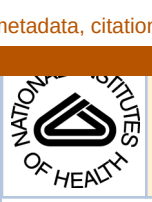

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Quantification of Arsenic, Lead, Mercury, and Cadmium in Newborn Dried Blood Spots

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The heavy metals, arsenic (As), lead (Pb), mercury (Hg), and cadmium (Cd) are ubiquitous environmental toxicants that are listed as the 1st, 2nd, 3rd, and 7th most important hazardous substances on the 2011 *CERCLA* priority list of 275 substances, respectively. Exposures to heavy metals during fetal and early postnatal development are of particular concern. Although the placenta acts as a partial barrier for Cd (Lin, 2010), As, Pb, and Hg readily cross the placental barrier and can expose the developing fetus (Agrawal, 2012). Yet, the health impacts of exposures to toxic metals during these early stages of human development are not well understood due to the paucity of *in vivo* human data. Additional methods for assessing exposures during these sensitive periods of susceptibility are needed.

Newborn dried blood spots (DBS)—drops of whole blood collected on filter paper following a simple heel stick within 24-48 hours of birth—are a potential resource for non-invasively investigating perinatal exposures to toxic metals. Dried blood spots are routinely collected from virtually all newborns in the US to screen for inherited metabolic errors and other disorders. Several state public health departments archive residual DBS and make them available for additional research, including exposure assessment (Olshan, 2007). Here we report an improved method for quantifying As, Pb, Hg, and Cd in newborn DBS to facilitate epidemiologic research on the health effects of early exposures to toxic metals.

Based on a recently reported method by Langer et al. (Langer, 2011), a targeted extraction and analytical procedure was developed and optimized for specifically quantifying As, Pb, Hg, and Cd in DBS specimens. Our major improvements to this method included 1) the use of a one-batch extraction procedure to avoid contamination and analyte loss during transfer and filtration steps, 2) the addition of gold (Au) in the extraction solution to amalgamate Hg and enhance recovery, 3) normalization of dried blood mass to more precisely estimate relative blood volumes, and 5) the use of paired-filter paper blanks for all DBS samples to

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account for background contamination in the Whatman #903 protein-saver cards (VWR, Atlanta, GA). To validate this improved method, forty-nine randomly selected and unidentified DBS samples were obtained from the North Carolina State Laboratory of Public Health with collection dates ranging from 2003-2009. All samples were stored at room temperature prior to analysis.

All samples were excised with a ceramic blade (VWR, Atlanta, GA) that was acid-washed in a 5% nitric acid (v/v) solution overnight prior to use. Samples were handled with acid-washed Teflon tweezers and were excided on an acid-washed Teflon working surface. Between each sample and paired card blank, the blades were washed with 18.2 mΩ deionized water for 30 seconds and air dried. One-half of an intact DBS was removed from each sample using the printed guidelines on the blood collection cards as a guide. The volume of blood in one-half of an intact DBS is approximately 30 μl. However, to account for blood volume variation between samples, the dried mass of each sample was normalized to the mean mass of all of the excised samples. A similar approximate size of a blank filter paper was excised from each card near each blood sample to account for background metal contamination in the filter paper. The mass of each blank was also normalized to the mean mass of all of the excised blanks. DBS and filter paper blanks were weighed in 15 ml polypropylene centrifuge tubes (VWR, Atlanta, GA) that were acid washed for one week using 5% ultrapure grade nitric acid (Sigma-Aldrich, St. Louis, MO). An extraction solution was prepared using 5% ultrapure grade nitric acid and 0.01% ultrapure grade Triton X-100 (Fisher Scientific, Pittsburgh, PA) in 18.2 mΩ deionized water. Two hundred ppb of Au was added to amalgamate Hg and prevent analyte loss throughout the procedure (Inorganic Ventures, Christiansburg, VA). Ten ml of extraction solution, determined gravimetrically, was added directly to each vial to avoid contact with additional labware. Five ppb of indium, bismuth, and yittrium were added as internal standards (Inorganic Ventures, Christiansburg, VA). DBS samples and filter paper blanks were vortexed briefly and incubated for 90 minutes at room temperature. After 90 minutes of incubation, samples were sonicated for 10 minutes. Samples were centrifuged at 3500 rpm for 10 minutes prior to analysis. Filtration was avoided to prevent contamination and analyte loss.

Concentrations of As, Pb, Hg, and Cd were quantified using a Perkin Elmer Elan II Inductively Couple Plasma Mass Spectrometer (ICP-MS). The instrument detection limits were determined to be in the low ppt range for each element. Metal concentrations were quantified based on a five-point calibration curve for each analyte. For Pb, three isotopes were scanned and summed (*m/z*: 206, 207, and 208). Arsenic, Hg, and Cd, were quantified using single isotopes with *m/z* of 75, 202, and 114, respectively. In addition to the samples and paired card blanks, four quality control samples were run along with each batch, consisting of a matrix blank, low calibration standard, quality control standard, and a spiked low control standard. These procedures are consistent with US Environmental Protection Agency's standard operating procedures (methods 200.8 and 6020a). Arsenic and Cd were run in both standard and dynamic reaction cell (DRC) modes to evaluate polyatomic interferences using oxygen as the reaction gas.

Median concentrations of As, Pb, Hg, and Cd are provided for the DBS samples, the filter paper blanks, and for pair-wise subtracted samples in Table 1. Concentrations were converted to ppb using the normalized masses, with the assumption that each half DBS consists of 30 ul of blood. While the actual blood volume may differ slightly from this assumed volume, this approximation allows for meaningful comparisons with gold standard methods, and provides an accurate relative blood volume for comparison between samples. Pair-wise subtracted 90^{th} percentile values are also provided, along with the percentages of each element that were at detectable levels. While there are no well-established heavy metal exposure guidelines for newborns, action and warning levels are provided in Table 1 as a

general reference. Quantification of As in DRC mode resulted in interfering ions at *m/z* 91 (AsO), and lacked analytical sensitivity for assessment of Cd. As a result, only data collected using standard operation mode are reported. To assess the assay reliability and precision, between-assay variance was evaluated as the sum of the four metals within each run. The low calibration and quality control standards between-assay %CVs were 10.2% and 10.0%, respectively. Spiked recoveries ranged from 81-118% for As, 93-105% for Pb, 48-230% for Hg, and 87-113% for Cd.

Analyte stability and recovery were evaluated across collection year to assess the effects of storage time. Mean heavy metal concentrations in the pairwise-subtracted DBS are plotted by year of collection in Figure 2. Mean concentrations of heavy metals in the DBS samples and filter paper blanks were also evaluated. Arsenic concentrations were elevated in the 2008 and 2009 DBS specimens in a minority of the samples, and Hg spikes were detected in a minority of samples in 2003 and 2009. These results suggest that contamination may be an issue during blood collection and storage. However, since no overall trends were observed as a function of storage time, recovery does not appear to decrease over time.

Arsenic concentrations were low in the DBS samples, with the majority of the samples below the limit of detection (82%<LOD). However, As concentrations in the filter paper blanks were all undetectable (Figure 1). As a result, pair-wise subtraction of the filter paper blanks from the DBS samples is not necessary in studies that are interested in exposures to As alone. Consequently, this would reduce the number of samples in half and substantially reduce the cost and time required for analysis. Future studies should focus on increasing the analytical sensitivity for As to reduce the number of non-detectable samples. This could be accomplished by increasing the sample concentration, either by using more sample volume (e.g. more than one-half of a DBS, or using a pooled sample design), or by decreasing the volume of the extraction buffer. It should also be noted that, while the majority of the samples were below the LOD in this study, these samples were from individuals from the general population that had no known exposures to As. In specifically defined studies including exposed populations this current method may have greater utility.

Lead was detectable in all of the DBS samples and had a median concentration of 13.3 ppb. However, unlike As, high concentrations of Pb were detected in the blank filter paper samples (median = 5.7 ppb)(Figure 1). These findings are consistent with previous studies that have reported Pb contamination in Whatman #903 blood collection cards (Chaudhuri, 2009). When the filter paper blanks were pair-wise subtracted from the DBS samples, the median Pb concentration was 7.0 ppb. In 16% of the samples the filter paper blanks had higher Pb concentrations than the DBS sample, suggesting a non-homogenous distribution of Pb across the cards. Lead in the filter paper blanks and DBS samples were weakly correlated (Pearson R= 0.04, p= 0.81). This finding suggests that, while the Whatman #903 collection cards contain significant Pb contamination, pair-wise subtracting the filter paper blanks from the DBS samples may not increase the precision of the assay.

Median Hg concentrations in the DBS samples and filter paper blanks were all below the detection limit. The 90^{th} percentile concentration after pair-wise subtraction was 1.9 ppb and was detectable in 33% of the samples (Figure 1). The correlation between Hg in the DBS samples and filter paper blanks was significant (Pearson R= 0.44, p= 0.002), suggesting that in the case of Hg using a pair-wise subtraction may provide a better estimate of Hg blood levels in DBS samples. While Hg was largely undetectable in these 49 newborn DBS samples, these concentrations were all low compared to the EPA reference dose of 5.8 ppb.

Cadmium concentrations were 1.3 ppb in the DBs samples and 0.2 ppb after subtraction of the Cd in the filter paper blank (Figure 1). After pair-wise subtraction Cd was detectable in

67% of the samples. As with Hg, the correlation between Cd in the DBS samples and filter paper blank was significant (Pearson R= 0.60, $p<0.001$), suggesting that subtracting the Cd concentrations in the pair-wise filter paper blanks may provide an improved estimate of Cd in the DBS samples.

This improved method provides a non-invasive means for assessing exposures to As, Pb, Hg, and Cd using state-archived newborn DBS samples. Although many of the DBS samples contained heavy metal concentrations that were below the level of detection in this study, these validation samples were obtained randomly from individuals that did not have any known exposures to heavy metals, and contained low heavy metal concentrations compared to action and warning levels that have been established for adults and children. In studies that include exposed populations metal concentrations would be expected to be higher and above the detection threshold. In such studies, normalization of the sample masses to mean sample values will also provide a better estimate of the relative amount of blood in each sample. Given these advantages, we report an improved method for quantifying heavy metal exposures that can facilitate epidemiologic research on the effects of fetal and perinatal exposures heavy metals.

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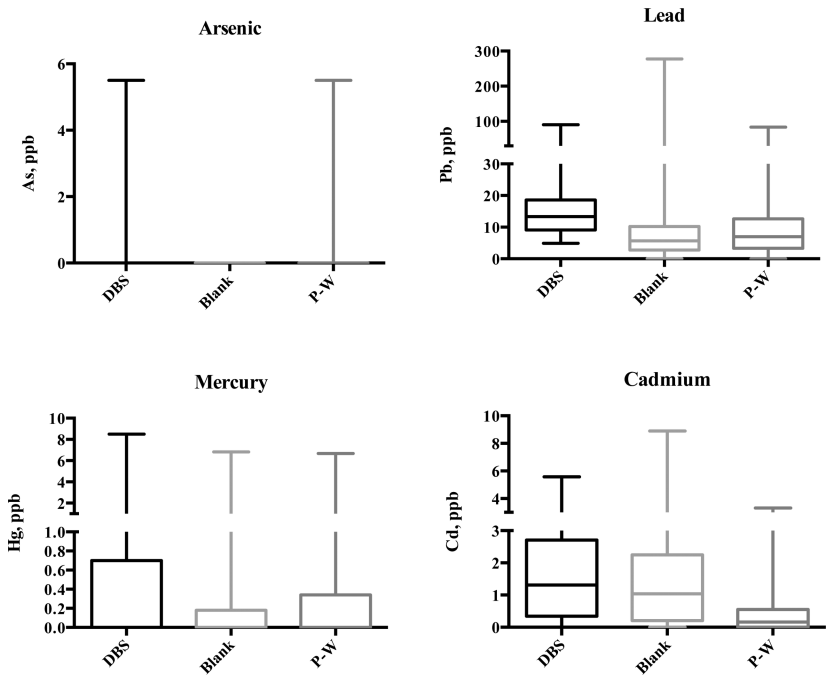

Figure 1.

Box-and-whisker plots of As, Pb, Hg, and Cd in DBS, filter paper blanks, and pair-wise (P-W) subtracted samples. Lines within the boxes represent 25th, 50th, and 75th percentiles, and whiskers illustrate the maximum and minimum values.

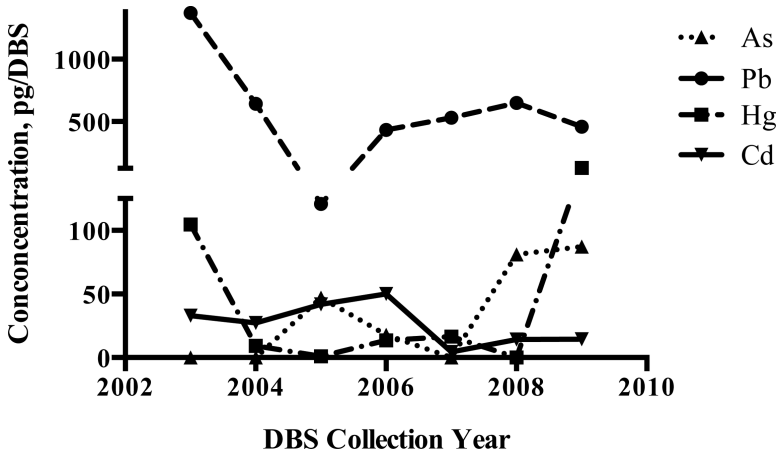

Figure 2.
Mean heavy metal concentrations in pair-wise subtracted newborn DBS samples plotted by year of collection.

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Table 1

Summary statistics for metal concentrations in the DBS and filter paper blank samples. While action/warning levels have not been established during fetal and perinatal development, various guidelines are provided for context.

Heavy metal	DBS, median	Blank, median	Pair-wise subtracted, median	Pair-wise subtracted, 90th percentile	Percentage above LOD	Action/warning level
Arsenic, ppb	0.0	0.0	0.0	2.5	18%	[1] >100
Lead, ppb	13.3	5.7	7.0	18.9	84%	[2] 100
Mercury, ppb	0.0	0.0	0.0	1.9	33%	[3] 5.8
Cadmium, ppb	1.3	1.0	0.2	1.0	67%	[4] NA

[1] Considered abnormal, ATSDR

[2] Action level, CDC

[3] Reference dose, US EPA

[4] Generally recognized guidelines for blood levels of Cd have not been established