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Assessing the stability of Cd, Mn, Pb, Se, and total Hg in whole human blood by ICP-DRC-MS as a function of temperature and time

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Abstract

Background: Comprehensive information on the effect of time and temperature storage on the measurement of elements in human, whole blood (WB) by inductively coupled plasma dynamic reaction cell mass spectrometry (ICP-DRC-MS) is lacking, particularly for Mn and Se.

Methods: Human WB was spiked at three concentration levels, dispensed, and then stored at five different temperatures: −70°C, −20°C, 4°C, 23°C, and 37°C. At 3 and 5 weeks, and at 2, 4, 6, 8, 10, 12, 36 months, samples were analyzed for Pb, Cd, Mn, Se and total Hg, using ICP-DRC-MS. We used a multiple linear regression model including time and temperature as covariates to fit the data with the measurement value as the outcome. We used an equivalence test using ratios to determine if results from the test storage conditions, warmer temperature and longer time, were comparable to the reference storage condition of 3 weeks storage time at −70°C.

Results: Model estimates for all elements in human WB samples stored in polypropylene cryovials at −70°C were equivalent to estimates from samples stored at 37°C for up to 2 months, 23°C up to 10 months, and −20°C and 4°C for up to 36 months. Model estimates for samples stored for 3 weeks at −70°C were equivalent to estimates from samples stored for 2 months at −20°C, 4°C, 23°C and 37°C; 10 months at −20°C, 4°C, and 23°C; and 36 months at −20°C and 4°C. This equivalence was true for all elements and pools except for the low concentration blood pool for Cd.

Conclusions: Storage temperatures of −20°C and 4°C are equivalent to −70°C for stability of Cd, Mn, Pb, Se, and Hg in human whole blood for at least 36 months when blood is stored in sealed polypropylene vials. Increasing the temperature set points on sample storage freezers can lead to large energy savings. The best analytical results are obtained when storage time at higher temperature conditions (e.g. 23°C and 37°C) is minimized because recovery of Se and Hg is reduced. Blood samples also lose volume over time and develop clots at higher temperature conditions (e.g., 23°C and 37°C), making them unacceptable for elemental testing after 10 months and 2 months, respectively.

1. Introduction

The effects of time and temperature on the stability of toxic and essential elements in human WB have been investigated for a number of elements, predominantly Pb. The storage conditions under which Pb in human WB samples is stable has been fairly well established. In a study of Pb stability in heparinized, unspiked, human WB, Subramanian et al. found no significant change in Pb concentrations in polyethylene and polypropylene containers for up to 2 weeks at 4°C, while polycarbonate containers were suitable for up to 60 days at −10°C [2]. Pb was found to be stable in both heparinized and EDTA preserved patient blood, regardless of tube type (glass or polypropylene) at −20°C, 4°C, and 22°C over a 10 week period [3]. A far more recent study of Pb stability in blood concluded that the variations in concentrations of lead-exposed heparinized patient samples stored at −85°C, −20°C, 4°C, and 22°C over a 15-month period were negligible [4]. The sample concentrations for Pb in these graphite furnace atomic absorption (GFAA) studies were 10.5 μg/dL – 616 μg/dL [2–4]. By comparison, the total geometric mean for blood lead in the updated tables of the Centers for Disease Control and Prevention's (CDC) *Fourth Report on Human Exposure to Environmental Chemicals* was 0.858 μg/dL [5].

Less is published on the stability of Cd and Hg in human WB, however. Subramanian et al. used GFAA to conclude that Cd (1.20 μg/L) in heparinized human WB stored in polycarbonate containers was stable up to 60 days at −10°C and in polyethylene and polypropylene vessels up to 2 weeks at 4°C[2]. Hg (11.2 μg/L) in EDTA-preserved bovine blood stored in borosilicate glass vials was stable for 2 years at 4°C and −20°C, as determined using atomic absorption[6]. More recently, Sommer et al. used ICP-MS with isotope dilution to measure Hg species in bovine blood (species sum in low and high pools were ~3.6 μg/L and ~13.7 μg/L, respectively). They concluded that species were stable up to 1 year at −70, −20, and 23°C, but no more than 2 weeks at 37°C because of changes in sample viscosity [7].

Similarly, not much is published on the stability of Se in human WB. Mestek et al. noted a 9% decrease in Se results on the fifth day after sampling using hydride generation atomic absorbtion spectroscopy [8]. Samples were unspiked, whole, human, heparin-preserved blood stored at 4°C. They concluded the decrease in concentration was due to adsorption of Se to the vial, but it did not indicate vial material. In a review of Se in human blood by electrothermal atomic absorbtion, Sabe et al. provide a brief summary of Se stability in human blood and particularly plasma [9]. They noted that typical storage temperature for whole blood, serum, and plasma in previous studies was −18°C to −25°C, but comparative stability studies were lacking.

Information on the stability of Mn in human WB is also absent in the literature, but we can extrapolate from the stability of serum. Hudnik et al. investigated the stability of Mn in serum using atomic absorption spectroscopy [10]. They concluded that storage of serum at −20°C was preferable to storage at room temperature, after noting that Mn results markedly increased at room temperature when stored in soft soda and pyrex glass vessels over 30 days.

Our laboratory uses ICP-DRC-MS to measure Cd, Mn, Pb, Se, and Hg in blood for technical assistance to our state and federal partners, epidemiological aid requests, and national health surveys, such as the National Health and Nutrition Examination Survey (NHANES) [1]. Quality analysis depends on the assumption that the concentration of elements in the sample has not changed during transit; however, not all patient samples are collected and shipped under rigorously controlled conditions—particularly epidemiological aid requests from other countries where infrastructure is lacking. In the pre-analytical phase, depending on the distance from the collection site to the clinical lab, patient samples may undergo shipping, handling, and storage in elevated temperature conditions prior to analysis. Blood samples may be drawn into glass vacuum tubes which cannot be frozen. Sample transfer into a plastic vessel that can be frozen risks introducing contamination if not done in a clean setting. Sample transfer also offers an opportunity for mislabeling patient specimens. These drawbacks make the long-term stability of human WB at 4°C particularly important to clinical chemistry. Post-analysis, WB elements samples may be stored far colder than necessary out of an abundance of caution and lack of data supporting stability at the concentrations of interest for all elements of interest. Storing biologic samples at low temperatures in mechanical freezers creates high energy costs [11]; therefore, establishing higher temperatures at which samples can be stored and for what length of time with no significant change in results could significantly reduce these costs. Even a small increase of 5°C in set point temperature saves energy [12]. This study aims to fill some of the gaps in the literature on the general stability of Mn and Se in human WB; to expand the literature on the stability of Cd, Pb, and Hg to include low element concentrations relevant to biomonitoring; and to provide a comprehensive comparison of storage temperatures from −70°C to 37°C.

As public health interest in toxic and nutritional elements has increased, WB elements panels have expanded to include elements beyond Cd, Pb, and Hg, while the literature available on stability has not. The assumption has been that the analytical uncertainty for most elements is too high to discern differences in concentration over time resulting from storage [13]. However, as techniques for trace analysis become more sensitive and method limits of detection (LOD) improve, it is important to revisit stability and determine if this assumption holds true for all the elements in the expanded WB elements panel and lower concentrations observed in biomonitoring. In this work, we prepared three WB pools at three concentrations (low, high, and elevated), stored them at five different temperatures, and periodically analyzed the samples using ICP-DRC-MS.

2. Materials and methods

2.1 Sample creation and dispensing

We screened bags of potassium ethylenediaminetetraacetic acid-preserved human WB (Tennessee Blood Services, Memphis, TN) and combined them to make low (LB), high (HB), and elevated blood (EB) pools. Purchasing human blood with native elemental concentrations in the ranges of interest for all five elements in the panel is not practically feasible, especially since a long-term stability study also requires a large volume of blood. When possible, we combined blood bags in a manner that limited the need for spiking.

When the native elemental concentration was not high enough, elements were spiked to the desired concentration using National Institute of Science and Technology (NIST)-traceable single element standards (Inorganic Ventures, Christiansburg, VA). Supplementing elemental concentrations by spiking is a common approach taken to prepare internal bench QC material for ICP-MS methods that use simple dilution sample preparation prior to testing. The plasma of the ICP-MS destroys the molecular structure of the sample introduced to it leaving mostly singly charged ions whether they were initially bound or free within the sample. Pools were stirred overnight and then tested for levels of Cd, Mn, Pb, Se, and Hg. Samples were dispensed using a Micromedic Digiflex-CX Automatic (Titertek, Huntsville, AL) as 1.8 mL into 2.0 mL polypropylene cryogenic vials (Thermo Fisher Scientific, Rochester, NY). Sample vials were screened to ensure acceptable levels of background contamination for the analytes of interest based on criteria such as population reference limits and LOD. The vial manufacturer recommends vial storage temperatures of ≤4°C. Sample containers specifically designed for higher temperatures were not evaluated. After dispensing, samples were stored in one of five temperatures: −70°C, −20°C, 4°C, 23°C (on the benchtop of a temperature-controlled lab), or 37°C.

2.2 Sample analysis using ICP-DRC-MS

At 3 and 5 weeks and at 2, 4, 6, 8, 10, 12, and 36 months, 3 vials of blood were analyzed per storage temperature, per pool, for a total of 45 samples per event. Samples were inspected prior to analysis. Compromised samples (visibly diminished sample volume or clots, for example) were replaced with an identical sample whenever possible. Sample vials were discarded after analysis.

Sample order was randomized prior to analysis on an ELAN® DRC II ICP-DRC-MS (PerkinElmer, Norwalk, CT) equipped with a DXi-FAST and ESI SC4 autosampler (Elemental Scientific Inc., Omaha, NE) using a previously described multi-element method [1]. Each analytical run included internal bench quality control (QC) samples prepared and characterized at CDC to establish that the run was in control [14, 15]. We also analyzed levels 1, 2, and 4 of standard reference material (SRM) 955c, Toxic Metals in Caprine Blood (NIST, Gaithersburg, MD) in each run.

2.3 Statistical analysis using an equivalence test

We evaluated the study results using an equivalence test modeled after the Food and Drug Administration's guidance for determining bioequivalence [16]. First, we evaluated the interaction terms of time and temperature (Table S1 in Supplemental Data). We then used a multiple linear regression model to fit the data. The model included time (categorical) and temperature (categorical) as covariates, and the measurement value was the outcome.

We used the model estimates from −70°C as the reference and compared the model estimates from different temperature conditions to them independent of time. The model estimate from −20°C, 4°C, 23°C or 37°C is μ_{Test}, and the model estimate for the reference temperature of −70°C is μ_{Ref}. We also used the equivalence test to compare the model estimates from different time points against the estimate for the reference time of 3 weeks

independent of temperature. In this case, μ_{Test} is the model estimate from 5 weeks and from 2, 4, 6, 8, 10, 12, 18, 24, 30, or 36 months, and μ_{Ref} is the model estimate from 3 weeks.

An equivalence test was performed using the following hypotheses:

H0 $\mu_{Test}/\mu_{Ref} < 0.8$ or $\mu_{Test}/\mu_{Ref} > 1.25$

H1 $0.8 \leq \mu_{Test}/\mu_{Ref} \leq 1.25$

If $\mu_{Test}/\mu_{Ref} < 1$, we test the hypothesis of $\mu_{Test}/\mu_{Ref} < 0.8$. If $\mu_{Test}/\mu_{Ref}, > 1$, we test the hypothesis of $\mu_{Test}/\mu_{Ref} > 1.25$. If $p < 0.05$ for the hypothesis testing, then we reject the null hypothesis, accept the alternative hypothesis ($0.8 \leq \mu_{Test}/\mu_{Ref} \leq 1.25$), and conclude that the test storage condition yields results statistically equivalent to the reference storage condition.

3. Results

3.1 Comparison limitations

We calculated the mean, standard deviation (SD), and coefficient of variation (%CV) for the samples kept at −70°C for three years and compared them to statistics from our characterized bench quality control (QC) material (Table 1). This comparison ensured that the −70°C storage temperature results obtained during the stability experiment met or exceeded expected method precision and therefore were reasonable for use as the reference temperature in the equivalence tests. In most cases, the differences between the CVs of the method QC and the reference samples were small, and we determined that the −70°C reference results were representative of typical method performance (Table 1).

WB samples stored at 37°C clotted at 4 months, and the samples stored at 23°C clotted at 12 months, so no data were collected for these storage conditions beyond the 2-month and 10-month analysis times, respectively. Therefore, only the equivalence of storage at −20°C and 4°C was compared to storage at −70°C for the full 36 months.

3.2 Equivalence testing

3.2.1 Temperature—The test storage temperature results (−20°C, 4°C, 23°C, 37°C) were statistically equivalent to the reference storage temperature results (−70°C) for all elements and pools. Estimates of μ_{Test} and μ_{Ref} from the model, ratios of μ_{Test}/μ_{Ref}, and p-values for all elements and pools at each temperature are in Supplemental Data.

3.2.2 Low pool—Results typical of μ_{Test}/μ_{Ref} for all elements in the low pool are in Figure 1: the ratios of μ_{Test}/μ_{Ref} deviate very little from 1.0 for all four temperatures tested and ratios are well within the equivalence boundaries.

3.2.3 High and elevated pools—Results for μ_{Test}/μ_{Ref} in HB and EB pools were also within the equivalence margins and therefore not significantly different from the reference temperature for all elements. However, plots of μ_{Test}/μ_{Ref} vs temperature for Hg in the EB and HB pools trended downward at 23°C and 37°C (Figure 1). We also noted this trend in the HB and EB Se pools (data not shown). The downward trend was greatest in the elevated

pool at 37°C for both of these elements. The magnitude of the trend was larger in the Hg pools than in the Se pools.

3.3 Time

The test storage time results were statistically equivalent to the reference storage time results for all elements and pools, except LB Cd (Figure 2). For comparison, the same plots of μ_{Test}/μ_{Ref} vs time for HB and EB Cd are shown in Figure 2. Estimates of μ_{Test} and μ_{Ref} from the model, ratios of μ_{Test}/μ_{Ref}, and p-values for the LB Cd pool are in Table 4. Similar tables for the remaining elements and pools are in Supplemental Data. We tabulated instrument results from HB and EB samples for Hg (Table 3) to see if the trend seen in the model estimates was visible in the non-modeled instrument results.

4. Discussion

4.1 Temperature

Results showing equivalence of storage at −70°C with warmer storage temperatures over long periods of time for all elements are consistent with our experience storing internally-prepared QC samples at −20 °C long-term, 4°C in the medium-term, and room temperature in the short-term with no notable issues. The decrease in μ_{Test}/μ_{Ref} at 37°C relative to the other test temperatures for Hg and Se in the EB and HB pools may be due to a temperature-related increase in adsorption or volatility of Hg and Se spiked into the HB and EB pools to achieve the desired concentration [17, 18]. We did not spike LB Se and Hg pools and did not observe the trend of decreasing elements concentration with increasing temperature. However, one limitation of spiking blood pools is that we can't say whether the apparent concentration decrease with increasing temperature is concentration-dependent or due to the presence of element that is not native to the matrix. Regardless, the estimates from all three Hg and Se pools stored at 37°C were statistically equivalent to storage at the reference temperature. Means and SDs of the non-modeled instrument results for Hg (Table 2 and Table 3) trends similarly to the model (decreasing mean and increasing SD with increasing temperature), with the greatest decrease occurring in the Hg EB pool at 37°C. The larger SD at 37°C may be due to inhomogeneity in the samples from small blood clots that were not clearly visible.

4.2 Time

The variation in ratios of estimates at different times was much greater than the variation in ratios of estimates at different temperatures. This large variation is due to the experimental design, where the model estimate for μ_{Ref} comes from results obtained in a different analytical batch than μ_{Test} for time, but in the same analytical batch for temperature. Placing these samples in the same analytical run removes the large source of variation in analytical factors that are subject to change, including instrument sensitivity, instrument operator, or calibrator lot. We noted this design flaw shortly after we evaluated our results and found it difficult to assess stability over time for the LB Cd pool. We observed that LB Cd μ_{Test}/μ_{Ref} was outside the lower equivalence boundary at 6 weeks and 10 weeks but within the boundaries at 8 weeks (Figure 2 and Table 4). If Cd adsorbed to the container walls or a change in concentration occurs by some other mechanism, we might expect the changes in

results to be directional, increase over time, and possibly show poorer inter-sample measurement precision. Instead, we observed that the estimate ratios were fairly consistent over time for the LB pool, hovering closely around a μ_{ref}/μ_{test} ratio of 0.8. The pattern of the ratios in the LB pool is consistent with the HB and EB pools but is shifted downward relative to the other pools which are both positioned at μ_{ref}/μ_{test} ratio of around 0.95.

We concluded that this apparent instability in LB Cd is likely an artifact of the study design. The estimate at each time is ratioed to a single estimate (μ_{ref}) at three weeks. Because we ratioed all estimates to that single time, a bias in μ_{ref} can skew the results. Samples analyzed at 3 weeks had the largest non-modeled average LB Cd results for samples stored at −70°C (0.639 μg/L, results not shown) and also the largest model estimate, 0.636 μg/L. By comparison, the non-modeled average for samples stored at −70°C over the total storage time ranged from 0.461 μg/L – 0.557 μg/L. The model estimate ranged from 0.442 μg/L – 0.548 μg/L (Table 4). Because μ_{Ref} from 3 weeks is skewed high, the LB Cd ratios are skewed low and the estimates at 6 and 8 weeks fall outside the low boundary. We compared our results for Cd in SRM 955c level 1 from the batch of 3-week stability samples to the reference value. SRM 955c level 1 has a reference concentration closest to the concentration of our LB pool (2.14 μg/L ± 0.24 μg/L [k=2.1]). We obtained a Cd result of 2.28 μg/L, which is within the uncertainty of the reference value, but is higher than the target concentration. This small bias in the SRM result suggests that the Cd results in the 3-week results may also be biased high relative to subsequent batches, leading to non-equivalence in weeks 6 and 8.

An isochronous approach to stability study design addresses this flaw by reducing variability that arises from the use of multiple operators, instruments, calibrator lots, and run-to-run variation in instrument response. Samples are kept in the reference storage condition (e.g. −70°C) and then moved to the test storage conditions for a specified length of time [19]. Once the experiment end date arrives, all samples are removed from test and reference conditions and analyzed close in time (preferably in one analytical batch) using the same instrument, operator, and calibrator lot. The disadvantage of this approach is that all samples must be stored for the duration of the study until a time when they can all be analyzed together. Furthermore, in a large, long-term, stability study with multiple pools, it may not be feasible to test all the samples in a single analytical run. The length of the stability study must also be established in advance.

5. Conclusion

We used an equivalence test to examine the stability of Cd, Mn, Pb, Se, and Hg in human WB using ICP-DRC-MS. This study adds information about Mn and Se stability in human WB that was difficult to find or missing from the literature. Storage temperatures of −20°C and 4°C are equivalent to −70°C for stability of Cd, Mn, Pb, Se, and Hg in human WB stored in sealed polypropylene vials for at least 36 months. We found this equivalence to be true for both low and elevated concentrations of these elements. The best analytical results are obtained when time is minimized at higher temperature conditions (e.g. 23°C and 37°C) because recovery of Se and Hg is reduced over time. Clots, changes in viscosity, and loss of volume may make samples unacceptable for elemental testing after 10 months and 2 months,

respectively. The trend observed here that higher temperatures incur additional problems in elemental recovery and sample integrity suggests that −20°C or −70°C would be the better storage temperature options if samples will be stored for more than 3 years. However, storing human WB samples at −20°C or 4°C up to 3 years prior to testing for these elements saves energy.

An isochronous experimental design would have simplified interpretation by reducing run-to-run variability on which our equivalence statistical test was based. We interpret the failure of the equivalence test of Cd in the LB pool at weeks 6 and 10 as a complication of our current experimental design rather than a true indication of lack of stability. We make this conclusion because of the quality assurance indicators from the analysis at 3 weeks and the flat estimate ratio profile over time for Cd in the LB pool. An isochronous experimental design would reduce the run-to-run variability such as instrument sensitivity, operator, and calibrator lot.

supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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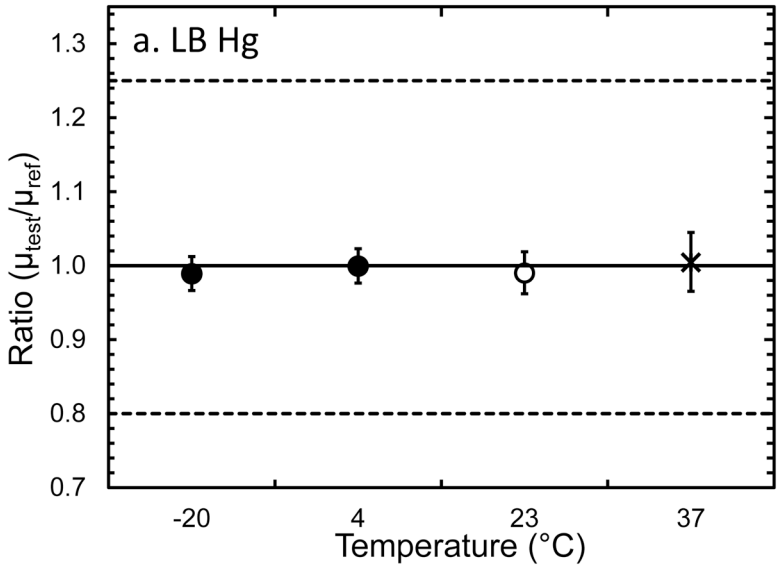
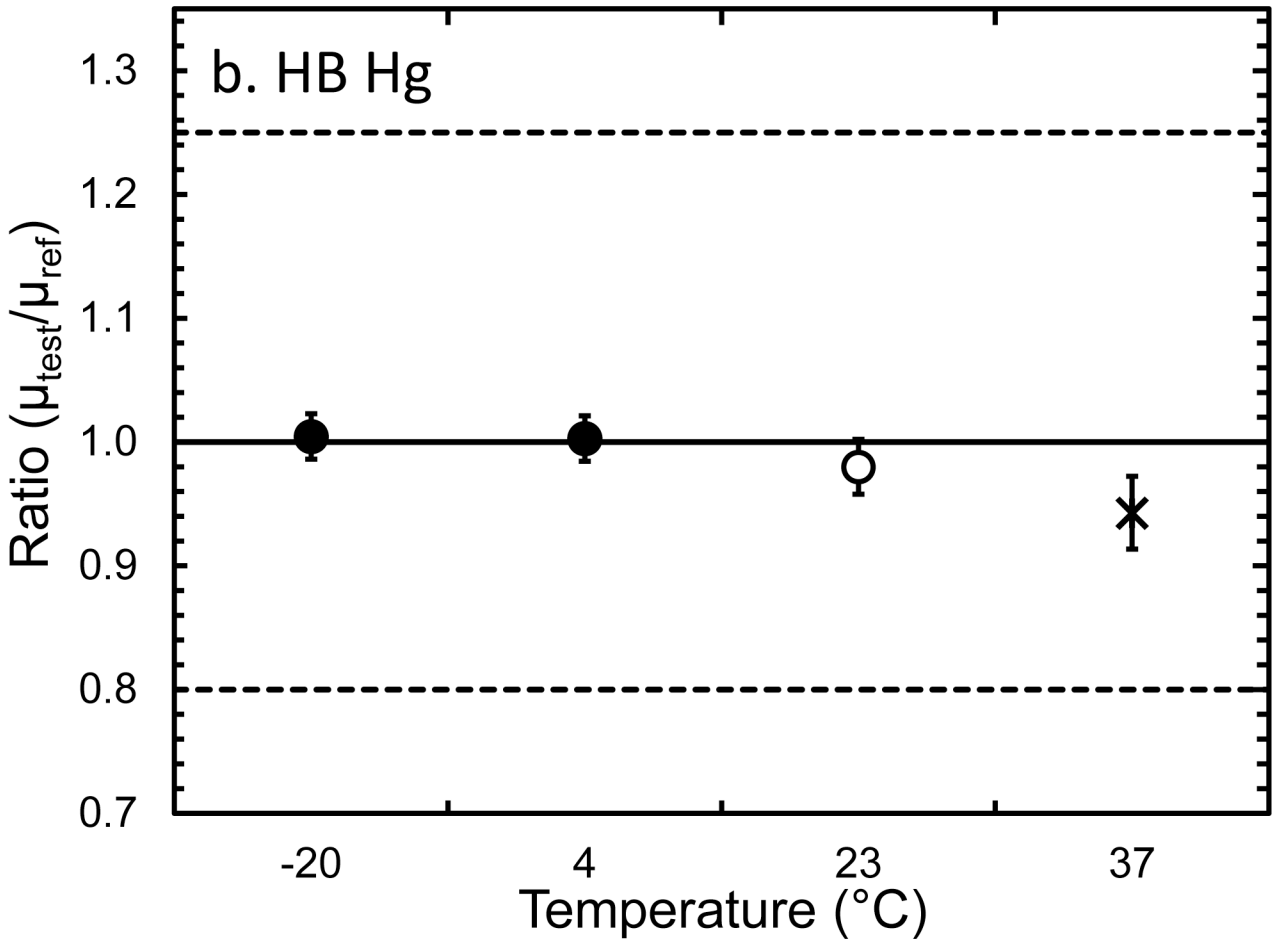




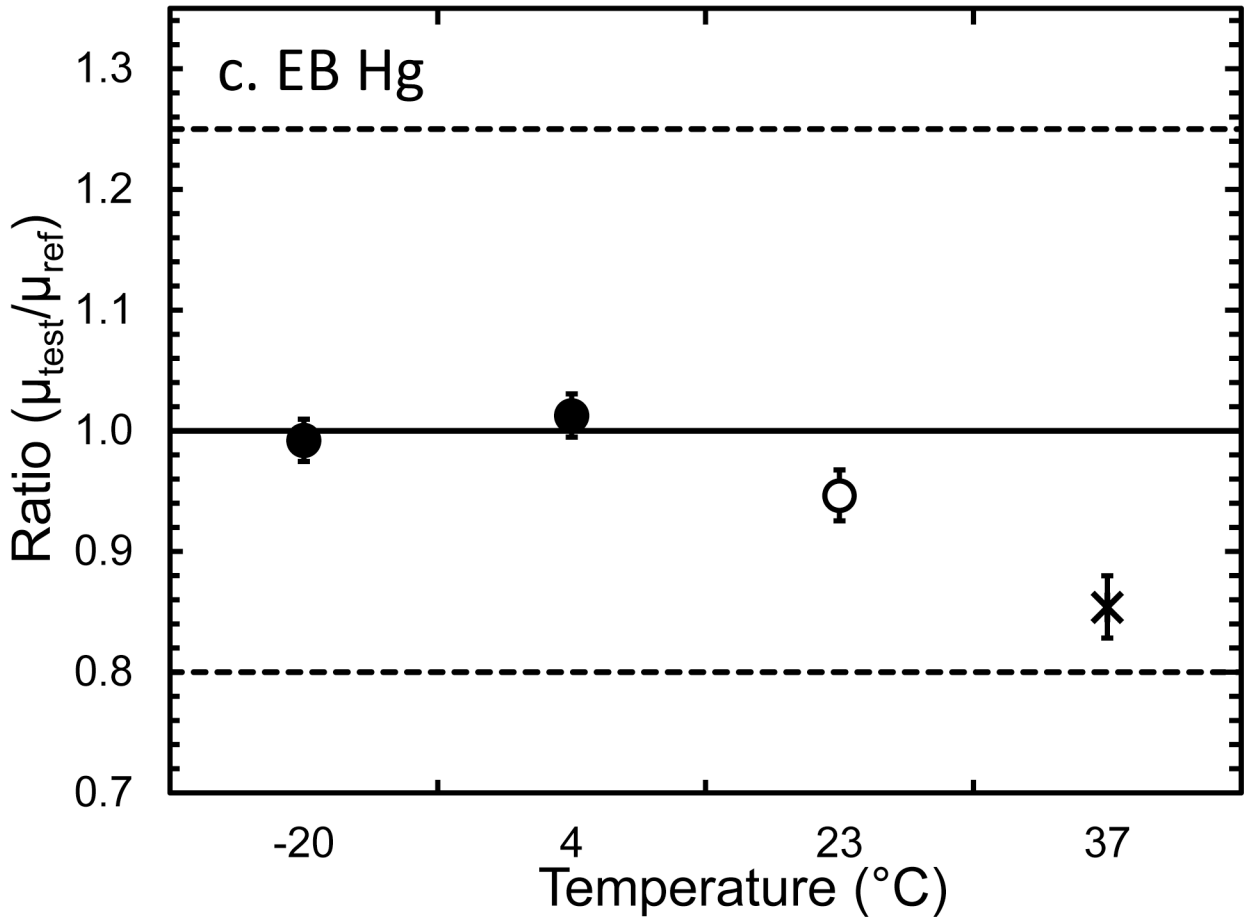
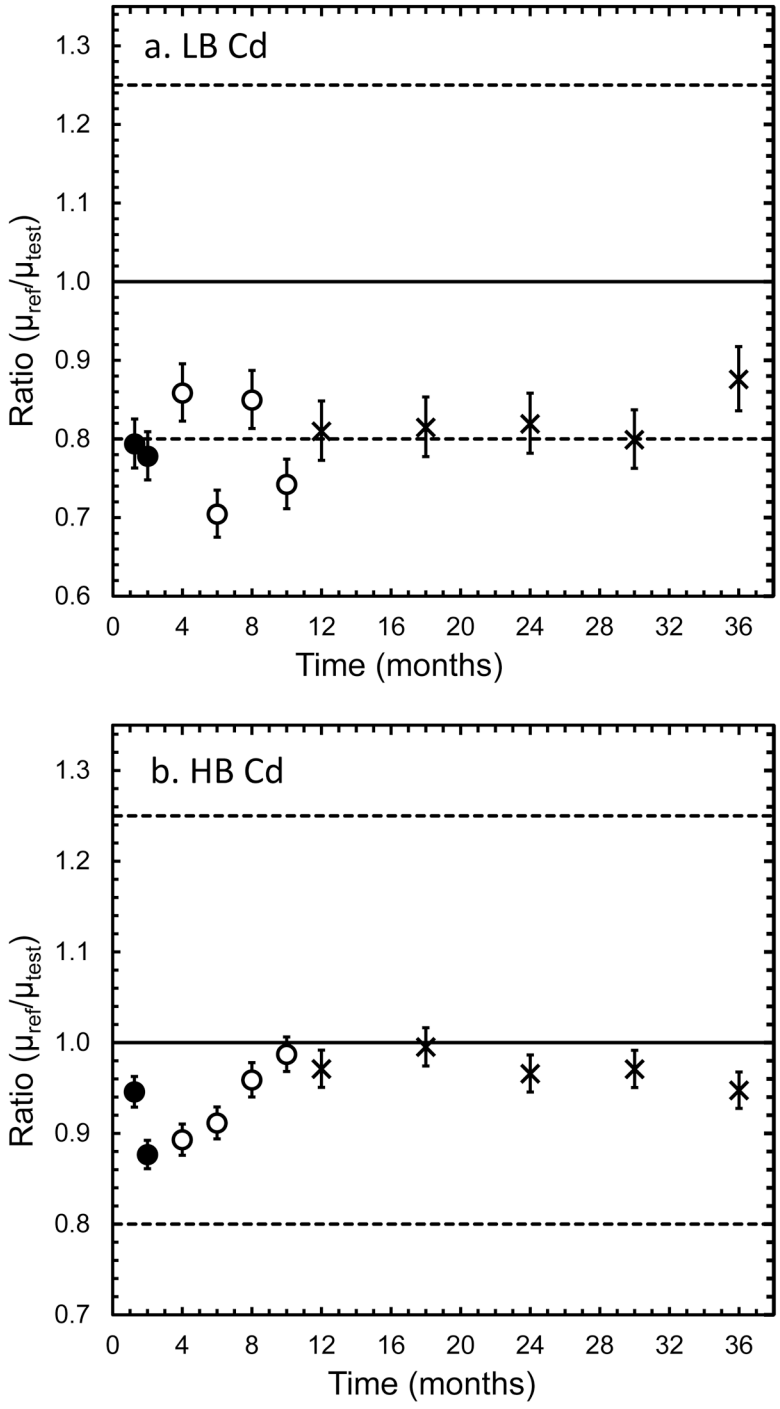


Figure 1.
Ratio of μ_{Test}/μ_{Ref} vs temperature for Hg in LB (a), HB (b), and EB (c) pools for (●) 3 weeks to 36 months, (○) 3 weeks to 10 months, and (×) 3 weeks to 2 months.



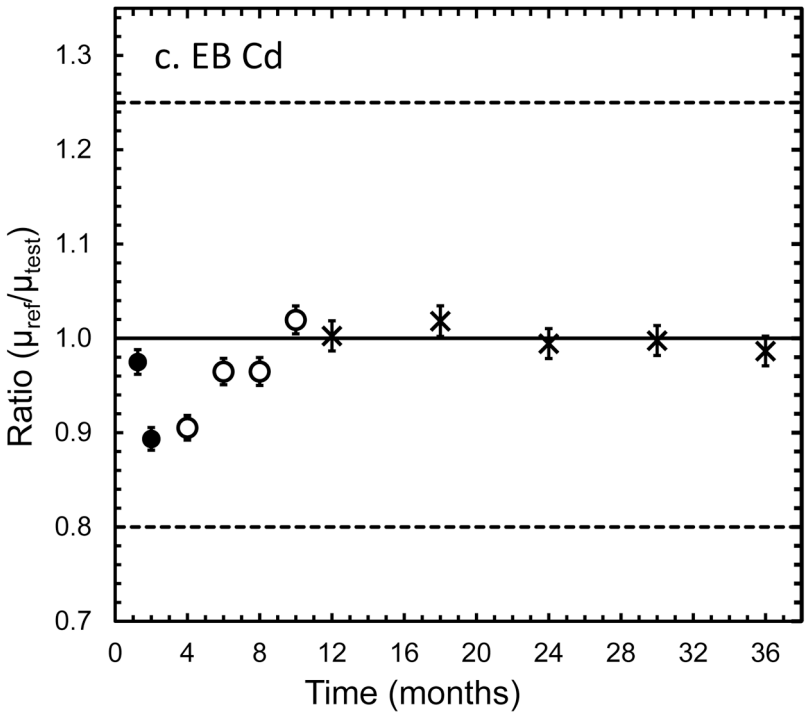


Figure 2.

Ratio of μ_{Test}/μ_{Ref} vs time for Cd in low, high and, elevated pools. (●) includes data from −20°C, 4°C, 23°C and 37°C, (○) includes data from −20°C, 4°C, and 23°C, and (×) includes data from −20°C and 4°C.

Table 1

Descriptive statistics from the −70°C reference samples (n=36) and method QC by element and pool level.

		Reference Samples			Method QC			
Element	**Pool**	**Overall Mean (µg/L)**	**SD**	**CV (%)**	**Overall Mean (µg/L)**	**SD**	**CV (%)**	**LOD**
Cd	Low	0.521	0.063	12%	0.459	0.041	9%	0.100
	High	3.04	0.14	5%	3.05	0.09	3%	
	Elevated	10.2	0.5	5%	44.8	1.2	3%	
Mn	Low	9.49	1.10	12%	8.04	0.45	11%	0.990
	High	26.7	1.9	7%	14.6	0.6	8%	
	Elevated	203	13	6%	42.9	1.9	8%	
Pb** *(µg/dL)	Low	0.974	0.053	5%	2.11	0.07	6%	0.070
	High	4.37	0.16	4%	10.0	0.1	3%	
	Elevated	8.65	0.29	3%	88.2	5.9	13%	
Se	Low	173	13	8%	190	6	7%	24.5
	High	236	15	6%	252	8	6%	
	Elevated	580	48	8%	2660	100	7%	
Hg	Low	0.443	0.046	10%	0.603	0.056	18%	0.280
	High	5.61	0.20	4%	5.89	0.15	5%	
	Elevated	192	7	3%	41.8	5.9	28%	

Table 2

HB Hg non-modeled instrument results (μg/L). Mean and SD are from n=3 sample vials, except ǂn=2.

	Mean ± SD				
Time	**−70°C**	**−20°C**	**4°C**	**25°C**	**37°C**
3 weeks	5.53 ± 0.19	5.55 ± 0.02	5.68 ± 0.08	5.75 ± 0.26	5.25 ± 0.19
5 weeks	5.79 ± 0.18	5.75 ± 0.02	5.81 ± 0.25	5.60 ± 0.58	4.82 ± 0.15
2 months	5.80 ± 0.11	5.74 ± 0.15	5.53 ± 0.05	5.56 ± 0.03	6.10 ± 0.08
4 months	5.70 ± 0.08	5.65 ± 0.11	5.46 ± 0.09	5.45 ± 0.11	-
6 months	5.78 ± 0.06	5.98 ± 0.16	5.97 ± 0.15	6.27 ± 0.29	-
8 months	6.04 ± 0.35	5.75 ± 0.07	6.08 ± 0.10	5.82 ± 0.03ǂ	-
10 months	5.54 ± 0.06	5.53 ± 0.06	5.49 ± 0.17	4.82 ± 0.44	-
12 months	5.55 ± 0.06	5.69 ± 0.07	5.89 ± 0.26	-	-
18 months	5.54 ± 0.07	5.72 ± 0.17	5.49 ± 0.30	-	-
24 months	5.67 ± 0.12	5.71 ± 0.12	5.61 ± 0.15	-	-
30 months	5.75 ± 0.27	5.64 ± 0.05	5.68 ± 0.22	-	-
36 months	5.56 ± 0.05	5.82 ± 0.45	5.73 ± 0.15	-	-

Table 3

EB Hg non-modeled instrument results (μg/L). Mean and SD are from n=3 sample vials, except *n=1.

	Mean ± SD				
Time	**−70 °C**	**−20 °C**	**4 °C**	**25 °C**	**37 °C**
3 weeks	187 ± 0	186 ± 1	189 ± 1	189 ± 1	150 ± 12
5 weeks	199 ± 2	191 ± 2	204 ± 7	196 ± 7	160 ± 5
2 months	188 ± 4	190 ± 1	193 ± 1	196 ± 5	193 ± 10
4 months	192 ± 3	188 ± 3	193 ± 1	178 ± 5	-
6 months	197 ± 1	201 ± 2	201 ± 4	186 ± 9	-
8 months	206 ± 0	204 ± 4	208 ± 7	164*	-
10 months	186 ± 3	187 ± 2	188 ± 3	160 ± 16	-
12 months	195 ± 4	192 ± 2	203 ± 5	-	-
18 months	189 ± 5	186 ± 3	186 ± 6	-	-
24 months	187 ± 5	187 ± 5	189 ± 1	-	-
30 months	187 ± 5	185 ± 3	187 ± 5	-	-
36 months	192 ± 3	190 ± 1	194 ± 7	-	-

Table 4

LB Cd estimates of μ_{Test} and μ_{Ref} from the model, ratios of μ_{Test}/μ_{Ref}, with 95% confidence interval (CI), and p-values. Estimate ratios with a significant p-value (p >0.05) are bolded.

Sample type	Time	Estimate (μg/L)	95% CI (μg/L)	Ratio	95% CI	p-value
μ_{Ref}	3 weeks	0.653	0.636–0.670	–	–	–
	5 weeks	0.519	0.501–0.536	0.794	0.763–0.825	0.002
	2 months	0.510	0.493–0.528	0.778	0.748–0.809	0.017
	4 months	0.561	0.541–0.581	0.858	0.823–0.896	<0.001
μ_{Test}	**6 months**	**0.462**	**0.442–0.482**	**0.704**	**0.675–0.735**	**0.950**
	8 months	0.555	0.534–0.576	0.849	0.813–0.887	<0.001
	10 months	**0.486**	**0.466–0.506**	**0.742**	**0.711–0.774**	**0.351**
	12 months	0.529	0.505–0.552	0.810	0.773–0.848	<0.001
	18 months	0.534	0.510–0.557	0.815	0.777–0.853	<0.001
	24 months	0.535	0.511–0.558	0.819	0.782–0.858	<0.001
	30 months	0.522	0.499–0.545	0.799	0.763–0.837	0.001
	36 months	0.572	0.548–0.595	0.876	0.836–0.918	<0.001