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Feasibility of Lead Exposure Assessment in Blood Spots using Energy-Dispersive X-ray Fluorescence

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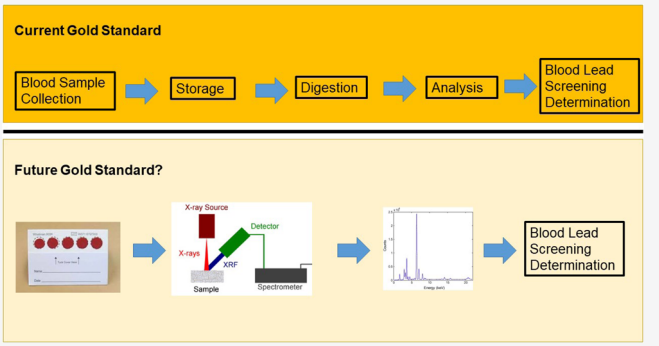
ABSTRACT: Collecting blood spots from newborns is a common procedure used to diagnose multiple health conditions. Fingerstick blood samples are routinely collected from children to diagnose elevated blood lead levels. In our study, we wanted to test the feasibility of using a high-power energy-dispersive X-ray fluorescence (EDXRF) device to accurately measure the concentration of lead in blood spots. We created spotted standards of a known concentration of lead on a filter paper at different volumes and concentrations. We determined the detection limit for lead through repeated measurements of our standards and calibration line slopes. We also tested the variability of the measured lead concentration across procedures and spotted blood volumes and found no significant additions to uncertainty in measurements. Finally, we compared blood lead concentrations measured by EDXRF and atomic absorption spectroscopy (AAS) and found EDXRF to be a significant predictor of blood lead (n = 22, R = 0.98, p value <0.001) with an average detection limit of 1.7 μg/dL of blood lead. This detection limit is similar to that of the AAS technique, which is commonly used in clinical testing laboratories for blood lead surveillance. These findings provide a proof of concept that blood spots measured by EDXRF may be used as a surveillance tool for lead exposure, even at elevated blood lead levels of 2−3 μg/dL.



1. INTRODUCTION

Accurately measuring metals in biological tissues is an essential part of environmental and occupational monitoring efforts and health studies. Blood samples are a primary biomarker of exposure. Levels of metals in blood in children have been widely measured since the 1970s with increasing emphasis beginning in the 1990s across the United States as part of standard lead surveillance.[1] The gold standard for evaluating children's blood lead levels is a venipuncture sample.[2] After collection, this sample is analyzed using some form of spectroscopy such as atomic absorption spectroscopy (AAS) or an inductively coupled plasma (ICP)-based method.

Graphite furnace atomic absorption spectroscopy (GFAAS) has been used for decades as the standard for metal measurements in blood, but AAS can only assess one metal at a time and has a practical limit of detection of about 1−2 μg/dL.[3] Inductively coupled plasma−mass spectrometry (ICP-MS) has largely replaced AAS for blood metal measurements as it has a consistently lower detection limit, in the parts per trillion range with current instrumentation, and multielement detection capabilities.[4] AAS and ICP-MS techniques requir-etake a substantial amount of time with typical sample read times (~3−5 min) only accounting for a small portion of the total time for sample preparation, calibration standards (run between samples), and quality control steps all adding a significant amount of time to the process that can take days in total. In addition, both AAS and ICP-MS require substantial laboratory instrumentation for blood collection, storage, and analysis.

Methods that simplify analysis of samples collected in the field do exist. For example, anodic stripping voltammetry can be conducted in the field on capillary blood,[5] but this has a detection limit of 2−3 μg/dL (20−30 ppb) with read times of about 5 min.[3] However, the equipment cannot determine levels above 65 μg/dL and is less accurate for levels >8 μg/dL.[3] Also, portable lead testing kits are known to have higher readings compared to venipuncture tests.[6−8]

An alternative field-friendly approach for blood metal analysis is to collect capillary blood spots using a filter paper. This has been used in lead screening as an alternative approach for measuring venous blood lead levels.[9−12] The benefit of using a filter paper is that the samples can be collected in the

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field, stored at ambient temperatures, and then analyzed at a later date. However, the most frequently used analysis methods require some form of digestion of a small punch from the blood spot or laser ablation to quantify the metal in the small part of the blood spot.[11,13,14] These sampling techniques face challenges such as the hematocrit level influencing blood wicking and drying and therefore potentially causing variation in the metal concentration depending on where in the spot is sampled.[15,16]

Energy-dispersive X-ray fluorescence (EDXRF) is a nondestructive approach for quantifying metals in samples and is routinely used for the assessment of metals on air filters in air monitoring and exposure assessment.[17] EDXRF has also been used in the past to analyze biologic tissues including whole blood, serum, water, liver, and other tissue samples although typically with rather high detection limits.[18−23] EDXRF has been utilized to analyze metals in blood spots but has primarily not focused on trace metals like Pb because of the high detection limits and instead considered metals such as Ca, Cu, and Zn that are found at much higher concentrations.[24]

Advances in technology, however, have substantially reduced the detection limits for EDXRF. Thus, EDXRF has the potential to improve on many of the analytic problems of other approaches for the analysis of metals in blood spots, which could then make analysis of metals in blood spots far more useful for screening and research purposes. EDXRF has many advantages over other approaches including ease of sample preparation and analysis, which reduces the need for complicated equipment requirements, consumable costs, and time needed for analyses. Importantly, EDXRF being a nondestructive analysis offers tremendous benefits as it can measure a wide area such as an entire blood spot, which reduces the potential biases from hematocrit-induced variation in metal concentrations across the blood spot. Furthermore, EDXRF has steadily improved both in size and functionality due to drastic improvements in detector and X-ray generation capabilities.

Given these improvements in the current EDXRF technology, we aimed to assess the capabilities of the current generation of EDXRF systems to assess metals in blood. To do this, we used an EDXRF to measure blood Pb concentrations of blood spots made from blood samples that had been previously measured by other standard approaches and focused on four analytical outcomes: (i) determining optimal measurement parameters, (ii) comparing measured concentrations with standard approaches, (iii) establishing detection limits, and (iv) assessing the effect of the blood spot volume on measured concentrations. A feasible EDXRF approach for metal measurements in blood would provide a new assessment opportunity to a larger research community and offer potential advantages to field studies, freeing them from collection, storage, and shipping requirements typical of blood samples.

2. METHODS

2.1. Epsilon 3XLE EDXRF Spectrometer. In this study, we used the PANalytical (Westborough, MA) Epsilon 4 measurement system. The system uses a silver anode X-ray tube with a maximum energy of up to 50 kV and a maximum power output of 15 W. Each instrument is equipped with a silicon drift detector with a resolution of about 145 eV. Measurements were made for varying times from 5 to 30 min. Measurements of dried blood spots were carried out by cutting out the entire spot using a cleaned 20 mm arc punch and placing the spot face down in a standard 32 mm XRF cup (Premier Lab Supply SC4131) with a 2.5 μm Mylar film (SPEX SamplePrep 3518).

2.2. Blood Spot Calibration. We prepared simulated blood spot standards using deionized (DI) water doped with different concentrations of lead. We used the same spotting procedures and materials for the water standards as was used for blood (see Sections 2.3 and 2.4 below). We spotted 150 μL of ultrapure type 1 DI water spiked with 0, 5, 10, 50, and 100 μg/dL of lead (Fisher Chemical, certified reference standard solution) on standard blood spot cards inside a clean room facility. We used Whatman 903 protein saver cards (GE Healthcare for Life Sciences) for spotting the standards and blood in this study. These standards were used to develop a calibration line under ideal conditions and to quantify results from other blood spot measurements. We also calculated the limit of detection from the calibration line (see Section 2.4).

We measured the standards using the air filtration analysis setting in Epsilon 4 spectrometer software for an initial measurement time of 5 min each. The standard air filtration analysis uses traditional peak fitting methods to extract the counts for individual elemental peaks. This method uses Gaussian elemental peak fits for each of the elements in the energy range and a background fit for any residual spectral components not accounted for from the elemental peaks themselves. Based on the fitted net counts from the Pb peak area and known concentrations, we produced the calibration line. For all subsequent results, this calibration line was used to determine the concentration from the fitted Pb peak net counts for the blood measurements, which were obtained from deconvolution of the spectral components that comes as a standard on the instrument.

2.3. Blood Samples. *2.3.1. Normative Aging Study (NAS).* In order to determine the effects of card loading volume, detection limits, and procedural reproducibility of the EDXRF approach, we used blood samples from participants in the NAS, a cohort of men in the Greater Boston area.[25] ICP-MS measurements on the blood samples were carried out using a PerkinElmer Elan DRC II. On average, 1.02 g of blood was digested using 1 mL of trace metal-grade nitric acid and 1 mL of hydrogen peroxide and then diluted to a final volume of 10 mL. The detection limit for Pb determined by 10 replicate quality control standards was <0.01 ppb, and the percent recovery was between 100 and 105% for all quality control standards. An initial calibration was done each day using eight standards ranging in concentration between 0 and 5 μg/dL.

For analysis with the EDXRF, the NAS blood samples were spotted onto Whatman 903 protein saver cards in June, dried in a fume hood, and stored in individual bags with a desiccant for about 2 months prior to an XRF analysis. The detection limit was calculated as twice the standard deviation of repeated measurements of the same blood spot or standard, which is routinely used as a standard in XRF for detection.[26−30] For procedural reproducibility testing, the same blood sample was spotted on multiple cards and we compared the results of EDXRF analyses of those cards.

2.3.2. Boston Children's Hospital. To have blood samples with relatively high blood Pb concentrations, we obtained 22 blood samples from children who were tested for blood Pb at Boston Children's Hospital. The whole blood samples were analyzed using a GFAAS as part of the lead testing protocol of the Hospital and we collected left over blood for EDXRF analyses. Sample concentrations measured by AAS ranged

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from 3 to 40 μg/dL and were used to test the analytical range of the EDXRF. For EDXRF analyses, we spotted the blood samples at a volume of 150 μL on Whatman 903 protein saver cards in June (the same batch of cards from the NAS blood), dried them in a fume hood, stored them in individual bags with a desiccant, and stored them for 6 months prior to the measurement (due to instrument availability).

2.4. Statistical Analysis. We determined a minimum detection limit (MDL) from the measurements of the standards using the equation

$$\mathrm{MDL} = \frac{2\sqrt{C_{\mathrm{BKG}}}}{\mathrm{slope}} \approx \sigma$$

where σ is the uncertainty associated with the measurement, C_{BKG} is the background counts (from the EDXRF) estimated using the 0 μg/dL standard, and slope is the beta from the calibration line. To estimate the EDXRF measurement detection limits at other measurement times than the 5-min measurements used to create the calibration line, we used the equation

$$\sigma_{\mathrm{unknown}} = \frac{\sigma_{5\mathrm{min}}}{\sqrt{\frac{t}{5_{\mathrm{min}}}}}$$

where t is the new EDXRF measurement time, $\sigma_{\mathrm{unknown}}$, is the new uncertainty, and $\sigma_{5\mathrm{min}}$ is the uncertainty as estimated by the calibration line slope. We additionally estimated empirical detection limits from repeated measurements of our 10 μg/dL standard and actual blood spots. In this case, the MDL was calculated as twice the standard error of the repeated measurements.

We used repeated measures analysis of variance (ANOVA) and correlations to determine whether the spotted sample volume affected measurements. We used linear regression and a Pearson correlation coefficient to examine the agreement between measurements made with AAS and EDXRF. The statistical significance was set at 95% level of confidence. We used *R* version 3.4.2 for all analyses.

3. RESULTS

3.1. EDXRF Reproducibility for Blood Spot Measurement. We tested the influence of the volume of blood used for spotting on EDXRF measurements by spotting the same blood sample on different cards using different volumes. We spotted two blood samples of 7.1 and 8.8 μg/dL Pb as measured by ICP-MS using 75 (two cards), 150 (seven cards), and 300 (five cards) μL of blood. The correlation between EDXRF-quantified results as a percentage of known ICP-MS concentrations and the known volume of spotted sample showed no significant relationship between volume and the measured signal from XRF (p value = 0.47 and R = 0.21). Similarly, a repeated measures ANOVA showed no differences by volume (p value = 0.40 and F value = 3.309) with sphericity and normality not violated in this data.

3.2. Calibration Line and Detection Limit. Figure 1 shows the calibration line for Pb measurements of our 0, 5, 10, 50, and 100 μg/dL DI water standards. Using the calibration line, we estimated a detection limit of 7.2 μg/dL for a 5 min measurement or 2.9 μg/dL for a 30 min measurement. We also calculated an empirical detection limit from repeated measures of the same spot made from a 10 μg/dL lead standard and two NAS blood samples with Pb concentrations of 7.1 and 5.5 μg/dL as measured by ICP-MS. The distribution of those repeated measurements is provided in Table 1, from which we calculated MDLs of 1.0, 1.0, and 3.2 μg/dL.

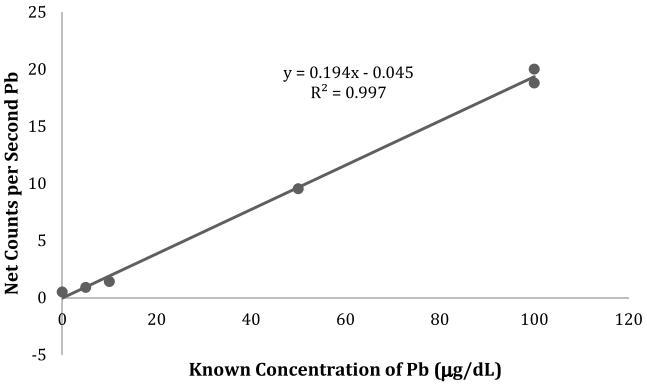

Figure 1. Calibration line of lead counts vs known lead concentrations in water using 5 min measurements (N = 6, error bars shown).

Table 1. Distribution of Repeated 30 min Measurements of Blood Spots

sample	number of measurements	concentration[a] (μg/dL)	coefficient of variation	MDL (μg/dL)
blood spot standard	22	10	0.05	1.0
blood spot 1 (150 μL)	20	5.5	0.09	1.0
blood spot 2 (300 μL)	30	7.1	0.23	3.2

[a]As measured by ICP-MS.

3.3. Procedural Repeatability. To test the error inherent in the whole procedure involved in EDXRF measurements, we used a 7.1 μg/dL blood sample from the NAS to make four separate blood spots with 300 μL of blood and a 8.8 μg/dL blood sample from the NAS to make six blood spots with 150 μL of blood. The distribution of 30 min EDXRF measurements made on separate spots from the same blood sample is provided in Table 2. The intraclass correlation coefficient for this data was 0.49 (p value = 0.003), indicating relative agreement with repeated blood spot cards.

Table 2. Procedural Repeatability Tested by Making a Number of Blood Spots using the Same Blood Sample

sample	number of samples	ICP-MS (μg/dL)	mean (μg/dL)	coefficient of variation	standard deviation (μg/dL)
blood spot 1 (300 μL)	4	7.1	5.1	0.27	1.36
blood spot 2 (150 μL)	6	8.8	9.1	0.08	0.74

3.4. Comparison between AAS and EDXRF Pb Measurements. The Boston Children's Hospital blood sample concentrations ranged from 3 to 40 μg/dL with an average of 21 ± 12 μg/dL as measured by AAS. We then compared these to measurements conducted with the EDXRF on blood spots made from the whole blood samples. The correlation between AAS measurements and 30 min EDXRF measurements was 0.99 (95% CI 0.97, 1.00) and in a linear

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regression the beta term was 1.00 (SD = 0.04), indicating that the two measurement approaches gave nearly identical readings on average (Figure 2).

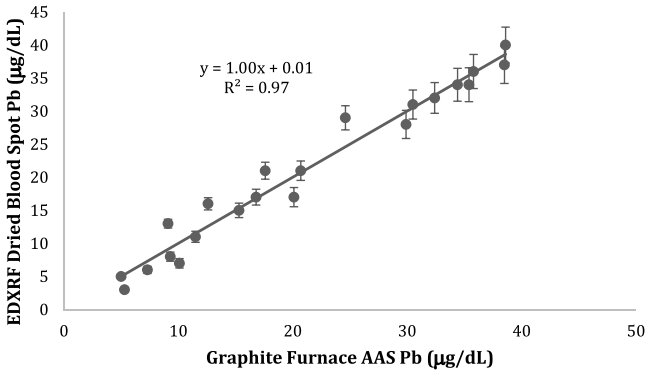

Figure 2. Comparison of XRF-measured blood spots to the same blood sample measured by AAS (N = 22).

4. DISCUSSION

In this study, we (i) found optimal parameters and procedures for measurement of lead in blood spots using an EDXRF, (ii) found excellent agreement between AAS measurements of whole blood samples and EDXRF measurements of blood spots from that whole blood sample (R = 0.99, 95% CI [0.97,1.00], and beta = 1.00 ± 0.04), (iii) determined the MDL for a 30 mi EDXRF measurement to be 2.9 μg/dL from our calibration line and empirical estimates to be as low as 1 μg/dL from repeated blood spot measurements, and (iv) identified no significant effect of spotting sample volume on EDXRF measurements ranging from 75 to 300 μL (ANOVA p value = 0.4) although a volume insufficient to create a spot at least the diameter of the EDXRF beam (~8 mm) would likely affect measurements as the beam would capture areas without any blood. Finally, repeated measurements of different spots made from the same blood sample also showed excellent agreement (coefficient of variation = 0.16), indicating good reliability of the EDXRF blood spot measurement procedure.

We created a calibration line using DI water standards. While this is not the best phantom for blood, we believe this was suitable for our purposes of quantification of blood spots and identification of the Pb detection limit. The analysis quantification performed exceptionally well against AAS results, which, again, shows the promising potential of using the EDXRF as a versatile method in the future.

In comparison to other studies using the EDXRF for biological tissue or water analysis, the detection limit was much lower in our study. Past studies typically had detection limits for most metals closer to a level of 100 μg/dL.[21,23] One study using an EDXRF claimed a detection limit of 0.4 ppb (0.04 μg/dL), but this study used a 500 W X-ray tube, which is much larger than the system used in the analysis presented in this study and would likely need an entire room dedicated to its operation.[31]

The detection limit we calculated ranged from 1.0 to 3.0 μg/dL for the various tests we performed. The detection limit calculated for the EDXRF is on the order of what was previously used for blood analysis using a GFAAS (MDL ~1−2 μg/dL) and is enough to capture exposure levels in many settings and certainly at the CDC reference level for elevated lead levels of 5 μg/dL in children. Of note, the detection limit we measured can be modified using different systems and measurement times to decrease the detection limit. The detection limit determined using the XRF counting statistics is proportional to the square root of the factor increase in time and power from the device. The system in these measurements had a 15 W output, but utilizing a higher output should give a reduction proportional to the square root of the increase in power.[32] For example, a 50 W system would then lower the detection limit by a factor of 1.8 and 50 W systems are readily available in the same form factor as the device used here. Similarly, doubling of the time would reduce the detection limit by a factor of 1.41.

Of note, previous attempts at analyzing blood spots demonstrated significant issues in the quantification.[16,33] This is mainly due to the sampling techniques used for collecting blood[34] and the use of small punches from the larger blood spot. This sampling method is subject to variability if metal concentrations vary across the blood spot as can occur because of the hematocrit effect, which is related to the drying pattern of blood on the card.[15,16] While new approaches for blood spot collection and making punches could improve on this problem,[35−38] the EDXRF largely avoids this problem by measuring essentially the whole blood spot, and the beam diameter can be adjusted to further aid with this issue. Furthermore, EDXRF measurements have the great benefit of being nondestructive, so the blood spots can still be retained for other analyses.

Measurements of similar samples at different spotted volumes (70−300 μL) produced similar results to our XRF methodology. The quantification of lead did not appear to vary with large changes in volume, but a further study is needed to definitively put this issue to rest. Thus, a further study including more samples at higher and lower volumes needs to be conducted to accurately determine whether the sample volume was indeed not having a significant impact on the outcome of the measurement. Lower sample volumes typical of neonatal fingersticks may have more likelihood of impacting the results rather than the higher sample volumes used in the analysis included here. Standard blood needs to be used in order to test this effect to produce the number of spots necessary to make a conclusion either way.

The procedural repeatability found that laboratory methods did not have an impact beyond what we saw already with the detection limit of the device. Creating blood cards in the lab will always increase the level of uncertainty from measuring the samples directly, but the main finding here demonstrated that this increase in uncertainty was lower than what we already identified with the detection limit. Thus, we can successfully create samples in the lab without harming the integrity of the measurement irreparably. In order to quantify the level of uncertainty introduced through laboratory preparation of the samples, a much larger controlled study of these effects would need to be undertaken.

However, more studies on potential contamination in field settings should be conducted to rule out other sources of variability that could be introduced during the measurement. Further studies at lower levels of blood lead, less than 3 μg/dL, with more optimized settings could provide lower detection limits and better indicate the device capabilities in real-world settings. Most studies in the United States currently report blood lead levels closer to 1 μg/dL, so this method would be most valuable in its current state as a screening tool for likely

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exposed populations rather than the general population but could potentially be improved in the future. This could also be an issue with previously collected blood spots. We potentially could get around this by measuring portions of the spotted paper free of blood and subtracting concentrations. This approach will need to be explored in further studies.

Measuring samples at increasingly lower levels of lead brings up a further question of contamination of the blood cards themselves. Previous studies have shown lead to be present in the blood cards at levels that would potentially influence results <1 μg/dL.[39,40] The calibration used here has a nonzero lead signal from the blank source material and thus already takes this contamination into account. Utilization of a background subtraction method can be used more effectively in future measurements in tandem with corrections for contamination by collecting blanks from each card used during the collection process and identifying the zero point specific to each card. The variability of this would not impact the overall conclusions found here that the EDXRF works well at quantifying blood spots with levels greater than 1−3 μg/dL. However, this is something that should be explored in the future to verify the result integrity for spots collected from different lots of cards with potential for varying contamination inherent in the raw materials.

This technique could be used similarly to measure other metals in blood at levels comparable to what was shown with lead. XRF devices generate a spectrum that encompasses a wide range of elemental excitations and any of those within a similar optimized range of lead could be identified and fitted likewise to what we have done with lead in this study. However, adequate calibration and testing would need to be carried out to ensure disentanglement from surrounding elemental peaks, which is a possibility with some common trace elements that rely only on a single (alpha) peak rather than two peaks (alpha and beta).

In summary, the desktop EDXRF could be easily applied using a standard spotting procedure for the measurement of blood lead and similarly for multielement quantification using an analysis for about 20−30 min to get a reasonable detection limit close to what has been used in the past with AAS (1−2 μg/dL), which would be viable for measurement of large-scale studies to reduce costs and sample preparation burden. Future studies can evaluate blood spot standards collected in field conditions, for example, global public health lead surveillance programs. Additional work on optimization, sample preparation, and contamination correction could help explore the feasibility of using the EDXRF for blood lead screening programs and how these financial, personnel, and equipment costs compare to the existing screening systems.

The EDXRF effectively quantified blood Pb in blood spots with a detection limit on the order of 1.0 to 3.0 ppb with good agreement with blood samples measured by AAS. The procedural stability was shown to be within the instrumentation uncertainty and the volume of the spotted sample was shown to have little effect on the measurement using the EDXRF. EDXRF would be ideal for quick and inexpensive population screening for metal exposures including blood lead. Additional analyses could consider how sample collection location, such as within a clinic or collected in the field, affects the sample quality and quantification.

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Notes

The authors declare no competing financial interest.

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