reviewer now deem it worthy of publication in Pharmaceuticals.

Questions for General Evaluation

1) Does the introduction provide sufficient background and include all relevant references?

Reviewer's Evaluation: Must be improved

➔ **Response: Thank you for the constructive comment. According to your comments, we have modified introduction and included additional relevant references to strengthen the background in the revised manuscript. We have summarized previously published simultaneous quantifying bioanalytical methods in Table 1, allowing a comparison with our methods to ensure accurate understanding of its analytical advantages.**

2) Is the research design appropriate?

Reviewer's Evaluation: Can be improved

➔ **Response: We would like to express our gratitude for the reviewer's insightful remarks. We have revised the manuscript by incorporating relevant content and corresponding tables to improve clarity.**

3) Are the methods adequately described?

Reviewer's Evaluation: Can be improved

➔ **Response: Thank you for your critical assessment and suggestions. We have carefully reviewed the Methods section and revised it to provide a clearer description throughout the revised manuscript.**

Comments and Suggestions for Authors

Huge effort was exerted, nevertheless lacking novelty and some main points.

1. The work unfortunately lacks novelty where several papers published similar work and authors did not show convincing comparison to them, additionally, they missed some of previously published work for this mixture and did not compare to it like:

1. Simultaneous Determination of Amoxicillin and Clavulanic Acid by LC-MS/MS in Human Plasma

Ji Shunli et al., Published in 2019 in Journal of China Pharmaceutical University

This research focused on developing a sensitive LC-MS/MS method for the simultaneous determination of amoxicillin and clavulanic acid in human plasma. The method was successfully applied to a bioequivalence study.

https://pesquisa.bvsalud.org/portal/resource/pt/wpr-807918

2. A 2D HPLC-MS/MS Method for Several Antibiotics in Blood Plasma and Tissue Samples

Rehm et al, Published in 2020 in Analytical and Bioanalytical Chemistry

This study presented a two-dimensional high-performance liquid chromatography–tandem mass spectrometry (2D HPLC-MS/MS) method for quantifying various antibiotics, including amoxicillin and clavulanic acid, in human plasma and tissue samples.

https://pubmed.ncbi.nlm.nih.gov/31900530/

➔ **Response:** **We are grateful to the reviewer for the careful evaluation and helpful suggestions. Initially, we refrained from citing this reference due to the language barrier, as only the abstract was available in English while the full text was in Chinese. Nevertheless, following your suggestion, we have translated the relevant sections and have now added the citation to the revised manuscript (Line 66). We have summarized previously published simultaneous quantifying bioanalytical methods in Table 1, allowing a comparison with our methods to ensure accurate understanding of its analytical advantages (Line 93). While we acknowledge that the referenced studies [1] and [2] offer similar works, we respectfully point out that they do not present the full set of validation parameters, including dilution integrity, incurred sample reanalysis (ISR), and/or anticoagulant effects. Thus, we believe that our method, which comprehensively addresses these parameters, offers bioanalytical novelty and better compliance with current regulatory validation criteria compared to the referenced studies. We hope this addresses your concern.**

2. The mobile phase used is typically not green, where formic acid is used, and it is common now to present ecofriendly analyses and care for this. Green analyses are the trending topics of analysis now. Authors should present greenness assessments of their new method e.g. using AGREE metric.

➔ **Response:** **We thank the reviewer for the insightful comments. We fully agree that green analysis is a current and important trend in analytical chemistry. Accordingly, we have added a section on greenness assessment to the revised manuscript. In particular, we evaluated the eco-friendliness**

of our analytical method using the AGREE metric, and the results demonstrate that our method aligns well with the principles of green analytical chemistry (Lines 309–316 and 497–505). We have also incorporated this into the graphical abstract as well. Thank you again for introducing the trending topic of analysis.

3. Application of the method is limited to just one product: (This method was sufficiently able to evaluate pharmacokinetics of Augmentin® immediate-release tablet (250/125 mg) in humans.), while there are several products and several formulas with different concentrations for this mixture.

➔ **<u>Response:</u> Thank you for your valuable comments. This analytical method was developed and validated to support future drug-drug interaction (DDI) studies of Augmentin® IR tablet as a victim drug. The clinical study presented in this manuscript was conducted as a pilot study to investigate the effect of gender and inter-individual variability on the pharmacokinetics of Augmentin® IR tablet. Our proposed method was sufficiently able to characterize pharmacokinetics for amoxicillin and clavulanate after oral dosing of lowest dose of Augmentin® (250/125 mg) IR tablet in humans. The dose ratio of amoxicillin to clavulanate increases in higher-dose formulations, although the clavulanate dose remains fixed at 125 mg. Given that the amoxicillin calibration curve in plasma spans from 10 to 15,000 ng/mL (considering dilution integrity, up to 60,000 ng/mL) and the observed mean C_{max} of amoxicillin was 5050 ng/mL after oral administration of the lowest dose Augmentin® IR tablet. Furthermore, it was reported that amoxicillin exhibited non-linear pharmacokinetic properties after oral dosing in humans, with less than dose-proportional increase in AUC and C_{max} values. Accordingly, we ensured that the calibration range of our method would adequately cover the expected plasma concentrations across various amoxicillin/clavulanate dosage regimens. We have described briefly this point in section 2.2.10. of the revised manuscript (Lines 288–295).**

4. Authors mention: linear concentration ranges were 10–15,000 ng/mL for amoxicillin ($r \geq 0.9945$) and 20–27 10,000 ng/mL for clavulanate, despite showing their method as very sensitive, they mixed up meanings of linearity with calibration range, where ICH guidelines mention them as two separate terms and the practical range must shows high precision for all its values, while 10 and 20 ng for sure will lack this precision on replication.

➔ **<u>Response:</u> We sincerely thank the reviewer for the detailed and meaningful input. To make it clear, we have added Table 2 in the revised manuscript (Line 191). As shown Table 2, the accuracy and**

precision of back-calculated LLOQ levels from six calibration curves met the acceptance criteria, and the slopes exhibited very low CV, indicating high consistency across runs (Line 191).

5. No clear method validation protocol like ICH guidelines or USP and not all validation parameters were covered in this study.

➔ **<u>Response:</u> We appreciate the reviewer's thoughtful and constructive comments. We had indeed evaluated all validation parameters according to the regulatory guidelines (USFDA and ICH M10) in this study. In response to the reviewer's comment, we have thoroughly reviewed our manuscript. We have revised the Methods sections to eliminate ambiguity and tabulated the corresponding results in a more organized manner in Results and Discussion sections of the revised manuscript (Lines 344). We have also cited additional references to support our revision (Lines 385–391). We hope this addresses your concern.**