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Pilot study for utilization of dried blood spots for screening of lead, mercury and cadmium in newborns

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The exposure of pregnant women and young children to environmental pollutants is an ongoing concern of state and local public health departments. Of primary concern is the exposure to lead in lead-based paints, methyl mercury in contaminated fish and cadmium present at mining sites. The feasibility, utility and methodology of using blood spot cards collected for new born health screening purposes was studied for use in conducting routine state-wide surveillance of blood lead, mercury and cadmium levels in infants. Homogeneity of different lots of blank filter paper was examined. Mass measurements (weights) of filter paper punches were taken across three different lots of filter paper. Statistical analysis of the data was performed using one-way ANOVA, which indicated no significant difference in the means of all three lots, but high variances were noted. The three metals were examined in three different lots of filter papers purchased from the manufacturer. The lots had measurable amounts of cadmium and lead, but not mercury. Lead spike values were observed for roughly about 7% of the blank samples, indicating heterogeneous distribution of this metal. Statistical analysis of the data was also performed using a two-way ANOVA calculation with Tukey's pairwise comparisons. The results found that total mean metal loadings across the three lots were different. The concentration of the metals can be different from each other and the concentration of any one metal can differ across lots. Stability at different concentrations of the heavy metals in blood spotted onto filter paper with time and storage conditions was examined. Results indicate acceptable performance for at least 8.5 months for lead (near CDC's concern level) and for mercury (near NRC's concern level). The filter paper and blood spots were analyzed for metals using an acid extraction, followed by analysis using an inductively coupled plasma mass spectrometer (ICP-MS). Blood spot cards were studied from four different states across the Rocky Mountain region. Internal blank punches adjacent to the blood spot and actual dried spot punches from the same card were analyzed simultaneously. The blank punch indicated the amount of contamination present in the blood spot sample. Statistical analysis of the data was performed using MANOVA followed by calculations for each metal separately. This method was found to be suitable for assessing maternal exposure to lead and mercury using residual newborn screening specimens. Additional research into the applicability for cadmium is needed. Because of the intrinsic problem of contamination from the skin surface of capillary blood samples or other internal or extraneous sources, automatic re-analysis of elevated results assures minimal false positives are reported.
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Introduction

The exposure of pregnant women and young children to environmental pollutants is an ongoing concern of state and local public health departments. Of primary concern is the exposure to lead from lead based paints, mercury in contaminated fish and cadmium present at former mining sites. The Environmental Health Laboratory at Centers for Disease Control and Prevention (CDC)'s National Center of Environmental Health measured chemicals in blood and urine samples from a random sample of participants from the National Health and Nutrition Examination Survey (Centers for Disease Control and Prevention, 2005). Mercury, lead and cadmium were among the group of heavy metals measured. Mercury and lead can cause neurodevelopmental effects in the developing fetus. The kidney is the critical target organ for cadmium and cadmium may increase risk for low bone-mineral density (Centers for Disease Control and Prevention, 2005).

The geometric mean blood concentrations for the US population aged 1–5 years for lead was 1.90 μg/dl (1.8–2.1) and 0.318 μg/l (0.268–0.377) for mercury. The 75th percentile concentration for lead was 2.50 μg/dl, whereas that for

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mercury was 0.800 μg/l. When split by gender, female patients had a 75th percentile mercury concentration of 0.800 μg/l, whereas male patients had a corresponding concentration of 0.600 μg/l. The 95th percentile blood concentration for the same population aged 1–5 years for lead was 5.80 μg/dl and 1.90 μg/l for mercury. Again, when split by gender, female patients had a 95th percentile mercury concentration of 2.70 μg/l, whereas male patients had a corresponding concentration of 1.70 μg/l. Of the children tested, 1.6% had blood lead levels greater than or equal to 10 μg/dl, which in 1991, the CDC established as a blood lead concentration of concern in children (Centers for Disease Control and Prevention, 2005).

With regard to mercury, it is noted that a cord blood mercury level of 85 μg/l is associated with a 5% increase in the prevalence of an abnormal Boston Naming Test. The lower 95% confidence bound of 58 μg/l is the concentration associated with neurologic effects in the fetus (Centers for Disease Control and Prevention, 2005). Schober et al. (2003) acknowledge, however, ''To account for uncertainties in exposure measures and variability in individual response to the toxic effects of mercury, the NRC [(National Research Council)] recommended an uncertainty factor of 10 to calculate a reference dose, corresponding to a concentration of 5.8 μg/l mercury in cord blood.'' Hence, the authors of this publication have named this concentration level, ''NRC's concern level'' or just, ''concern level'' or ''level of concern'' with regard to mercury throughout the publication. Similar nomenclature was utilized for lead.

For cadmium, the geometric mean for ages 1 and older was 0.412 μg/l (0.378–0.449). The mean levels of blood cadmium in children ages 1–5 could not be calculated due to the proportion of results below the limit of detection (Centers for Disease Control and Prevention, 2005).

Although most state health departments conduct ongoing surveillance of childhood blood lead levels, there currently are no data on the prevalence of childhood mercury poisoning in children, and no baseline data on lead levels in newborns. Unborn children can be exposed to lead and mercury in the womb. Newborns can also be exposed to lead and mercury in the mother's breast milk (Agency for Toxic Substances and Disease Registry (ATSDR), 1999a, b). Women who consume mercury during pregnancy pass mercury to the unborn child. The level of blood mercury in unborn children and infants can be higher than the blood mercury levels in their mothers (Agency for Toxic Substances and Disease Registry (ATSDR), 1999b). Indeed, it has been noted that while the cord/maternal blood mercury concentration ratio has been assumed (in many instances) to be 1.0 (Rice et al., 2003; Stern and Smith, 2003), Rice et al. (2003) acknowledge that the ratio may be in the range of 1.5 to 2.0, whereas Stern and Smith (2003), in a detailed study, determined a ratio of 1.7 with a standard deviation (SD) of 0.9 and a 95th percentile of 3.4.

The Utah Department of Health, Public Health Laboratory (UPHL) assessed the feasibility, utility and methodology using residual dried blood spots collected for routine newborn screening for metabolic and genetic disorders as a source for testing maternal/fetal blood lead and mercury levels. First blood is blood taken from a newborn within 3 days of birth, usually with a heal stick. Newborn screening is the term used to describe a variety of tests performed from a blood sample taken during the first few hours of a newborn's life. This screening plays an important role in preventive medicine. Moreover, since lead and methylmercury crosses the placenta, blood lead and methylmercury levels in the newborn are comparable to those in the mother. The use of residual dried blood samples from these tests provide an excellent opportunity to estimate the blood lead and mercury levels in statewide populations of newborns and their mothers.

The use of blood samples collected on filter paper for lead testing in children has been studied extensively (Wang and Demshar, 1992; O'Broin, 1993; Schonfeld et al., 1994; Vereby et al., 1995; Wong et al., 1995; Yee and Holtrop, 1997; Stanton et al., 1999; Cizdziel, 2007; Clinical and Laboratory Standards Institute®, 2007). Advantages of using filter paper blood spots have been determined to be the following: a relatively small amount of blood sample, the use of readily available filter paper blood samples, stability and ease of storage and transportation. Some of the expressed concerns include contamination of the filter paper, sample handling, environmental impact, heterogeneous distribution of metals in the filter papers and spreadability of blood through the filter paper, which may bias the results (O'Broin, 1993; Schonfeld et al., 1994; Wong et al., 1995; Mei et al., 2001; Cizdziel, 2007; Clinical and Laboratory Standards Institute®, 2007).

This study used excess neonatal blood spot cards and analyzed them for lead, mercury and cadmium. Over 1000 samples, which include unexposed blank filter papers, blood spots from newborns and internal blanks taken adjacent to the blood spot were evaluated over a period of 3 years. Samples were obtained from Utah and three other states in the Rocky Mountain region of the United States. Some of the unique aspects of this study were blood spots from neighboring states, which provided inter-state data from geographically similar states, and simultaneous determination of metal concentration in both dried blood spot and internal blank allowed inferences to be made regarding contamination for each sample along with state-to-state contamination.

This study presents (1) examination of a single physical parameter, allowing inferences to made regarding intra- and inter-lot homogeneity of filter papers used for blood collection; (2) analysis of the extent of contamination in

unused filter paper as a control study; (3) analysis of pairs of dried blood punches and internal blanks; (4) performance of the analytical method; (5) evaluation of storage conditions with time and temperature and (6) statistical analysis of data and interpretation of the results.

Methods

Filter Paper Punch Mass Distribution

The mass distribution within filter paper cards was determined from two lots of Grade 903 filter paper cards and one additional lot (BFC 180) of filter paper cards. Cards from each lot were selected at random using numbers generated from an online random number generator (Research Radomizer; www.radomizer.org/form.htm). The order in which the cards were punched was also randomized as was the printed circle/area selected and weighed. A total of 20 punches (each $\frac{1}{4}$ inches (6.35 mm) in diameter) were made from several cards from each lot into a tared, clean 15-ml polypropylene centrifuge tube and weighed on a Mettler Toledo AX205 DeltaRange® balance (Mettler-Toledo Inc., Columbus, OH, USA). Masses were determined to nearest 0.1 mg. Mass determinations were performed on two different days so that the inter-day variance component would be included within the variance calculations.

Extraction Procedure

Filter paper punches, each $\frac{1}{4}$ inches (6.35 mm) in diameter, were extracted for analysis of heavy metals. The extraction procedure was applied to punches from unexposed blank filter paper, internal card blanks and filter paper containing the actual dried blood sample. Unexposed blank filter paper is defined as filter paper material obtained from the manufacturer, but has had minimal environmental exposure. Internal card blanks are those blank punches that are adjacent to the actual blood samples. These punches are assumed to have been exposed to the same environmental conditions as the actual blood samples, and hence are used to assess extraneous environmental contamination from the hospital, contamination during transit to the laboratory, storage contamination and contamination during laboratory handling. The authors note, in passing, however, that random contamination may also occur within the blood spot itself. Thus, the internal blank punch near the blood spot will only estimate some, and hence, in many instances, not the full extent, of the contamination.

Four punches (two sets in duplicate) were made from a blood spot card directly into 15-ml polypropylene tubes (VWR International Inc., San Diego, CA, USA). One set of punches was from the card adjacent to the newborn's blood sample spot (internal blank) and served as an indicator for overall environmental contamination of the card. The other set of was from a portion of the newborn's blood sample. In a consultation with the Minnesota Department of Health and followed by UPHL's own verification calculations, it was found that each punch contains 11.5 μl of blood (the actual calculated value by the authors was 11.44 μl, assuming homogenous spreading of the blood through the filter paper). This value (11.5 μl) was used for all calculated data presented in this study. Furthermore, this value was used by the UPHL in its participation in the Wisconsin State Laboratory of Hygiene proficiency-testing (PT) program for filter paper blood lead over the past three years. The fact that the UPHL has passed every testing event utilizing said value further attests its accuracy.

An empty 15-ml polypropylene tube from the same lot as the other samples tubes was used as a tube control. This ''blank'' tube was filled with the same extraction solution as the tubes containing the actual samples and carried through the entire extraction procedure to assess contamination from the actual procedure.

The extraction solution was composed of a 2% double-distilled hydrochloric acid solution (GFS Chemicals®, Columbus, OH, USA) containing 0.05% 2-mercaptoethanol (Fluka, Milwaukee, WI), 0.001% L-cysteine (Fluka) and 10 p.p.b. iridium (Ir) and rhodium (Rh) (Spex Industries Inc., Edison, NJ). A 1.5-ml volume of the solution was added to each tube and then vortexed for 15 min. The tubes were then allowed to stand for overnight (about 16–18 h), and then vortexed for another 15 min, and finally centrifuged for 5 min at 5000 r.p.m. in an Eppendorf 5804 centrifuge (Brinkman Instruments Inc., Westbury, NY). The tubes were then placed into the autosampler of the inductively coupled plasma mass spectrometer (ICP-MS) for analysis. The autosampler contains a special plastic cover box designed and built by the UPHL to minimize environmental contamination of the extracted samples.

Quality Control Sample Preparation

Reconstituted reference material (RRM) samples were prepared from a freeze-dried, human whole-blood toxicology control (level 1, lot number 9081) purchased from Utak Laboratories Inc. (Valencia, CA). The material had a verified lead mean value of 2.3 μg/dl, with an expected range of 2.0–2.5 μg/dl. The material also had a verified mean mercury value of 1.20 μg/l, with an expected range of 1.02–1.38 μg/l. The dried reference material was stored at 2–8°C and reconstituted by adding 3 ml of 18-MΩ water with a volumetric pipette (Rainin, Oakland, CA), vortexing gently for 5–10 min and then allowing 1 h for equilibration and subsequent warming to room temperature. The reconstituted control was then spotted dropwise with a pasture pipette onto Grade 903, lot W011 filter paper. The blood was added until the dotted, printed circle was filled, which corresponds to a total blood volume of about 75 μl (Clinical and Laboratory Standards Institute®, 2007). This value has also been calibrated during the present study. The spotted filter paper

cards were allowed to dry for several hours. The spotted filter paper cards were then placed in TearZone Safeguard Specimen bags (VWR International Inc.), stored under refrigerated conditions and analyzed in the same manner as patient samples. Two sets of RRM internal blank and blood punch pairs were analyzed after every 10 sample pairs to ensure proper quality control of the analysis. It should be noted that although patient samples were stored with a desiccant, these samples were not due to the rate at which they were used. Specifically, these samples were consumed with such rapidity that storage time was minimal.

Additional quality control samples were prepared using whole human blood provided by the Priority Metals Quality Assessment Scheme (PMQAS, Québec, Canada) as part of the PT program at the CDC. The CDC PT blood sample (PMQAS 0719) was stored at refrigeration temperatures of 2 to 8°C. The sample was vortexed immediately before being spotted onto Grade 903, lot W011 filter paper and stored in the same manner as the RRM filter paper samples. The values assigned to this sample were 26.8 μg/dl lead and 4.01 μg/l mercury. These values are the medians of all values reported by the participating laboratories. The authors of this publication note that this central tendency measurement (as opposed to the arithmetic mean) was probably used because of its robustness in response to outlier observations and is normally used with skewed distributions. Should the observations be normally distributed however, the arithmetic mean and median values are equal to each other. This QC sample was analyzed approximately on a once-a-week basis and was used as a mercury medium concentration quality control sample. These samples were also stored without desiccation, as it was unclear what, if any, impact moisture might have on metal concentrations. Indeed, the authors note that while concerns have been raised in this regard "…for DNA analysis…for research and confirmation of other conditions…[,]" (Clinical and Laboratory Standards Institute®, 2007), presumably due to microbial degradation, little information seems to be available that would suggest direct impact on metal concentrations.

Preparation of Storage Time and Conditions Samples

Additional CDC PT samples PMQAS 0625 (2.03 μg/dl lead, 0.71 μg/l mercury and 0.62 μg/l cadmium) and PMQAS 0628 (16.6 μg/dl lead, 2.60 μg/l mercury and 5.36 μg/l cadmium) were spotted onto Grade 903, lot W031 filter paper, using the same procedure as described for the quality control samples. Ten internal blank and dried blood punch pairs were analyzed to obtain initial mean values and recoveries. The remaining filter paper cards were divided and placed in TearZone Safeguard Specimen bags (VWR International Inc.). Half of the samples were then stored in a well-lighted room under room temperature and the remaining samples stored were under refrigeration in the dark. Punches from these cards were taken periodically and analyzed to test the effect of storage condition and time upon the dried blood spots. Like the previously mentioned samples and for the same reason, these samples were also stored without desiccation.

Analysis

The samples were analyzed using an Elan DRC II ICP-MS (PerkinElmer, Shelton, CT, USA) equipped with a Meinhard nebulizer and a quartz cyclonic spray chamber. The dynamic reaction cell (DRC) was not used for this work. For lead, the three isotopes scanned and summed were m/z 206, 207 and 208. For mercury, the three isotopes analyzed and summed were m/z 199, 200 and 202, whereas for cadmium, only the isotope with m/z 111 was analyzed. Arithmetic isobaric correction equations were used where appropriate and two replicate readings were taken for each mass. For each element, a calibration curve was constructed using aqueous-based samples and calculated using unweighted ordinary linear regression methods and the intercepts were not forced through the origin. Lastly, it should be noted that all mercury results represent total (organic and inorganic) mercury concentrations.

Statistical Analyses

One-way ANOVA calculations for filter paper punch masses were performed using Minitab Statistical software, version 13.31. Two-way ANOVA with Tukey's pairwise comparison calculations for the unexposed lots of Grade 903 blank filter paper was performed by fitting the data into a general linear model (GLM), using the said procedure with Minitab Statistical software, version 13.31. Those observations deemed outliers were excluded from the calculations. Furthermore, as all mercury concentrations were below the method detection limit (MDL) of 0.65 μg/l, values for mercury were also excluded from the calculations. Therefore, the factor of lot had three levels (W011, W031 and W041) and the factor of metal had two levels (Pb and Cd). Each lot had multiple replicates run on different days so as to include the inter-day variance component within the variance estimates; however, not all the lots were analyzed across the same days, and so an analysis examining the effect of analysis date on this particular data set was not performed. Additionally, it should be noted that although lead is traditionally reported in units of μg/dl and cadmium and mercury in units of μg/l, all units had to be homogeneous allowing valid statistical inferences. Hence, although every effort had been made to preserve this traditional reporting format, deviations were made when deemed necessary.

The effect of analysis date ("date bias" as connoted by the authors of the publication) was also investigated for this particular type of analytical procedure. Specifically, any day-to-day variability induced by different levels of instrumental performance may be present and is known as a "random effect." A random effect is one that is not part of the

experimental design, but is intrinsically part of the overall analytical procedure. The random effect of date induces a variance component such that measured values across different days have two variance sources. One variance source is the variance present for all measurements taken on a particular day, while the second source of variance is induced when measurements are taken on different days. (Ideally, the latter would be non-significant and the former homogeneous on different days.) If the latter variance component is indeed induced and is significant, it may be important to include this measure in all hypotheses testing where the variability would alter the observed values. Determination of this variance source was accomplished by using variance-component analysis. The measured values used for this particular analysis were all taken in late spring over a 2-month period. Consequently, the time period does not allow the effects of seasons, for example, summer, fall and winter, on the reproducibility of the analytical procedure. This study presents date bias data only for lead and cadmium. Date bias for mercury is not presented, but rather, was used to modify the analytical method for mercury.

In determining if there was a difference in metal concentrations across states, a multivariate analysis of variance (MANOVA) was performed. In effect, this determines whether there is a difference in any linear combinations of the measured values across states. The test procedures included Wilk's Lambda, Pillai's Trace, Hotelling–Lawley Trace and Roy's Greatest Root.

Correlation charts of the data were prepared and Pearson correlation coefficients were calculated to determine whether there exists a correlation between internal blank punches and the corresponding dried blood punches. If a correlation does exist, it simply means that as internal blank values rise, there is a corresponding tendency that dried blood spots values will also be raised. Simple bivariate regression analyses were also performed for each of the metals separately.

Results

Analysis of Blank Unexposed Filter Paper

Box plots of the mass data are presented in Figure 1. The red dots within the plots are the mean values, while the lines within the boxes are the calculated median values. The box bottoms and tops represent the first and third quartiles, respectively. The means, SDs and one-way ANOVA results are tabulated in Table 1a, while raw data are recorded in Table 1b.

Inclusion of outliers within the box plots (denoted by *) suggests symmetrical or near-symmetrical distributions about the means, indicative of normal or nearly normal distributions. All mean values reported in Table 1a (59, 60 and 62 mg) are close to each other and could not be statistically differentiated ($P = 0.509$). The spread of the data can be noted in Table 1b.

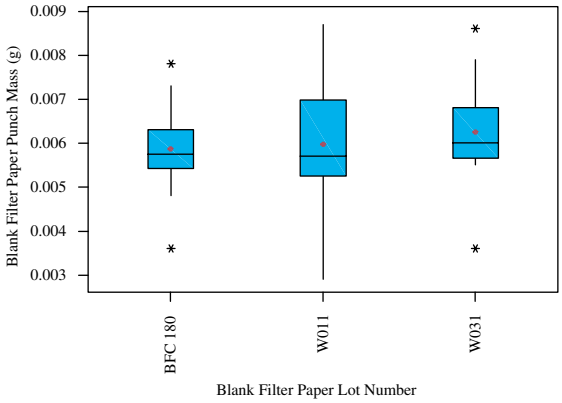


Figure 1. Box plots of observed punch masses stratified by filter paper lot.

Figure 2 are images of various blood spot specimens at the UPHL and are likely to be exemplary of various specimen types that might be encountered in practice. Inclusive within this figure is an example of a specimen that is amenable to this method and examples that are expected to contribute to the production of variable results.

Table 2 reports the results of the analysis of three different lots of unexposed Grade 903 filter paper for lead, mercury and cadmium. Unexposed in this particular case means cards that were shipped directly to the UPHL and were exposed (in theory) only to the laboratory environment and not other, extraneous environments, such as that of a hospital. This procedure was designed to provide the best estimates of contamination that is already in and on the cards and should include only minimal environmental contamination.

Tabulated within this table are the mean values for each metal within each lot, the total number of observation made for each metal in each lot, and the number of outliers observed for each metal as determined by Grubbs' test for outlying observations (Taylor, 1987), and application of the ± 3 SD criteria. It should be noted that the 3 and 4 outliers reported for lots W011 and W041, respectively, were all high values, while likewise, 3 of the 4 outliers reported for lot W031 were also high.

Two-way ANOVA calculations (after all units were homogenized) revealed that the effect of lot was statistically significant ($P < 0.001$), the effect of metal was statistically significant ($P < 0.001$), and the effect of metal*lot was also statistically significant ($P < 0.001$). Figure 3 illustrates the main effects plots for lot and metal. It will be noted that the red dashed line across both main effects plots is the least-squares (LS) grand mean or the LS mean of all observed cell values. Furthermore, the plot depicts LS mean values for each level of each factor. It should be noted that LS means are different from data means in that LS means are the expected mean values that would be obtained if the data set were balanced (i.e., each cell of the model contained an equal

Table 1a. Parameter estimates and one-way ANOVA results for filter paper punch masses stratified by lots.

Filter paper lot identification	Mean mass (g)[a]	Standard deviation	f[b]	Prob > |t| (P-value)[b]
BFC 180	0.0059	0.0009	0.68	0.509
Grade 903 W011	0.0060	0.0012		
Grade 903 W031	0.0062	0.0010		

[a]$n = 20$ for each mean determination.
[b]Values computed using a pooled standard deviation of 0.0011.

Table 1b. Raw mass data of blank filter paper punches.

BFC 180 (mass, g)	Lot W011 (mass, g)	Lot W031 (mass, g)
0.0057	0.0059	0.0079
0.0056	0.0054	0.0086
0.0036	0.0051	0.0055
0.0062	0.0066	0.0074
0.0063	0.0074	0.0036
0.0064	0.0087	0.0065
0.0065	0.0060	0.0059
0.0058	0.0056	0.0069
0.0057	0.0064	0.0060
0.0078	0.0071	0.0072
0.0048	0.0073	0.0060
0.0054	0.0054	0.0060
0.0054	0.0056	0.0062
0.0073	0.0075	0.0056
0.0056	0.0029	0.0056
0.0054	0.0058	0.0059
0.0063	0.0052	0.0065
0.0059	0.0054	0.0063
0.0061	0.0052	0.0055
0.0055	0.0049	0.0058

number of observations). It should be noted further, however, that although the LS means are different from the data means in theory, in practice they were nearly identical, having values that differed $< 0.1\ \mu$g/l. Figure 4 shows the interaction plot of metal*lot for the 2 metals across the 3 lots of filter paper.

Cadmium

Table 3 lists the variance component induced by different analysis days and the associated 95% confidence interval for cadmium for the internal blanks. It should be noted that the variance component induced by different analysis days was not significant for the blood spot samples and hence, not listed. Included in this table are the mean value differences (blood spot−internal blank) and standard errors for each state for said metal. It should be noted that for the purposes of formality, using MANOVA calculations, Wilk's Lambda indicated there was a significant difference ($\alpha = 0.001$) in metal concentrations across states for the difference values. Pillai's Trace, Hotelling–Lawley Trace and Roy's Great Root test procedures all resulted in the same conclusion, specifically that there was an overall significant difference in the metal concentration vectors across states.

Figure 5 shows the correlation chart for the cadmium values obtained from both the dried blood spots and internal card blanks with the corresponding 50%, 90%, 95% and 99% bivariate ellipses. Table 4 lists the bivariate regression parameter estimates and Table 5 lists the values obtained for cadmium in proficiency testing samples by whole blood and filter paper analyses.

Lead

Listed in Table 6 is the variance component induced by analysis date and the associated 95% confidence interval for lead in the internal blanks. It should be noted that the upper limit of the confidence interval is essentially non-calculable and the variance component induced by analysis date for the dried blood spots was not statistically different from zero and hence, not listed. Also tabulated in the table are the mean value differences (blood spot−internal blank) and associated standard errors for each state. What is particularly interesting to note is that all difference values are statistically different from zero with the exception of state 1.

Shown in Figure 6 is the correlation plot for the dried blood spots and corresponding internal blank values. Tabulated in Table 7 are the point bias estimates for lead calculated from the literature, using the procedure of Martin (2000), between dried filter paper blood spots and whole venous blood. Table 8 contains the bivariate regression parameter estimates and Table 9 the values obtained for lead in proficiency testing samples by whole blood and filter paper analyses.

Table 10 lists the results from the storage time and condition study and Figure 7 contains a graphical representation of said data. Table 11a contains the summary QC data covering approximately a 1.5 month time period along with an estimate of the MDL of this method. The MDL was estimated as three times the SD for the lot W031 data listed in Table 2. It is interesting to note that irrespective of which lot data was utilized, all would have produced similar MDL estimates.

Table 11b contains the summary data for 18 patient samples, analyzed in duplicate, covering a 1 month time period to demonstrate the reproducibility capability of the method for lead.

Mercury

Shown in Figure 8 is the correlation plot for mercury for the internal blanks and blood samples. The authors have stretched this picture to first, allow closer examination of the data points, and second, to accentuate the circular nature of the ellipses. Table 12 contains the bivariate regression

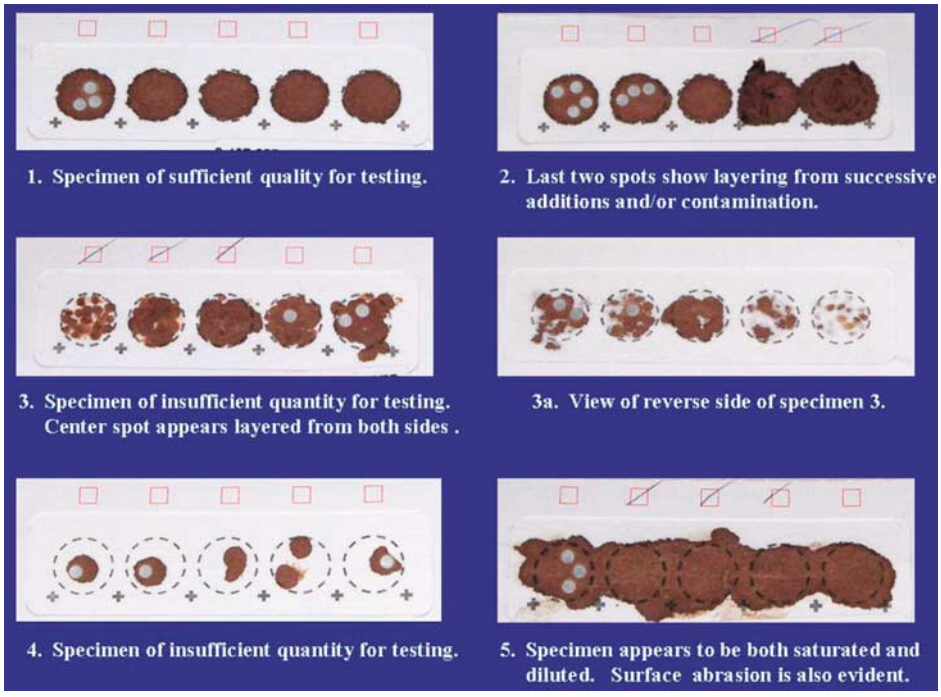


Figure 2. Photographs of various Utah blood spot specimens observed by the UPHL.

Table 2. Grade 903 blank filter paper metal concentration determinations stratified by lot.

Element	Lot number	Number of observations	Number of outliers[a]	Mean blood equivalent concentration[b]
Lead	W011	55	3	$0.41 \pm 0.10\,\mu g/dl$
Mercury	W011	55	0	$<0.65\,\mu g/l$
Cadmium	W011	55	0	$1.41 \pm 0.25\,\mu g/l$
Lead	W031	50	4	$0.82 \pm 0.12\,\mu g/dl$
Mercury	W031	50	1	$<0.65\,\mu g/l$
Cadmium	W031	50	1	$2.51 \pm 0.26\,\mu g/l$
Lead	W041	55	4	$0.32 \pm 0.07\,\mu g/dl$
Mercury	W041	55	0	$<0.65\,\mu g/l$
Cadmium	W041	55	1	$2.35 \pm 0.28\,\mu g/l$

[a]Outliers defined as those observations that were removed as determined by Grubbs' test for outlying observations or those that exceed ± 3 SDs from the mean.
[b]Mean values determined with outliers excluded. Values reported as $\mu \pm 1\sigma$.

parameter estimates and Table 13 the values obtained for mercury in proficiency testing samples by whole blood and filter paper analyses.

Table 14 lists the results from the storage time and condition study and Figure 9 contains a graphical representation of said results. Table 15a contains the summary QC data covering approximately a 1.5-month time period along with an estimate of the method detection limit of this method. This particular MDL was estimated by taking a subset of the 124 observations listed in Table 15a for the RRM data. Outliers were removed resulting in final subset of 84 observations. The SD of these 84 observations was then estimated and the MDL, defined as three times the SD, was calculated. The detection level was further confirmed using aqueous standards near and at the MDL level.

Table 15b contains the summary data for 18 patient samples, analyzed in duplicate, covering a 1 month time period to demonstrate the reproducibility of the method for mercury.

Discussion

Blank Filter Paper Evaluation

Although the mean masses are close to each other and cannot be statistically differentiated (illustrated in Figure 1 and

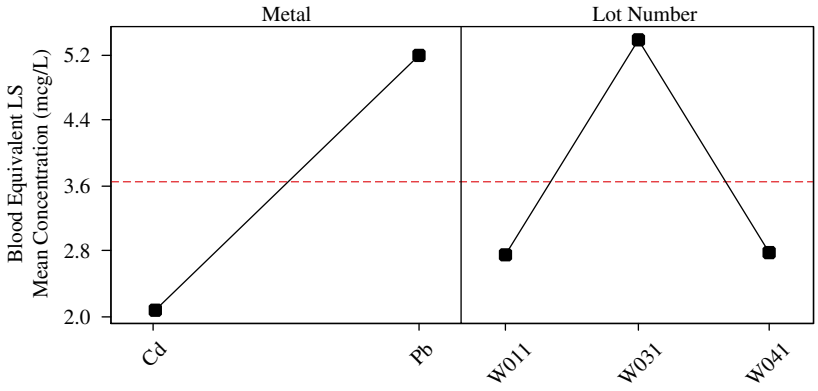
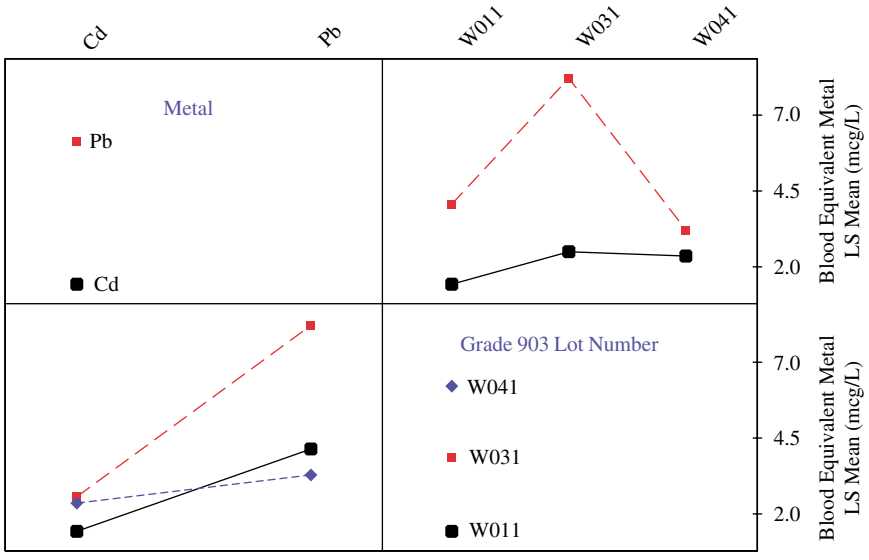


Figure 3. Main effects plots of lot and metal for Grade 903 unexposed blank filter paper.



Note: All units are homogeneous allowing for valid statistical inferences.

Figure 4. Interaction plots for the effect of metal*lot for Grade 903 unexposed blank filter paper.

Table 3. Estimates of statistically significant variance components for blank and blood filter paper punches for cadmium and associated mean blood equivalent concentration (dried blood spot−internal blank) for each state.

Element	Blank sample variance component[a]	Blood sample variance component
Cadmium	2.6706 (0.8615, 36.145)	Not significant
State	Mean blood equivalent concentration (μg/l)	Standard error
Utah	−0.38	0.23
State 1	−0.28	0.35
State 2	0.15	0.35
State 3	1.62[b]	0.37

[a]95% Confidence intervals are given in parentheses.
[b]Statistically different from zero with $\alpha = 0.001$.

Table 1a), suggesting homogeneous masses across lots, it is clearly not the case when individual masses are examined (see Table 1b). Indeed, it will be noted that punch masses can range from about 30 to about 85 mg. Thus, if it is assumed that the punch sizes are homogeneous, the densities of the material might differ significantly and hence, the amount of blood that might be contained within each punch might likewise differ significantly. Because it is assumed that the amount of blood that is contained within each punch is homogeneous, the actual blood volumes that might actually be contained within each punch may be significantly different and hence, act as a source of variance.

Other factors have been identified as impacting the amount of blood that might be contained within each punch. O'Broin (1993), citing other references, included, paper batch-to-batch differences, volume of blood added to the paper and the location of the punch. The work of O'Broin (1993),

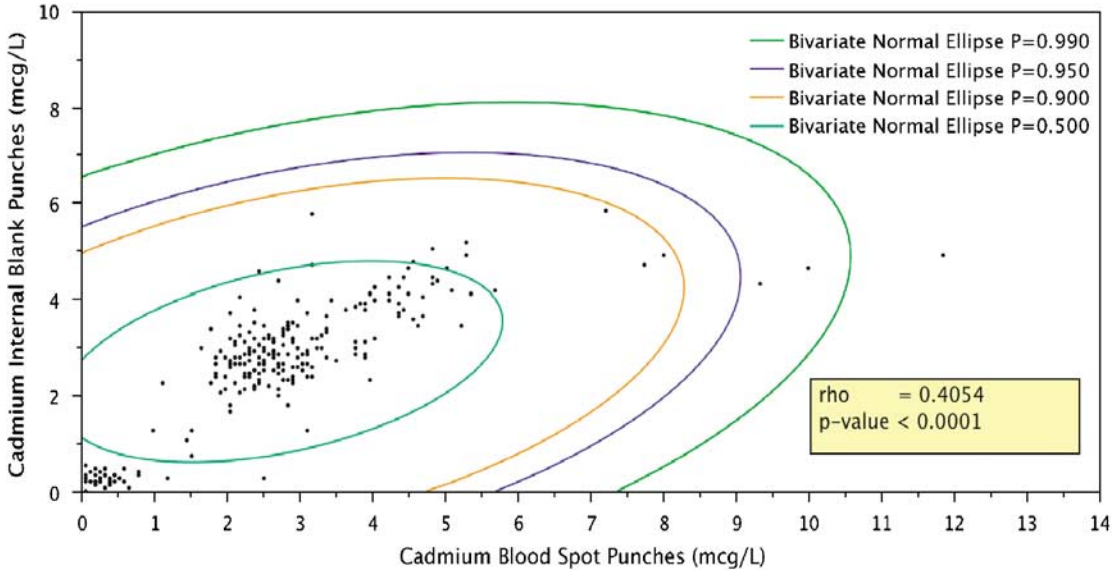


Figure 5. Correlation plot of internal blank punches and dried blood spot punches for cadmium from four Rocky Mountain area states.

Table 4. Bivariate regression parameter estimates for cadmium on blank filter paper punches modeled as a function of dried blood punches (blank punch = intercept + slope × blood punch).

Term	Estimate (μg/l)	Standard error	*t*-ratio	Prob > |*t*| (*P*-value)
Intercept	1.92	0.15	12.76	<0.0001
Slope	0.28	0.04	7.03	<0.0001

Table 5. Cadmium in blood spots compared with CDC whole-blood nationwide PT values.

Element	Proficiency testing blood sample[a]	Nationwide median value (μg/l)	Mean blood spot value obtained by Utah (μg/l)[b]	Percent recovery after background subtraction[c] (%)
Cadmium	PMQAS 0625	0.62	2.84 ± 0.17	53
Cadmium	PMQAS 0628	5.36	7.18 ± 0.23	87

[a]PT blood spotted on Grade 903, lot W031, filter paper.
[b]$n = 10$; mean reported values do not include background subtractions. Values reported as $\mu \pm 1\sigma$.
[c]Value subtracted from mean values were those mean values listed in Table 2 for Grade 903, lot W031 blank filter paper values.

however, was primarily focused on the hematocrit content of the blood and the volume of blood added to the filter paper in relation to, "…the absorbency of blood on paper." Specifically, the work reports decreasing serum volume contained within each $\frac{1}{4}$-inch punch with a concurrent decrease in the hematocrit content. The work further reports that (for the most part) the calculated amount of blood contained within the punches was larger for 100-μl

Table 6. Estimates of statistically significant variance components for blank and blood filter paper punches for lead and associated mean blood equivalent concentration (dried blood spot−internal blank) for each state.

Element	Blank sample variance component[a]	Blood sample variance component
Lead	0.000034 (0.000007, --------)	Not significant
State	Mean blood equivalent concentration (μg/dl)	Standard error
Utah	0.64[b]	0.22
State 1	0.64	0.33
State 2	1.11[c]	0.33
State 3	1.17[b]	0.35

[a]Upper end of confidence interval cannot be calculated.
[b]Statistically different from zero with $\alpha = 0.01$.
[c]Statistically different from zero with $\alpha = 0.001$.

applications of blood relative to 75-μl applications. No replication was reported and hence the amount of error contained within the experiment could not be reported.

In a later, impressive, study, Mei et al. (2001) reported on various factors that might impact blood volume on punches. In apparent contradiction with the O'Broin study, it was (predominately) found that increasing the percentage of hematocrit resulted in decreased serum volumes in $\frac{1}{4}$-inch diameter punches. The study also reported that increasing the volume of blood applied to the filter paper increased the amount of serum in the punch, and hence, the amount of blood that might be contained within the punch. While the authors of this publication are of the opinion that statistical

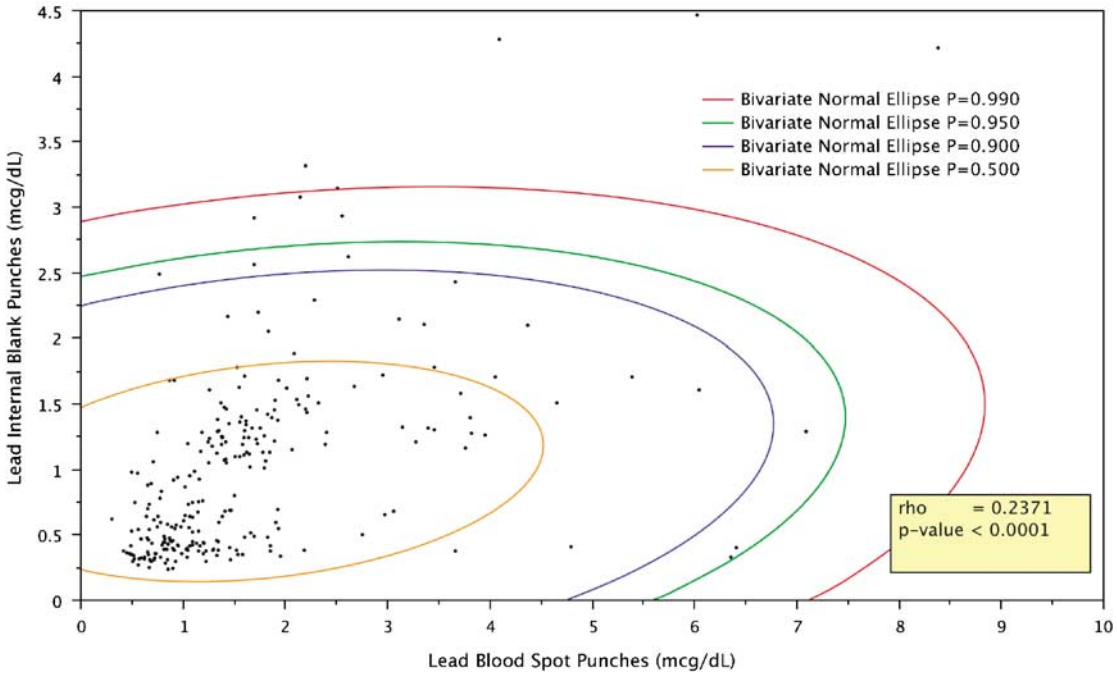


Figure 6. Correlation plot of internal blank punches and dried blood spot punches for lead from four Rocky Mountain area states.

Table 7. Point bias estimates of lead at the 10 μg/dl level of concern for dried blood spots *vs* whole blood calculated from the literature.

Number of observations	Correlation coefficient	Point bias estimate (μg/dl)	Utilization of blanking[a]	Reference
170	0.83[b]	+4.6	Unknown	Schonfeld et al., 1994
167	0.98	+1.8	Unknown[c]	Vereby et al., 1995
30	0.98	+8.0	Yes[d]	Wang and Demshar, 1992
32	0.92	+3.7	No	Wong et al., 1995
119	0.98	+1.2	Yes	Yee and Holtrop, 1997

[a]Standards prepared with filter paper blanks.
[b]Dried filter paper blood and venous blood analyzed by different laboratories.
[c]Laboratory analyzes blank filter paper punches if blood level exceeds 10 μg/dl.
[d]Laboratory used dried blood spot standards.

differentiation across the blood-volume data set might be difficult due to the amount of error contained within said data, the study does however, underscore the importance of collection, drying and storage techniques on the volume of blood that might be contained within punches.

Shown in Figure 2 are photographs of various dried blood spot specimens available to the authors of this publication for study. The $\frac{1}{8}$-inch punches shown in the photographs are punches taken earlier for the testing of other newborn disorders. In the first specimen, the spots fill the circles to the printed borders, indicating (as noted earlier) these spots contain about 75 μl of whole blood. (Clinical and Laboratory Standards Institute®, 2007) Furthermore, the spots appear to be homogeneous, thus indicating that this specimen is appropriate for testing. In the second specimen, the first three spots appear to be appropriate for testing, whereas the latter two, however, appear to be problematic, as indicated by the pen markings above the spots from the UPHL's newborn screening unit. The latter two appear to have been successively layered and/or contaminated, indicating blood spots that are heterogeneous. In the third specimen, too little blood appears to have been collected on the first and latter two spots. Indeed, also pictured is the reverse side of the specimen, indicating very little blood penetrated the filter paper. The center spot, however, appears to have been spotted on both sides, contrary to protocol (Clinical and Laboratory Standards Institute®, 2007), likely resulting in a heterogeneous spot. The fourth specimen simply has too little blood to obtain reliable, $\frac{1}{4}$-inch diameter punches. In the last specimen, too much blood has been applied to the filter paper. Possible "milking" or squeezing of the wound is evident in conjunction with possible dilution of the specimen. Surface abrasion or scratching is also evident. Thus, the specimen might not only be heterogeneous, there also is little

blank area available for testing. All these specimens displayed in the photograph, with the exception of the first, can likely contribute to variability in the amount of blood contained within the spots and hence, contribute significantly to the variability of the testing results.

The observations recorded in Table 2 are particularly interesting. It will be noted that the 3 and 4 out of the 55 observations (5% and 7%) for the lead values in lots W011 and W041, respectively, were deemed high outliers, as were 3 of the 4 out of the 50 observations (6%) in lot W031. These results seem to be in excellent agreement with Wong et al. (1995) who found earlier that "five out of 69 paper blanks (7%) had lead levels exceeding three SDs above the mean…" An important consequence of these outlier observations is that they would tend to suggest that lead concentrations are not homogeneously distributed on or throughout newborn blood filter papers, an assertion evidently supported by Cizdziel (2007) who also reported, "…several anomalously high signal spikes…of the…(filter paper)… blank area(s)…" It is important to note at this point in the study that these spikes seem to be primarily limited to lead and are not so problematic for cadmium or mercury.

The results of the two-way ANOVA calculations using data listed in Table 2 are also particularly revealing. The effect of lot being statistically significant indicates when lot observations are averaged across the metals, at least one of the lots is statistically different when contrasted against the other lots, while likewise the effect of metal, being statistically significant, indicates that when metal values are averaged across the lots at least one metal is statistically different from another or, in this specific case, the lead concentration is statistically different from the cadmium concentration. The main effects plots for both lot and metal are contained within Figure 3.

Table 8. bivariate regression parameter estimates for lead on blank filter paper punches modeled as a function of dried blood punches (blank punch = intercept + slope × blood punch).

Term	Estimate (μg/dl)	Standard error	t-ratio	Prob > |t| (P-value)
Intercept	0.85	0.055	15.29	<0.0001
Slope	0.007	0.002	3.86	0.0001

As illustrated in Figure 3, the LS mean metal concentration (Cd and Pb) of lot W011 was 2.76 μg/l (blood equivalent), the LS mean of lot W031 was 5.37 μg/l and the LS mean of lot W041 was 2.79 μg/l. Tukey's multiple comparisons at the 95% level of confidence indicated lot W031 as being statistically different from lots W011 and W041, but there was insufficient evidence to differentiate lot W011 from lot W041. Hence, in terms of unexposed blank filter paper LS mean metal loadings across both metals, lot W031 was statistically different from lots W011 and W041. The consequence of this find is that it would tend to suggest differences in metal loadings could exist across different lots of filter paper. Lastly, regarding the main effect of metal, the LS mean for blood equivalent lead was 5.20 μg/l, while the mean for blood equivalent cadmium was 2.09 μg/l and these metal concentrations are statistically different from each other when averaged across lots. The consequence of this find is that different metals can exhibit different concentrations. It should be noted that while the above noted lead value of 5.20 μg/l (0.52 μg/dl) is below the method reporting limit (MRL) of 20 μg/l (2.0 μg/dl), it is above the detection limit of 2.7 μg/l (0.27 μg/dl), indicating the results still have merit.

The interaction effect of lot*metal via two-way ANOVA calculations was also found to be statistically significant and the full interaction effects plots are displayed as Figure 4. (It will be noted that the plot in the lower left side of the figure depicts three lines that are not parallel, indicating indeed there is an interactive effect.) The significant interactive effect essentially means (in consequence for this study) that at least one LS metal mean for one lot is statistically different from the metal LS mean, for the same metal, from another lot. As illustrated in Figure 4, the blood equivalent lead LS mean concentrations for lots W011, W031 and W041 were 4.12, 8.24 and 3.24 μg/l (0.41, 0.82 and 0.32 μg/dl), respectively, and furthermore, Tukey's multiple comparisons at the 95% confidence level indicate all three LS means are statistically different from each other ($P<0.0001$ for each contrast). Again, while these noted values are below the MRL, they are above the MDL, indicating that lead concentrations across lots may still be statistically different and hence, different in practice. The blood equivalent cadmium LS mean concentrations for filter paper lots W011, W031 and W041 were

Table 9. Lead in blood spots compared with CDC whole-blood nationwide PT values.

Element	Proficiency testing blood sample[a]	Nationwide median value (μg/dl)	Mean blood spot value obtained by Utah (μg/dl)[b]	Percent recovery after background subtraction[c] (%)
Lead	PMQAS 0625	2.03	2.44 ± 0.07	80
Lead	PMQAS 0628	16.6	15.7 ± 0.46	90

[a]PT blood spotted on Grade 903, lot W031, filter paper.
[b]$n = 10$; mean reported values do not include background subtractions. Values reported as $\mu \pm 1\sigma$.
[c]Value subtracted from mean values were those mean values listed in Table 2 for Grade 903, lot W031 blank filter paper values.

Table 10. Effect of storage time and conditions on dried blood spot lead determinations.

Sample and storage condition[a]	Analysis date (2007)	Expected value (μg/dl)	Observed value (μg/dl)[b]	Percent recovery (%)
PMQAS 0625 (original value)	27 February	2.03	2.44	120.1
PMQAS 0625 (room temperature)	25 May	2.03	2.75	135.3
PMQAS 0625 (refrigerated)	25 May	2.03	2.92	143.7
PMQAS 0625 (room temperature)	14 June	2.03	2.63	129.6
PMQAS 0625 (refrigerated)	14 June	2.03	4.29	211.4
PMQAS 0625 (room temperature)[c]	12 September	2.03	2.72	134.0
PMQAS 0625 (refrigerated)[c]	12 September	2.03	2.52	124.1
PMQAS 0625 (room temperature)[d]	14 September	2.03	2.31	113.8
PMQAS 0625 (refrigerated)[d]	14 September	2.03	2.43	119.7
PMQAS 0625 (room temperature)[e]	15 November	2.03	2.41	118.7
PMQAS 0625 (refrigerated)[e]	15 November	2.03	2.51	123.6
PMQAS 0628 (original value)	27 February	16.6	15.69	94.5
PMQAS 0628 (room temperature)	25 May	16.6	17.53	105.6
PMQAS 0628 (refrigerated)	25 May	16.6	16.10	97.0
PMQAS 0628 (room temperature)	14 June	16.6	17.08	102.9
PMQAS 0628 (refrigerated)	14 June	16.6	15.86	95.5
PMQAS 0628 (room temperature)[f]	12 September	16.6	16.20	97.6
PMQAS 0628 (refrigerated)[f]	12 September	16.6	16.11	97.0
PMQAS 0628 (room temperature)[g]	14 September	16.6	15.40	92.8
PMQAS 0628 (refrigerated)[g]	14 September	16.6	16.04	96.6
PMQAS 0628 (room temperature)[h]	15 November	16.6	16.28	98.1
PMQAS 0628 (refrigerated)[h]	15 November	16.6	15.44	93.0

[a]Samples are CDC PT samples spotted onto Grade 903, lot W031 filter paper. Samples stored without desiccant.
[b]Blank values not subtracted.
[c]Room temperature internal blank and refrigerated internal blank values were 0.12 and 0.13 μg/dl, respectively.
[d]Room temperature internal blank and refrigerated internal blank values were 1.10 and 0.98 μg/dl, respectively.
[e]Room temperature internal blank and refrigerated internal blank values were 0.87 and 0.80 μg/dl, respectively.
[f]Room temperature internal blank and refrigerated internal blank values were 1.53 and 1.23 μg/dl, respectively.
[g]Room temperature internal blank and refrigerated internal blank values were 2.13 and 9.75 μg/dl, respectively.
[h]Room temperature internal blank and refrigerated internal blank values were 1.09 and 0.86 μg/dl, respectively.

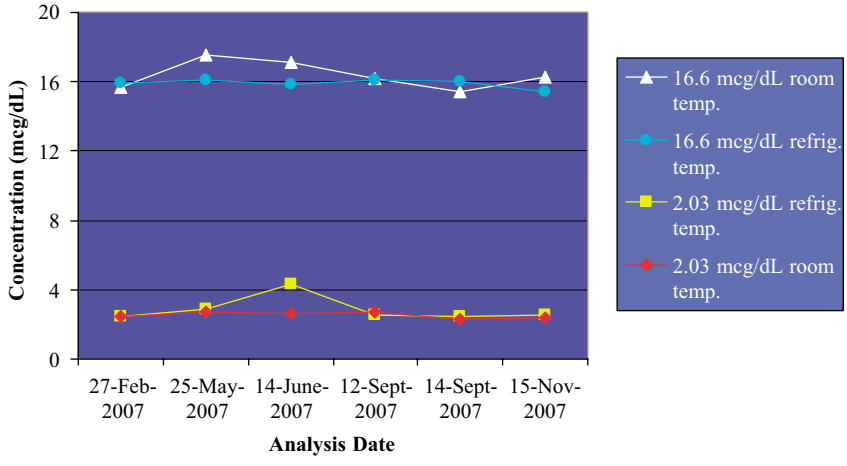


Figure 7. Plot of storage time and conditions on dried blood spot lead determinations.

1.41, 2.51 and 2.35 μg/l, respectively, and Tukey's multiple pairwise comparisons indicate that the LS mean cadmium concentration for lot W011 is statistically different from lots W031 and W041, but there was insufficient evidence to differentiate the cadmium concentrations across lots W031 and W041 ($P = 0.8491$). These results thus suggest that the different metals may have different concentrations across different lots of filter paper.

Table 11a. Performance summary for lead QC samples covering a 1.5-month time period.

Sample	N	Date range	No. of runs	Expected value (μg/dl)[a]	Mean value obtained (μg/dl)[b]	Percent recovery (%)	% RSD
RRM blood spotted on filter paper	126	18 April–08 June	15	2.3	2.39 ± 0.34	103.8	14.4
Aqueous check standard 1	8	18–24 April	2	0.13	0.14 ± 0.005	105.3	3.4
Aqueous check standard 2	53	19 April–08 June	12	0.33	0.35 ± 0.03	107.2	8.7
CDC PT blood on filter paper[c]	5	10 May–13 June	4	26.8	24.4 ± 1.49	91.1	6.1
Method detection limit (MDL) estimate = 0.36 μg/dl, based on three times the 0.12 μg/dl SD listed in Table 2.							
Method reporting limit (MRL) estimate = 2.0 μg/dl, based on recovery experiments at 2.0 μg/dl spike recoveries (see Table 9).							

[a]Expected range for lead is 2.0–2.5 μg/dl. Reconstituted blood spotted onto lot W011 filter paper.
[b]Blank values not subtracted. Values reported as $\mu \pm 1\sigma$.
[c]CDC PT Blood Sample PMQAS 0719. Blood spotted onto Grade 903, lot W011 filter paper.

Table 11b. Performance summary of 18 patient duplicate samples for lead covering a 1-month time period.

Sample number	Internal blank (μg/dl)	Dried blood spot (μg/dl)	Internal blank dup. (μg/dl)	Dried blood spot dup. (μg/dl)
1	0.32	0.65	0.36	0.79
2	0.77	1.26	2.06	1.10
3	0.32	1.00	0.49	0.87
4	0.41	0.81	0.34	0.86
5	0.46	0.78	0.34	0.69
6	0.43	0.78	0.52	2.10
7	0.40	0.84	0.44	0.80
8	0.55	0.80	0.69	0.90
9	0.34	1.26	0.40	1.42
10	0.54	0.71	0.43	0.74
11	1.20	1.25	0.84	1.25
12	0.18	0.71	0.16	0.72
13	0.27	0.91	0.24	2.10
14	0.46	0.77	0.46	1.07
15	0.37	0.78	0.38	0.92
16	0.28	0.64	0.40	0.71
17	0.99	1.07	0.80	1.88
18	0.57	1.84	0.63	1.34

Note: Values highlighted in red denote "spike" or "hot spot" values.

Internal Blank and Dried Blood Punch Pair Evaluation

Cadmium It is denoted in Table 3 that the blank samples exhibited a date bias, as indicated by the statistically non-zero variance component estimate of 2.6706. When the blank values are subtracted from the dried spot values, two results are obtained. First, the resulting difference values exhibit date biases that are not statistically different from zero (or, in other words, the date bias disappears), and second, the only statistically significant non-zero difference value was for state 3, indicating something was different with regard to these samples relative to the other state samples. It will also be noted that Utah and state 1 have difference values less than zero. While these values are not statistically different from zero, it does reflect the finding that on many occasions the internal blank values exhibited higher cadmium values relative to their respective dried blood spot pair. The authors are of the opinion that this observation can be explained by considering a non-conventional scenario.

If it is assumed that the cadmium metal was present on the filter paper prior to blood introduction, then it can be reasonable to assume that the introduction of blood onto the filter paper may cover some of the metal-containing active sites on the filter paper and drying in this state, rendering some of the metal sites resistant to the extraction procedure. Hence, the resulting solution from the extraction may not contain a representative sample of the background contamination. Given the fact that the dried blood punches contain blood material, even after overnight extraction, would indeed suggest some sites would be extraction resistant. Thus, upon subtraction, one may, in fact, be subtracting too much, resulting in a biased low blood concentration, or, in the cases of Utah and state 1, negative blood cadmium concentrations. Admittedly, the model is not perfect and is confounded by numerous additional factors, but it has some utility and can be used to explain similar-type observations for lead and mercury.

The correlation plot (see Figure 5) of the internal blank and blood punch pairs indicates that there is a correlation between the pairs, as evidenced by the elongated ellipses and the statistically non-zero Pearson correlation coefficient. The correlation is further illustrated that the slope resulting from the bivariate regression (see Table 4) is also statistically non-zero ($P < 0.0001$). It is also noteworthy that the intercept is also statistically different from zero ($P < 0.0001$), and provides a gross estimate of the overall background contamination for all the state samples, being specifically 1.92 μg/l (Table 4), and is certainly within the range the authors found within the blank, unexposed filter paper, recalling that range being 1.41–2.51 μg/l (see Table 2).

These high background levels can be problematic, however, as illustrated in Table 5. In human blood spiked with 0.62 μg/l cadmium, only a 53% recovery was realized after

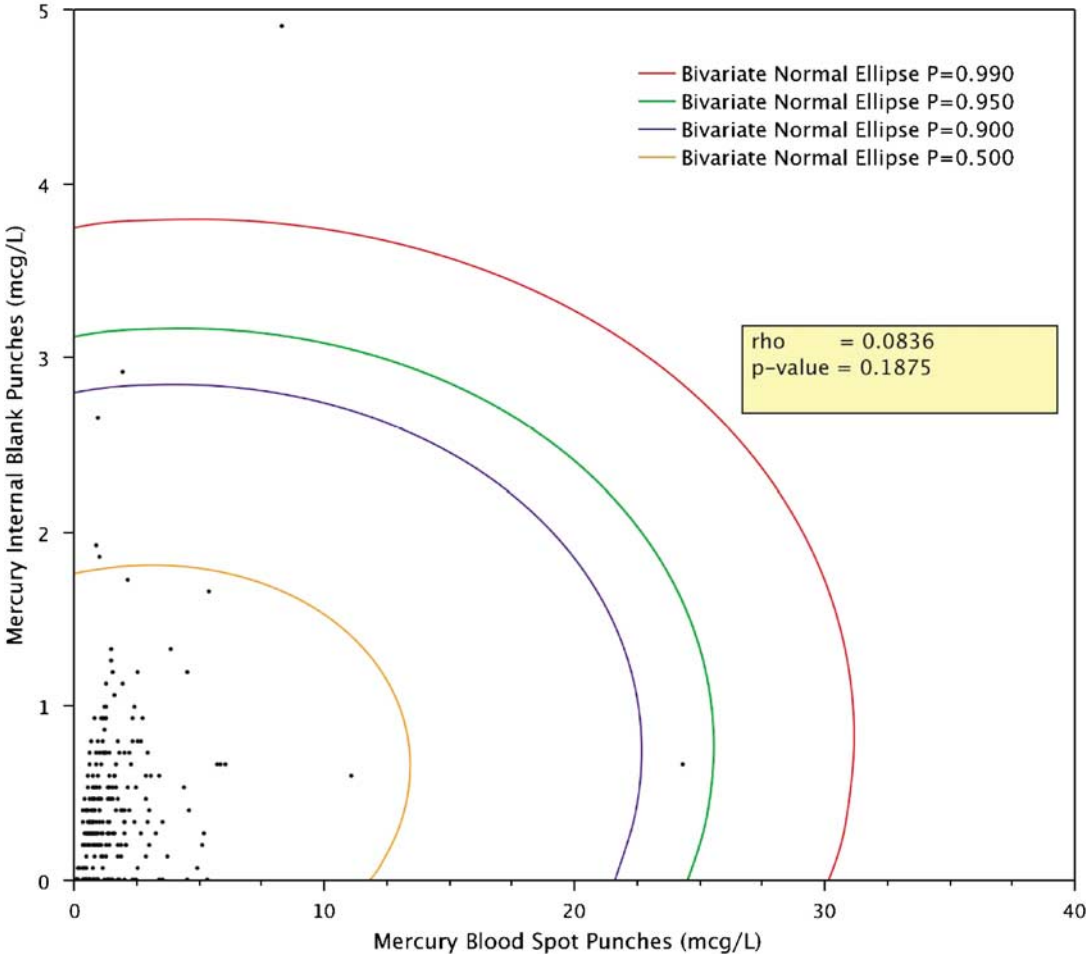


Figure 8. Correlation plot of internal blank punches and dried blood spot punches for mercury from four Rocky Mountain area states.

Table 12. Bivariate regression parameter estimates for mercury on blank filter paper punches modeled as a function of dried blood punches (blank punch = intercept + slope × blood punch).

Term	Estimate (μg/l)	Standard error	*t*-ratio	Prob > |*t*| (*P*-value)
Intercept	0.53	0.07	7.60	<0.0001
Slope	0.01	0.01	1.32	0.1875

background subtraction. If background subtraction is not used, then the value returned was 2.84 μg/l, which represents a 458% recovery. The situation is improved when the concentration is increased to 5.36 μg/l and a concurrent 87% recovery is realized.

The high background values proved to be problematic in producing reliable values. It was concluded that further research into using this method for cadmium determinations was necessary. Consequently, storage time and conditions studies for this metal were not conducted.

Lead

Background Although statistical analysis indicates (see Table 6) a date bias, upon close examination it is clear that the bias ($\sim$0.006 μg/dl) is insignificant for the purpose of the study goal. All difference values (blood spot−internal blank) listed in Table 6 are statistically non-zero, with the exception of state 1, indicating for the most part the ubiquitous nature of lead. (It should be noted that while both Utah and state 1 have the same mean difference values, the standard error of the latter is larger thus resulting in the null hypothesis not being rejected for that particular mean.) These results suggest that contamination may vary across states.

In examining the effects of background contamination more closely, the authors of this study calculated point bias estimates from data available in the public literature (Wang and Demshar, 1992; Schonfeld et al., 1994; Vereby et al., 1995; Wong et al., 1995; Yee and Holtrop, 1997), and comprise the tabulated values in Table 7. It should be noted that the relatively low correlation coefficient for the first

Table 13. Mercury in blood spots compared with CDC whole-blood nationwide PT values.

Element	Proficiency testing blood sample[a]	Nationwide median value (μg/l)	Mean blood spot value obtained by Utah (μg/l)[b]	Percent recovery[c] (%)
Mercury	PMQAS 0625	0.71	0.71 ± 0.05	100
Mercury	PMQAS 0628	2.6	2.57 ± 0.12	99–100

[a]PT blood spotted on Grade 903, lot W031, filter paper.
[b]$n = 10$; mean reported values do not include background subtractions. Values reported as $\mu \pm 1\sigma$.
[c]Mercury background values not subtracted.

Table 14. Effect of storage time and conditions on dried blood spot mercury determinations.

Sample and storage condition[a]	Analysis date (2007)	Expected value (μg/l)	Observed value (μg/l)[b]	Percent recovery (%)
PMQAS 0625 (original value)	27 February	0.71	0.71	100.0
PMQAS 0625 (room temperature)	25 May	0.71	1.23	173.4
PMQAS 0625 (refrigerated)	25 May	0.71	1.29	181.8
PMQAS 0625 (room temperature)	14 June	0.71	0.73	102.7
PMQAS 0625 (refrigerated)	14 June	0.71	0.82	116.2
PMQAS 0625 (room temperature)[c]	12 September	0.71	1.40	197.2
PMQAS 0625 (refrigerated)[c]	12 September	0.71	0.96	135.2
PMQAS 0625 (room temperature)[d]	14 September	0.71	1.11	156.3
PMQAS 0625 (refrigerated)[d]	14 September	0.71	1.10	154.9
PMQAS 0625 (room temperature)[e]	15 November	0.71	1.58	222.5
PMQAS 0625 (refrigerated)[e]	15 November	0.71	1.24	174.6
PMQAS 0628 (original value)	27 February	2.6	2.57	98.8
PMQAS 0628 (room temperature)	25 May	2.6	3.06	117.7
PMQAS 0628 (refrigerated)	25 May	2.6	2.81	108.1
PMQAS 0628 (room temperature)	14 June	2.6	2.66	102.3
PMQAS 0628 (refrigerated)	14 June	2.6	2.41	92.8
PMQAS 0628 (room temperature)[f]	12 September	2.6	3.04	116.9
PMQAS 0628 (refrigerated)[f]	12 September	2.6	2.87	110.4
PMQAS 0628 (room temperature)[g]	14 September	2.6	2.86	110.0
PMQAS 0628 (refrigerated)[g]	14 September	2.6	2.80	107.7
PMQAS 0628 (room temperature)[h]	15 November	2.6	3.31	127.3
PMQAS 0628 (refrigerated)[h]	15 November	2.6	2.94	113.1

[a]Samples are CDC PT samples spotted onto Grade 903, lot W031 filter paper. Samples stored without desiccant.
[b]Blank values not subtracted.
[c]Room temperature internal blank and refrigerated internal blank values were 0.35 and 0.16 μg/l, respectively.
[d]Room temperature internal blank and refrigerated internal blank values were 0.22 and 0.16 μg/l, respectively.
[e]Room temperature internal blank and refrigerated internal blank values were 0.56 and 0.33 μg/l, respectively.
[f]Room temperature internal blank and refrigerated internal blank values were 0.40 and 0.23 μg/l, respectively.
[g]Room temperature internal blank and refrigerated internal blank values were 0.44 and 0.11 μg/l, respectively.
[h]Room temperature internal blank and refrigerated internal blank values were 0.51 and 0.17 μg/l, respectively.

calculated value likely resulted from the venous blood and dried filter paper spots being analyzed by different laboratories. The results indicate all point bias estimates to be positive and range from 12% to 80% at the 10 μg/dl concern level, even when blanking was employed, thus suggesting background contamination (and in some cases severe contamination) in earlier studies.

Figure 6 shows the correlation plot for the dried blood spot and internal blank pairs from this study. The elongated ellipses indicate a positive correlation between the pairs as does the statistically significant non-zero Pearson correlation coefficient, although the data appear to be quite scattered. The slope resulting from the bivariate regression, also being statistically different from zero ($P = 0.0001$), indicates a correlation. The intercept reveals a very rough background estimate of 0.85 μg/dl for all state samples and is in good agreement with the estimated value of 0.82 μg/dl found in lot W031 of the unexposed filter paper, but considerably higher than the estimated values for unexposed lots W011 and W041 (see Table 2). These results further confirm that with regard to lead, background contamination in patient samples may still be a significant issue.

Table 9, listing the results of PT samples spotted onto filter paper, offers some particularly interesting insights. The blood

containing a median value 2.03 μg/dl lead returned a mean value of 2.44 μg/dl (without background subtraction) or 120% recovery, but when the total background concentration was subtracted, the resulting value was only 1.63 μg/dl, which tabulated to an 80% recovery. This would tend to suggest that too much background may have been subtracted and that indeed, the application of blood onto the filter paper would make some metal-containing sites resistant to extraction. The blood sample containing 16.6 μg/dl lead returned a value of 15.7 μg/dl, which represented a 94.5% recovery without background subtraction. When total background is subtracted, the return value represented a 90% recovery. Although these recoveries are excellent, there is a consistent negative bias. It is understood that losses can occur from spotting, drying, extraction and instrumental analysis of the extraction solution.

The concept of blood covering and masking some metal-containing sites resistant to extraction on filter paper is intriguing and has some merit, and indeed can be used to explain these additional observations. However, this issue requires further study. Given this, in light of the above discussion, the authors of this study (at present) have elected not to use background subtraction as others have suggested (Cizdziel, 2007), but instead recommend that the background data be used to evaluate overall, the extent of background contamination associated with the blood spots. It is recognized other laboratories may choose differently depending upon their individual data quality objectives.

Storage and method performance Table 10 records the results of the storage conditions and times study section. Figure 7 represents the data graphically. An interesting feature of the data is that the low lead concentration samples spiked at 2.03 μg/dl all appear to be high, part of which might be attributable to background. The high value of 4.29 μg/dl value obtained on the !4 June 2007 likely represents an artificial spike value. It should be noted, however, as no internal blank was taken with this sample,

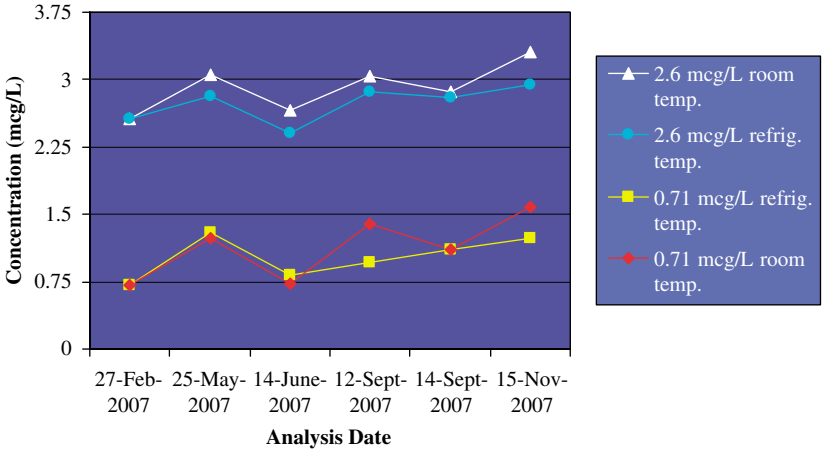


Figure 9. Plot of storage time and conditions on dried blood spot mercury determinations.

Table 15a. Performance summary for mercury QC samples covering a 1.5-month time period.

Sample	*n*	Date range	No. of runs	Expected value (p.p.b.)[a]	Mean value obtained (p.p.b.)[b]	Percent recovery (%)	% RSD
RRM blood spotted on filter paper	124	18 April–08 June	15	1.2	1.08 ± 0.39	87.3	36.9
Aqueous check standard 1	8	18–24 April	2	1.3	1.29 ± 0.05	99.5	3.9
Aqueous check standard 2	53	19 April–08 June	12	3.3	3.29 ± 0.15	99.6	4.5
CDC PT blood on filter paper[c]	5	10 May–13 June	4	4.01	4.03 ± 0.37	100.5	9.2

Method detection limit (MDL) estimate = 0.65 μg/l, based on three times the standard deviation (0.22 μg/l) calculated from a subset ($n = 84$) of the reconstituted blood data with four outliers removed. Two were removed via two iterations using the three SD criteria. The remaining two points were removed by applying Grubbs' test twice allowing a false rejection probability of 10%. The Grubbs' critical value was estimated by linear interpolation. The MDL has been confirmed by using aqueous standards.

Method reporting limit (MRL) estimate = 0.71 μg/l, based on 0.71 μg/l spike recoveries (see Table 13).

[a]Expected range for mercury is 1.02–1.38 μg/l. Reconstituted blood spotted onto lot W011 filter paper.
[b]Blank values not subtracted. Values reported as $\mu \pm 1\sigma$.
[c]CDC PT Blood Sample PMQAS 0719. Blood spotted onto lot W011 filter paper.

Table 15b. Performance summary of 18 patient duplicate samples for mercury covering a 1-month time period.

Sample number	Internal blank (μg/l)	Dried blood spot (μg/l)	Internal blank dup. (μg/l)	Dried blood spot dup. (μg/l)
1	<0.65	<0.65	<0.65	<0.65
2	0.74	1.02	0.56	1.21
3	<0.65	0.99	<0.65	0.93
4	<0.65	<0.65	<0.65	<0.65
5	<0.65	<0.65	<0.65	<0.65
6	<0.65	0.67	0.77	0.64
7	<0.65	0.88	<0.65	0.88
8	<0.65	1.12	<0.65	1.20
9	<0.65	0.63	<0.65	0.77
10	<0.65	0.78	<0.65	1.09
11	<0.65	1.17	<0.65	1.27
12	<0.65	1.27	<0.65	1.25
13	<0.65	1.42	<0.65	1.39
14	<0.65	0.73	<0.65	0.74
15	<0.65	<0.65	<0.65	<0.65
16	<0.65	2.83	<0.65	3.91
17	<0.65	1.90	<0.65	2.56
18	<0.65	<0.65	<0.65	<0.65

Note: Values highlighted in blue, while less than the MDL, are added for comparison purposes only.

further inferences are not plausible. The last measurements obtained after about 8.5 months of storage are both (room temperature and refrigerated) reasonably close to the original value, indicating little additional contamination overall had taken place during the study time. Examination of Figure 7 demonstrates excellent agreement between all observed values with exception of the June 14 data. Furthermore, the nearly flat profiles further strengthen the information conveyed by the numerical values, specifically, the excellent stability of the specimens over the $8\frac{1}{2}$-month study period.

The higher concentration samples prepared at 16.6 μg/dl (nearer the 10 μg/dl level of concern) appear to have been impacted very little, all exhibiting acceptable, albeit slightly biased, low recoveries. This suggests that lead in blood spots are stable for at least 8.5 months whether stored at room temperature or refrigerated. Lastly, because these samples were stored without desiccation, it can be further inferred that for metals determination, such treatment might not be necessary.

Recorded in Table 11a are the performance summaries for the QC samples analyzed in a time period that covers approximately 2 months. The aqueous check standards all had good recoveries. It will be noted that contrary to the findings of Cizdziel (2007), the RRM used with a certified value of 2.3 μg/dl returned an excellent agreement mean value of 2.39 μg/dl. Again, the background values were not subtracted. It will also be noted, however, that a fairly significant amount of error is associated with this sample, being 14.4%RSD, albeit given the low concentration of the metal (near the MRL; see Table 11a), it should not be altogether unexpected nor construed to be unusual. The CDC PT sample at 26.8 μg/dl exhibited a mean recovery of 91.1%.

Recorded in Table 11b are the performance summary results for 18 patient samples analyzed in duplicate over a 1-month period. Overall, the results are comparable with most of the replicates exhibiting relative SDs within a 20% range. The three values highlighted in red are observations believed to be "spike" (unexplained high) values. Out of the 36 total paired observations, this would represent an 8% occurrence, in agreement with 5%–7% outlier observation frequency recorded in Table 2. Notwithstanding the spike values, the method demonstrates good reproducibility. Taken together, all these points indicate overall good performance of the analytical method.

Furthermore, the UPHL has established a policy that when elevated lead values are detected, the samples are automatically re-analyzed to determine if the observed, elevated level was a "spike" value or an actual reflection of lead blood levels. This helps to assure minimal reporting of false positives. It will be noted further that the authors had available to them 2–4 dried blood spots (as depicted in Figure 2) from which to obtain punches, thus allowing for replicate analyses.

Lastly, it should be emphasized that the UPHL has participated in the Wisconsin State Laboratory of Hygiene proficiency testing program for filter paper blood lead for the past three years utilizing the method outlined in this publication. Every testing event has been passed thus demonstrating further the utility and accuracy of this analytical method.

Mercury

Background Early evaluation of the method and statistical analysis indicated a statistically significant date bias in both the internal blank samples and dried blood samples. The estimated date bias for the internal blank samples was only 0.35 μg/l and the date bias for the dried blood samples was about 1.3 μg/l. Consequently, the method was modified to reflect the conditions currently discussed in this paper. Perusal of all subsequent data generated with the modified methodology presented in this study currently suggests minimal date bias. It does not however, definitively preclude its presence and further study of this area is necessitated.

Figure 8, showing the correlation plot of the internal blank values and the corresponding dried blood punch values, features nearly circular ellipses, in contrast to cadmium and lead, which indicates little correlation between the paired values, which is in agreement with the low Pearson correlation coefficient that is not statistically different from zero ($P = 0.1875$). The bivariate regression parameter

estimates likewise indicate little correlation between the internal blank and dried blood spot values in that the slope is not statistically different from zero ($P = 0.1875$) and the intercept, being statistically different from zero, reveals a gross background estimate of about 0.53 μg/l. This value is below our method detection limit.

The returned values for the PT samples (see Table 13) spotted onto filter paper, at concentrations of 0.71 and 2.6 μg/l, all returned recoveries in the 99%–100% range without background subtraction. It should be noted that all internal blank values for these samples were below the MDL. However, the authors have sporadically observed detectable amounts of mercury in internal blank samples of patient samples. Similar to the discussion on lead however, in general, the authors recommend not subtracting background values but utilize the data to evaluate the extent of contamination.

Storage and method performance Tabulated in Table 14 are the results of the storage time and conditions study. Figure 9 depicts the results graphically. The striking feature of the low concentration samples (0.71 μg/l) is that all of the data are biased quite high with exception of the data recorded for 14 June 2007, where values are quite reasonably close to the original value. Given that the internal blank values are quite low, indicating little extraneous contamination, these biased high observations remain unexplainable. The observation for the higher mercury concentration samples (2.6 μg/dl) are likewise, for the most part, biased high, but most of the values are within $\pm$ 10% of the expected value. The results provide little evidence for differentiating between room temperature storage and storage under refrigerated temperatures.

Table 15a lists the performance summaries for the QC samples analyzed over an approximate 2-month time period. The aqueous check standards all demonstrated excellent mean recoveries. The RRM demonstrated an acceptable mean recovery of 87.3%. The large relative standard deviation associated with this mean is not highly unusual given the low concentration of the element. A noteworthy finding is that the CDC PT sample with a concentration of 4.01 μg/l mercury (relatively nearer the level of concern) exhibited excellent recoveries, demonstrating that this methodology is suitable for screening purposes.

Table 15b lists the performance summary results for 18 patient samples analyzed in duplicate for mercury. The results overall are quite comparable. The values listed in blue, while being below the MDL (0.65 μg/l), have been listed to demonstrate the reproducibility of this method. For example, sample 6 had an original concentration value of 0.67 μg/l. The duplicate analysis produced a value of 0.64 μg/l, below the MDL of 0.65 μg/l but certainly very close to the original value. These results overall demonstrate that this method can produce reasonably reproducible results for mercury.

Lastly, like lead, the UPHL has established a policy that when elevated mercury values are detected, the samples are automatically re-analyzed to determine if the observed, elevated level was a ''spike'' value or an actual reflection of mercury blood levels. Again, this helps assure minimal reporting of false positives.

In summary, the study found that additional work needs to be conducted for cadmium. In all samples studied, which includes both unexposed filter paper and patient samples, the level of contamination for mercury was found to be significantly smaller than those observed for lead. Possible heterogeneous distribution of the metals (lead and mercury) was found, leading to unexplained ''spike values.'' However, the frequency of observed spikes was lower for mercury relative to lead. The study of newborn blood spot cards from the four different states indicates that contamination can vary across states. Lead and mercury values near the levels of concern were found to be stable in dried blood spots for up to 7 months. The method was found to be reproducible for both lead and mercury.

It is recommended that simultaneous analysis of both internal blanks and dried blood spots be performed. The blanks can serve as a guide to determine the extent of possible contamination although it can be confounded by ''spike'' values. Overall, the method was found to be suitable for routine screening of newborns for both lead and mercury. Automatic re-analysis of elevated samples assures minimal false positives is reported.

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