Author’s response to reviews

Title: Safety, Tolerability, and Pharmacokinetics of Repeated Oral Doses of 2-Hydroxybenzylamine Acetate in Healthy Volunteers: A Double-blind, Randomized, Placebo-controlled Clinical Trial.

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Version: 1 Date: 13 Dec 2019

Author’s response to reviews:

Thank for you the consideration of our manuscript and the opportunity to revise and resubmit it. We appreciate the reviewers’ comments and have addressed each below and in the manuscript. Relevant revisions to the text are quoted below. Please note that we have also added an author and grant funding sources that were erroneously omitted from the initial submission.
Reviewer 1:

Thank you for your complimentary review of our manuscript. Our response to your comment is below.

Comment 1) Describe the limits of your study as patients' numbers are low and to outline this in the conclusions.

Response 1) This paper describes one of the first-in-human studies of 2-HOBA. It was designed to evaluate safety, tolerability, and PK on multiple doses of 2-HOBA. The number of volunteers were studied was in line with standard practices for early-phase clinical trials as well as current recommendations for sample size in first-in-human studies (Shen, J., B. Swift, R. Mamelok, S. Pine, J. Sinclair and M. Attar (2019). "Design and Conduct Considerations for First-in-Human Trials." Clin Transl Sci 12(1): 6-19). Nevertheless, we have revised the manuscript to note the limitation of the sample size in the manuscript as follows:

(lines 392-3) As this study was conducted with a small number of healthy volunteers, the generalizability of the results is limited.

Reviewer 2:

Thank you for your careful review and critique of our manuscript. We have attempted to address each of your critiques thoroughly and thoughtfully below and in our revised manuscript text where applicable. We hope these responses and revisions adequately address your concerns with the manuscript.

Comment 1) Did the investigators prepare and submit an investigational new drug application to FDA? If so, mention in the manuscript. If not, explain why not.
Response 1) An IND was not submitted to FDA. 2-HOBA acetate is not a drug, as defined in section 201(g)(1) of the Federal Food, Drug, and Cosmetic Act. 2-HOBA is self-affirmed GRAS and will be marketed as a dietary supplement in accordance with the Dietary Supplement Health and Education Act of 1994 (DSHEA). The FDA has clearly stated that an IND is not required to evaluate a dietary supplement’s effect on the structure or function of the body [Guidance for Clinical Investigators, Sponsors, and IRBs Investigational New Drug Applications (INDs) — Determining Whether Human Research Studies Can Be Conducted Without an IND]. An IND application will be filed prior to any studies designed to test efficacy to prevent or treat disease.

Comment 2) Did the manufacturer provide 2-HOBA drug product dissolution and content uniformity data? If not, did the investigators perform CU on drug product? If not, why not? What acceptance criteria were utilized for the product tested?

Response 2) The manufacturer provided a specification for the test product. The average fill weight of a 250 mg (active) capsule was set at 416.3 – 483.8 mg/capsule. The uniformity of weight from 20 capsules was to be no more than 2 ± 7.5% and none ± 15% deviation from the mean. Disintegration was set at no more than 60 min. The 2-HOBA acetate range was set at 237.5 – 262.5 mg/capsule. The test product used in the study complied with these specifications as indicated on the COA and confirmed by our laboratory.

Comment 3) The rationale for Q 8 hr dosing is not clear. The authors describe the apparent elimination half-life, based on single dose data, is ~ 2 hours. The manuscript also describes that 2-HOBA is not detectable in plasma after 8 hours, yet, Q 8 hr dosing was selected to "maintain effective plasma levels". This needs to be clarified in the manuscript prior to publishing.

Response 3) The use of the word “effective” in the initial version of the manuscript was poor word choice. Rather, Q 8 hr dosing was selected as it represented an interval where we could maintain 2-HOBA exposure (detectable 2-HOBA levels in plasma) throughout the dosing interval. This has been clarified in the manuscript as follows:

(lines 146-150) Single doses of 2-HOBA acetate given to healthy volunteers resulted in 2-HOBA plasma concentrations in the range of 8.5 – 320 ng/ml at 8 hours and no detectable levels 24 hours following dose administration. Thus, an eight-hour dosing interval was selected for the multiple dose studies to ensure continued 2-HOBA exposure throughout the dosing interval.
Comment 4) Given 2-HOBA was administered orally, and absolute bioavailability is not known, nor are there data ruling out dose-dependent bioavailability, greater clarity needs to be incorporated into the manuscript that the volume of distribution and clearance estimates are not true measures of Vd and Cl.

Response 4) We have clarified and enhanced the discussion regarding unknown bioavailability in the manuscript as follows:

(lines 355-62) In addition, oral bioavailability of 2-HOBA has not yet been established in humans and may vary considerably based on dose, the gastrointestinal environment, processes regulating its absorption, concomitant medications, and other unknown individual-specific factors. Thus, the lack of a dose-dependent increase in 2-HOBA exposure in this study could be attributed to an unknown factor that increased bioavailability in the low dose group or decreased bioavailability in the high dose group. This unknown bioavailability in both the low and high dose groups limits the interpretation of both clearance and volume of distribution following oral administration of 2-HOBA.

Comment 5) The description of urine sample collection is not clear. Did the investigators collect clean catch samples at 4, 8, 12 and 24 hours or complete urine samples via urine collection intervals (e.g., 0-4, 4-8, 8-12 and 12-24 hr)? It's not clear from the data presentation.

Response 5) Clean catch urine samples were collected at 4, 8, 12 and 24 hours. This has been clarified in the manuscript (line 165).

Comment 6) Please describe in this manuscript under what conditions the plasma bioanalytical methods for 2-HOBA and salicylic acid (SA) were validated. As well, please describe the in-process performances of the two assays during routine analysis of samples.

Response 6) These have been described in the manuscript as follows:
Quantification of 2-HOBA was validated over the range of 5 – 5000 ng/mL, with within-run precision of 3.7 – 7.0%, bias -9.7 – 2.8%, and between run precision of 4.4 – 6.2%, bias -7.1 – 1.6%. In-process analytical performance of 2-HOBA during routine analysis of samples demonstrated an intra-assay precision of 1.1 – 14.8%, bias -4.0 – 17.1%, and inter-assay precision of 3.7 – 9.0%, bias 6.0 – 9.0%. Quantification of salicylic acid in samples was qualified over the range of 100-5000 ng/ml. In-process analytical performance of salicylic acid during routine analysis of samples demonstrated an intra-assay precision of 2.3 – 8.8%, bias -5.2 – 8.7 and inter-assay precision of 4.6 – 6.4%, bias -1.5 – 6.2. All standards and quality control samples for 2-HOBA and salicylic acid met acceptance criteria (standard curve R2 &gt; 0.90, 66.7% of all QC samples and at least 50% at each concentration within 15% of nominal concentration).

Related comments 7, 8, 9) The manuscript lacks a statement on whether the serial blood (plasma) PK sampling scheme utilized is sufficient given the PK of 2-HOBA and SA... It would be helpful to see plasma drug and metabolite concentration-time profiles presented as semilog plots to see whether, post Tmax, the declines in plasma drug/metabolite concentrations were mono- or bi-phasic.

Please clarify for the editor whether accumulation ratios were calculated correctly ... The description "mild" 2-HOBA accumulation" in the results/discussion section and "did not cause excessive accumulation" are not useful descriptions of accumulation. I'd like to see the authors focus on whether single dose pharmacokinetics predict steady-state pharmacokinetics...

Given the half-life and Q 8 hr dosing, please include in the 2-HOBA and SA tables average steady-state concentrations (over the dosing interval) as well as percent fluctuation parameters.

Responses 7-9) The 2-HOBA (original Figure 1, now Figure 1A) and salicylic acid (originally not shown, now Figure 1B) concentration-time profiles are now presented as semi-log plots in the manuscript figures.

We chose to maintain the PK sampling scheme utilized in the single ascending dose study of 2-HOBA acetate. However, we agree that the lack of a sampling point between 8 and 24 hours may have failed to fully capture the final elimination phase between 8 and 24 hours. This may have resulted in an underestimation of half-life following a single/initial dose and may partially explain the underprediction of 2-HOBA accumulation.
The accumulation ratios were calculated correctly; the calculation method has been clarified in the methods section (lines 208-210). Additional PK metrics (Cavg, Cmin, and %PTF) have been added to Table 3. We also calculated the effective half-life of 2-HOBA based on the dosing interval and the accumulation index from Day 1 to Day 15 (lines 214-218). Discussion on the prediction of steady-state pharmacokinetics has been added as follows:

(lines 337-61): The AUC increased from day 1 to day 15 in both dose groups, indicating 2-HOBA accumulation with both 2-HOBA acetate dosing regimens, yielding accumulation ratios that ranged from 1.19 – 1.94. The greater accumulation of 2-HOBA in the current multiple dose study was higher than predicted (1.06 – 1.22) in the previous single ascending dose study. Under-prediction of 2-HOBA accumulation can be attributed, in part, to a slightly longer estimated elimination half-life and an even longer effective half-life relative to the dosing interval used in the present study. The utility in calculating an effective half-life to better predict accumulation with multiple dosing strategies and modified dosage formulations have highlighted the importance of accounting for dosing regimen factors (route of administration, dose, and dosing interval) and unknown or complex disposition processes (absorption, distribution, and elimination) that impact overall exposure. The greater than predicted accumulation index in the current multiple dosing study further substantiates that dosing every 8 hours may be sufficient to maintain 2-HOBA exposure throughout the dosing interval. Unlike the dose-dependent increase in 2-HOBA exposure observed across a broader single dose range, average systemic exposure (Cmax and AUC) to 2-HOBA was similar for 500 and 750 mg doses. This may be related to considerable inter-subject and inter-study variability in response to 2-HOBA oral administration, as the 500 mg dose resulted in greater exposure on average than was observed in the previous single-dose study.

Comment 10) The similar drug exposures for the 500 and 750 mg doses are attributed by the authors to be due to considerable inter-subject variability. Is it possible 2-HOBA exhibits dose-dependent absorption?

Response 10) Addressed in response 4, above.
Comment 11) … the authors attribute the drastic differences in CSF/plasma ratios for 2-HOBA and SA to protein binding. This is a reasonable conclusion, however, I would like to see a discussion or at least consideration for pKa differences for parent and metabolite. Is it possible that the degree of ionization in plasma contributes to the differences? If unlikely, explain why.

Response 11) The drastic difference in CSF/plasma ratios for 2-HOBA and SA are most likely attributed to the large difference in plasma protein binding of 10% versus 80%, respectively. However, the discussion has been revised to also discuss the potential impact of pKa and ionization differences as follows:

(lines 374-82) However, both compounds also demonstrate a high degree of ionization at a physiologic pH of 7.4 which could increase the time required to reach distribution equilibrium in CSF. Predicted pKa values for the carboxylic acid group on 2-HOBA (parent) and the primary amine group on salicylic acid (metabolite) are estimated to be 2.79 and 8.63, respectively. As such, both compounds would be almost completely ionized in the systemic circulation with only about a 5% difference in the unionized species in favor of 2-HOBA. In addition to plasma protein binding and percent ionization, substrate specificity for efflux transporters at the blood-CSF barrier could also play an important role in establishing equilibrium concentrations in the CSF.