Author's response to reviews

Title: A Round Robin Approach to the Analysis of Bisphenol A (BPA) in Human Blood Samples

Authors:

Laura N Vandenberg (lvandenberg@schoolph.umass.edu)
Roy R Gerona (roy.gerona@ucsf.edu)
Kurunthachalam Kannan (kkannan@wadsworth.org)
Julia A Taylor (TaylorJA@missouri.edu)
Richard B van Breeman (breemen@uic.edu)
Carrie A Dickenson (dickenson@obgyn.ucsf.edu)
Chuynyan Liao (cxl12@health.state.ny.us)
Yang Yuan (yuanyang0819@gmail.com)
Retha R Newbold (newbold1@niehs.nih.gov)
Vasantha Padmanabhan (vasantha@med.umich.edu)
Frederick S vom Saal (vomsaalf@missouri.edu)
Tracey J Woodruff (woodrufft@obgyn.ucsf.edu)

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Author's response to reviews: see over
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Environmental Health

Dear Dr. Grandjean,

Attached please find our revised manuscript “A Round Robin Approach to the Analysis of Bisphenol A (BPA) in Human Blood Samples”, for publication in *Environmental Health*. In this paper, we report novel findings from a Round Robin to address issues with measuring BPA in human blood. This work is of high importance because while there is widespread human exposure to BPA, there are key concerns about whether BPA can be measured in human blood, which are addressed in this paper. The reviewers overall were extremely positive. We have modified the manuscript with some further details requested by the reviewers and the responses are at the end of this letter. We have attached the final manuscript, if you need a version with track changes, please let us know. It was not clear where to add that.

The article is an original work and has not been previous published whole or in part and is not under consideration for publication elsewhere. All authors have read the manuscript, agree the work is ready for resubmission to the journal, and accept responsibility for the manuscript’s contents.

We look forward to your review.

Sincerely,

Tracey J Woodruff
Program on Reproductive Health and the Environment
1330 Broadway St., Ste 1135
Oakland, CA 94612
web: www.prhe.ucsf.edu
Response to Reviewer 1:

This is an amazing work and a carefully prepared paper reporting data from a very important robin approach on analysis of BPA in human blood samples. This study is highly needed and contribute with crucial new argues to the ongoing discussion on BPA. The authors should be congratulated in their efforts. 
I will strongly recommend publication of the paper, and I have only few comments, questions and suggestions.

We appreciate this positive assessment from the Reviewer and will respond to the individual points raised below.

Major comments

1. Is the placement for glucuronic acid on the BPA molecule unique? Please describe uBPA and BPA-G and the labeled compounds used for spiking and control material more detailed – company, structure, labeling etc. What is an authentic standard?

BPA is a symmetrical molecule, thus although there are two sites for glucuronidation (the hydroxyl groups), they are equivalent. BPA-G is therefore a single isomer and a unique molecule.

We have added additional information about the sources of these materials to the manuscript. The Sigma-Aldrich BPA we used is >99% pure and labeled BPA from both Sigma-Aldrich and Cambridge Isotope Labs is 98% pure. The BPA-G was 95% pure.

An authentic standard is a highly characterized compound typically used as a performance calibrator. This information has been added to the manuscript.

2. Please describe the procedure for preparation of double-stripped serum (charcoal dextran).

Charcoal dextran stripping is a process that removes steroids and other compounds like BPA from serum. In this protocol, activated charcoal and dextran are combined, and added in equal volumes to serum. The mixture is heated for a period of time, and then centrifuged to remove the charcoal and isolate the serum.

For the production of double-stripped serum, this protocol is repeated (for a total of 2 treatments with activated charcoal-dextran). We also used triple-stripped serum. This information has been added to the manuscript.

3. It seems like the labs measured lower BPA-G levels in almost all spiked serum samples than the predicted concentrations in these samples (phase 2 and 3 experiment, fig 3 and suppl fig 4). For instance, in both experiment all four labs measured lower conc. in the highest spike levels than the predicted conc., but was 19.53 ng/ml the “though” conc.? How was the facit or through values of uBPA and BPA-G determined? Please comment.
The value of 19.53 ng/ml was the spiked concentration of BPA-G, i.e. the amount added to the serum samples. The amount of spiked uBPA and BPA-G was determined by serial dilutions of a stock solution of the specified compound.

The Reviewer asks whether the BPA-G levels in the spiked serum samples were reported to be lower than the actual amount spiked. Based on the data reported in Figure 3, Supplemental Figure 4, Figure 4 and Supplemental Figure 5, we have drawn a more nuanced interpretation.

In Phase 2 of the round robin [shown in Figure 3], a total of 20 measures of samples spiked with BPA-G were conducted. 9 under-estimated the spiked concentrations, and 3 over-estimated the concentration spiked. The remainder (8/20) were within 20% of the spiked value, although most under-estimated the concentrations spiked to a small degree. In Phase 3 of the round robin [shown in Supplemental Figure 4], 3/20 samples over-estimated and 3/20 samples underestimated the amount spiked. The remainder (14/20) were within 20% of the spiked value, with a mixture of over-estimates and under-estimates.

In the experiments designed to test inadvertent hydrolysis of BPA-G [shown in Figure 4 and Supplemental Figure 5], we observed a mixture of over-estimates and under-estimates, as well as labs that reported concentrations extremely close to the concentration spiked.

We have now added a few brief statements about these relationships to the results section. The manuscript text also noted that reported BPA-G levels were higher than the concentration spiked in the experiment described in Figure 4C.

Minor comments

1. In phase 1 serum was collected from human patients, but in phase 3 the serum was collected from multiple individuals also later on called donors. Was all blood samples taken from healthy volunteer donors?

   We thank the Reviewer for pointing out this discrepancy in terms. In Phase 1, the blood samples were collected from the clinical toxicology unit, so we cannot be certain of their health status. In Phase 3, the blood samples were taken from healthy volunteer adult donors. This is noted in the text.

2. Was the final conclusion on sample storing at different temperatures, that storing of both samples and control material was ok at -20#C? And for how long?

   Prior to starting the Round Robin, we wanted to ensure that the samples would not be affected by shipping conditions. Samples were sent on dry ice and immediately placed in the freezer upon arrival. The sample storage test indicated that these conditions should not affect the integrity of the sample. This result is consistent with much more detailed sample storage studies, which we have cited in the revised manuscript.

3. How was tubes for storing at -20#C tested BPA-free?
All materials that came in contact with serum were tested in the same manner: blank solutions (water, methanol, stripped serum) were placed in the materials and maintained there, then extracted and tested for BPA contamination. This protocol is described in detail in the Methods section.

4. It is somewhat confusing to distinguish between the phase 2 and first part of phase 3 experiment, was the difference that the labs had the possibility to use authentic standards including 13C-BPA-G in experiment 3? And did the labs actually used 13C-BPA-G in phase 3, and did this improved the results?

The Reviewer is correct; the major difference between Phase 2 and Phase 3 was the availability of the standards, which became available only for use in the final phase. All laboratories used the new standards that were provided; this is made clearer in the text. Laboratory performance improved from Phase 2 to Phase 3. This may be due to the additional standards available, or could be due to another factor. This is also mentioned in the revised text.

5. Second part of Phase 3, All donors were instructed to avoid sources for BPA, but which?

Donors were instructed to avoid polycarbonate plastics, canned foods, and contact with thermal papers. This is now indicated in the text.

6. Results from fig 1 and suppl fig 1B,C would be easier to read in on table instead of spread in two figures in both main paper and supplementary.

We appreciate this feedback from the Reviewer. We know it can be difficult for readers to switch from supplementary materials and data presented in the main manuscript, so we tried to be strategic and selective about the placement of data in either location. We believe the data is easier presented in a figure format since it involves two compounds (uBPA and BPA-G), numerous different types of samples, and results from the four independent laboratories. However, we agree with the Reviewer that it would be easier to have all of this data in one location, so we have moved the second part of Supplemental Figure 1 to Figure 1.

7. This paper contains a lot of tables and figures, so please try to simplify by use of the same terms, abbreviations etc. Especially in table 2, 3 and suppl. Table 1, for instance use h or hours not both, mL or µL and use the same notation for mass transitions etc. (supple fig 1).

We have carefully reviewed the terms and abbreviations used in the manuscript, tables and figures to ensure consistency.

Response to Reviewer 2:
This manuscript is well written, and addressed some issues (e.g., method validation for conjugated BPA, interlaboratory studies, etc.) identified during the 2010 Joint FAO/WHO Expert Meeting on Toxicological and Health Aspects of BPA. The authors are suggested to consider the following comments for the revision.

We thank the reviewer for this positive assessment. We have responded to the individual comments below.

A Round Robin study is similar to the inter-laboratory study with the major objective being to evaluate the performance of each participating laboratory in the analysis of a series of samples for some particular chemical(s). However, this objective may not be among the three goals addressed in this study, and thus should be added or revise goal #2.

The Reviewer is correct; a Round Robin allows for the evaluation of each individual laboratory in a series of analyses. One goal of this Round Robin was to allow each laboratory to improve in their performance. This is now articulated more clearly in the revised text.

The authors actually did all the work, analysed samples with different levels of uBPA and BPG-G (e.g., Figures 3 and 6), although the results, especially at lower levels, do not agree well among labs. The authors should discuss more on the performance of each participating laboratory in terms of the results; what are the possible causes for the large differences for some of the samples, how could they be improved, and what are the implications of the results uncertainty, etc.

The Reviewer has raised some important issues regarding the variability in the observed results between laboratories. We have added a brief discussion of this to the manuscript.

Other comments:
1. Table 3 title: should be four labs, not five?

We thank the Reviewer for catching this error. We have corrected this table.

2. Table 3: why the LODs for uBPA and BPA-G are so much different, especially for Labs 1 and 2? How were they determined?

Each laboratory used the methods their lab had previously developed for environmental chemicals (or BPA in particular). Thus, factors such as sample volume, the inherent ease of ionization, and background concentrations in blank materials can influence the LOD.

3. Provide info on chemicals (BPA and BPA-G in particular) used, source, purity etc.

This information has been added to the text, where available.

4. Manuscript title could be revised to better reflect contents.
Although we respect the Reviewer’s opinion, we elect to keep the manuscript title as is. A large number of prior studies have examined BPA concentrations in human serum. What distinguishes this study from prior publications is the use of the “round robin” approach. Thus, we believe both these pieces of information should be included in the title.