Author’s response to reviews

Title: Accidental intoxications in toddlers: lack of cross-reactivity of vilazodone and its urinary metabolite M17 with drug of abuse screening immunoassays

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Editor
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We submit a revised version of our manuscript entitled “Accidental intoxications in toddlers: lack of cross-reactivity of vilazodone and its urinary metabolite M17 with drug of abuse screening immunoassays” for consideration for publication as a Research Article in BMC Clinical Pathology.

In response to the reviewer and editor critiques and suggestions, we have done major revisions including: change in article title, removal of two tables, inclusion of case histories, placement of the chemical synthesis details in supplementary information (Additional File 1), testing of vilazodone and M17 on an additional amphetamines immunoassay and a serum tricyclic antidepressant assay, and rewriting of Discussion and Conclusions. The expanded cross-
reactivity testing required collaboration with another laboratory and an addition of 3 co-authors. In addition, the lead author changed surname due to marriage.

The manuscript now has 2 figures, 1 table, and 1 additional file.

Specific response to critiques are detailed below. We thank the 3 peer reviewers and editor for time in reviewing this manuscript.

The authors affirm that this work has not been previously published nor is under consideration for publication elsewhere. All authors approve the version submitted.

All authors declare no competing interests.

Thank you for your consideration,

Sincerely,

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Reply to editor and peer reviewers:

Technical Comments:

1. Please include the full postal address of the submitting author in the Title page.

This has been done.

Reviewer 1

1. In my opinion, the work should focus on the possible cross-reactivity of vilazodone in amphetamine immunoassays, since no date of suspected cross-reactivity with other immunoassays (cannabis, cocaine, barbiturates, ...) is reported.
We have revised the manuscript to focus on the cross-reactivity of amphetamines and TCAs, the only compounds with suspected cross-reactivity from computational predictions.

2. The case reports of vilazodone exposure on which this study is based should be described in the article, since the information available in the references provided is very scarce.

We have included descriptions of the case reports in the beginning of Results section. The clinical details of these case have not been previously reported.

3. Results of amphetamine immunoassays were reported in case report of vilazodone ingestions in young children cited in the manuscript?

The cases summaries now in the manuscript include description of drug testing performed.

Case #1 included presumptive positive amphetamine screens at the outside hospital and our hospital. These were both Roche Diagnostics assays (effectively the same assay run on two very similar Roche platforms). Our hospital sent out amphetamines confirmation by LC/MS/MS (ARUP Laboratories) which was negative. Urine vilazodone analysis was also sent out (NMS Laboratories) and showed a urine vilazodone concentration of 130 ng/mL.

Case #2 included positive amphetamine screen at our hospital (again Roche). Unfortunately, no confirmatory analysis was performed. Clinical history strongly suggested vilazodone exposure with access of child to vilazodone tablets in a blister foil in mother’s purse and without any known accessibility to amphetamines.

4. Cross-reactivity testing was performed up to a concentration of 100,000 ng/mL. What levels of vilazodone or its metabolites could be expected in the urine in cases of overdose?

We found no published literature on urine concentrations of vilazodone or its M17 metabolite. As noted above, case #1 had a urine vilazodone concentration of 130 ng/mL. We chose 100,000 ng/mL as the upper limit as it is nearly 3 orders of magnitude higher than the measured concentration in case #1 and was the highest we could reasonably test given limited amount of the custom synthesized M17. There is literature on serum concentrations of vilazodone and M17, both from adult pharmacokinetic studies and pediatric intoxications, to guide testing. The highest vilazodone serum concentration reported in the literature was 1,600 ng/mL in a young child who ingested up to 280 mg of vilazodone and had seizures. We reference these studies in the manuscript and tested as high as 2,000 ng/mL in serum for the cross-reactivity testing.

5. In discussion, the authors state “The similarity of vilazodone overdoses to amphetamines toxicity demonstrate the importance of confirmatory analysis to rule out amphetamine and methamphetamine exposure”. However, the study seems to rule out that positive amphetamine results were caused by vilazodone. Could you explain this point?
We have added more details on this. Our case #1 had negative LC/MS/MS for amphetamines (results available after hospital discharge). Without this confirmation, it was logically considered to be presumptive accidental ingestion of amphetamines.

6. An important limitation of the study is the lack of confirmation of positive amphetamine results by a reference method, such as GC-MS or LC-MS/MS. These methods would have allowed excluding causes of false positive other than viladozone.

In our case descriptions now in the manuscript, case #1 did have amphetamines confirmatory testing by LC/MS/MS which was negative. Case #1 also had urine vilazodone analysis which showed a concentration of 120 ng/mL. Unfortunately, confirmatory analysis was not performed for case #2 but did have history strongly suggestive of vilazodone overdose (young child with access to mother’s purse and vilazodone foil pack).

7. Table 1 does not provide additional information to the text and could be eliminated.

Table 1 has been eliminated as suggested.

8. The description of Chemical Synthesis of Vilazodone Metabolite M17 should be summarized.

The details on the synthesis of M17 has been moved to supplementary material (Additional File 1).

Reviewer 2

The authors present a study evaluating vilazodone and its metabolite for cross-reactivity in an amphetamines immunoassay. The manuscript is well-written and easily understood. Some suggestions for improvement are included below:

1. The title is somewhat misleading, as it implies that cross-reactivity was noted. Would revise to more clearly reflect the results of the study.

We have revised the title.

2. Abstract stated vilazodone & metabolite 'did not cross react on any immunoassays', which implies that multiple amphetamines immunoassays were evaluated whereas only the Roche cobas methods were tested. Given that the patient reports were only of false-positive amphetamines screens & the low predicted cross-reactivity with any of the other DOA tests included in the study, it makes more sense for the abstract to refer specifically to the one method tested.
The manuscript now includes testing of two different urine amphetamines immunoassays and a serum TCA immunoassay; these are referenced in the Abstract. These were the compounds that had potential cross-reactivity by computer predictions. The manuscript has been revised to this effect.

3. Line 109, "hydroxy" is missing the r
   This correction has been made.

4. The authors might add a few comments to describe why 2D modeling is adequate, when antibodies recognize 3D structures.

   This is a very good question and one we have explored in detail in previous studies. We have added information in the Discussion on this point. 2D methods offer the advantage that they are fast to calculate and do not require the generation of multiple 3D conformations for the comparator or test compounds. For the respective systems used, we do not have the 3D structures of all the antigens used in each case so we cannot absolute compare using 3D. This would be necessary to perform a 3D analysis based on shape. A 3D pharmacophore of a proposed molecule confirmation may be useful as an alternative but it would require several assumptions on the conformation. Our prior studies with amphetamines and amphetamine-like drugs showed limited utility of 3D methods except for very closely related compounds (e.g., amphetamine, methamphetamine). 3D studies did not perform well with less closely related structures, which is the case with vilazodone and its metabolites compared to amphetamines.

5. Line 261 the word screening is duplicated
   This correction has been made.

6. The discussion of haptens in the methods largely duplicates what's in the discussion section. Would keep in only one location; could probably remove from methods
   We have removed from the Methods section.

7. Lines 296-298, the sentence is awkward. "immunoassays" should be plural or the sentence should refer more specifically to a single immunoassay. Similar issue with lines 316-318
   The section containing the sentences has been replaced and revised.

8. Since vilazodone is commercially available and showed predicted cross-reactivity with TCA immunoassays, it would be interesting & provide additional support for the model's predictive
capacity if the drug were tested on TCA screens. Possibly could be its own publication, since it's a bit of a tangent from amphetamines.

We performed additional experiments to test vilazodone and M17 cross-reactivity with a serum TCA immunoassay. There was no cross-reactivity at concentrations up to 2,000 ng/mL, a concentration in excess of the highest vilazodone serum concentration reported in the literature (1,600 ng/mL), a young child that ingested up to seven 40-mg tablets.

9. Would strengthen the study to test the metabolite on other amphetamines immunoassay platforms as well, since each uses distinct antibodies for recognition.

We performed additional testing on the Siemens Emit II Plus Amphetamines immunoassay. Similar to the Roche immunoassay, no cross-reactivity was noted.

10. Have the authors investigated adult urine samples for false-positive results? Would strengthen the conclusion that the responsible factor was a childhood-specific metabolite if urine from adult overdoses did not provide positive results.

We have added data to this effect. We did not identify any additional false positive cases in children or possible false positives in adults.

Reviewer 3

1. The discussion is mostly repeating results, and should be rewritten, focusing on possible explanations why no cross-reactivity was found, in what way one could further examine possible cross-reactivity of vilazodone (urine samples from patients administered vilazodone, discovering other metabolites and testing cross-reactivity of these). The authors should also consider discussing potential consequences of false positive amphetamine screen in patients administered vilazodone. If former case reports with false positive amphetamine results originate from other amphetamine assays than the one used in the present study (Roche), this should also be discussed, and testing cross-reactivity of vilazodone and metabolites in other amphetamine assays should be encouraged.

The Discussion has been significantly rewritten. As noted above, we added cross-reactivity testing with a second amphetamines immunoassay (Siemens Emit II Plus Amphetamines).

2. The conclusion of the paper is almost identical to the conclusion in the abstract, and should also be rewritten.

The Conclusion has been rewritten.
REQUESTED REVISIONS:

Abstract:

3. Line 52: Hyphen "cross-reactivity"

This correction has been made.

Background:

4. Line 94-96: The authors could consider removing this sentence. QT-prolongation is not further referred to in the manuscript.

This sentence has been removed.

5. Line 116: "Reports" and "reported" in the same sentence, please consider alternatives.

This sentence has been edited to avoid duplication.

6. Line 119-124: Since immunoassays from different companies have different characteristics, the authors should present which immunoassay they are referring to here. In reference 18 it is the same as the one tested in the article. I have unfortunately not been able to access reference 17.

This information has been added. As noted above, we also added testing on the Siemens Emit II Plus Amphetamines Assay.

Methods:

7. Line 152: The first "or" should be "of".

This correction has been made.

8. The presentation of 2D molecular similarity analysis and chemical synthesis of M17 should switch place, presented in the same order as the study actually was performed.

This change has been made. In addition, details on the chemical synthesis are now in supplementary data (Additional File 1) as described below.
9. The description of the synthesis of M17 is comprehensive. Depending on the scope of the journal, this could be a part of supplementary material, and presented in a short form in the main manuscript.

The details on the synthesis of M17 has been moved to supplementary material (Additional File 1).

10. Line 257-258: "Drug of abuse assays" and "drug abuse assays" are both used in this manuscript. The authors could choose one writing style.

We have revised to use “drug of abuse assays" consistently throughout the manuscript.

11. Line 261: Screening screening…

This correction has been made.

12. Line 273-279: Characteristics of different immunoassays could rather be a subject of the discussion.

We have added discussion on this issue in the Discussion.

Results:

13. The first paragraph describes an interesting retrospective analysis, summarized in table 2. It is not obvious for me if this retrospective analysis is the same as the one referred to in "Background" from ref 17 and 18, though the legend of table 1 describes in more detail in what way the results are created (a combination of results from ref 17 and 18, with supplemental data from a new chart review?). The objective of doing a retrospective analysis should be described in "Background" since it is an important part of the work as well. More details of how it is performed (the combination of former results and a new chart review) should be incorporated in the first paragraph of "Method". Are the same immunoassay methods used in both ref 17, 18 and in the new chart review? Table 1 should be referred to in "Background", and not under the banner "Results", since this is not part of the present study.

We have made extensive revisions that address these points. We present the case histories of the two cases in detail (these were not presented in ref 17 and 18) and have eliminated Tables 1 and 2. Details on the immunoassays are also provided.

14. Line 316-318: This sentence should not be a part of the results section.
This sentence has been deleted from the Results section.

Discussion:

15. The first two paraphrases are mostly repeating information from "Background" and could be shortened/removed from the manuscript. Some of the text in paragraph two could be incorporated in a discussion on possible clinical consequences of false positive amphetamine results in patients with intake of vilazodone. The authors should consider discussing if a false positive amphetamine assay could result in different patient treatment or follow-up. The authors could also discuss if positive amphetamine immunoassay results in children routinely should be confirmed with specific analytical methods.

We have added discussion on these topics.

16. Line 416-427: This paragraph is repeating information from the results section and could be shortened.

We have shortened this paragraph.

17. Line 431: Cross-reactivity is tested up to a concentration of 100,000 ng/mL. This is a common upper concentration for cross-reactivity testing. Do the authors have any reason to believe that the concentration of vilazodone and/or metabolites could exceed this concentration in urine from patients with vilazodone overdose? This could be addressed in the discussion.

As discussed above, we found no published literature on urine concentrations of vilazodone or its metabolite. We chose 100,000 ng/mL as the upper limit as it is nearly 3 orders of magnitude higher than the measured level of 130 ng/mL in case #1 (with the same urine samples having a presumptive positive amphetamines screen) and also was the highest concentration practical with the limited amount of the custom synthesized M17 available to test.

Conclusion:

18. The conclusion of the paper is almost identical to the conclusion in the abstract. Studies that could further enlighten the question of cross-reactivity of vilazodone and its metabolites on amphetamine assays could be emphasized.

The Conclusion has been rewritten.

In accordance with BioMed Central editorial policies and formatting guidelines, all manuscript submissions to BMC Clinical Pathology must contain a Declarations section which includes the
mandatory sub-sections listed below. Please refer to the journal's Submission Guidelines web page for information regarding the criteria for each sub-section (https://bmcclinpathol.biomedcentral.com/).

These sections are in the revised manuscript