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Calibration of laser ablation inductively coupled plasma mass spectrometry for quantitative measurements of lead in bone

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Summary

Lead accumulates in bone over many years or decades. Accordingly, the study of lead in bone is important in determining the fate of ingested lead, the potential for remobilization, and for the application of bone lead measurements as a biomarker of lead exposure. Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) was used to measure the spatial distribution of lead in bone on the micrometer scale. In general, LA-ICP-MS studies are somewhat limited by the lack of matrix-matched standards and/or reference materials for calibration and validation purposes. Here we describe the application of pressed pellets prepared from New York State Department of Health candidate Reference Materials for Lead in Bone (levels 1 through 4), to provide a linear calibration for $^{208}\text{Pb}/^{43}\text{Ca}$ in the concentration range <1 to $30\text{ }\mu\text{g g}^{-1}$. The limit of detection was estimated as $0.2\text{ }\mu\text{g g}^{-1}$. The measured lead values for pelletized NIST SRM 1486 Bone Meal and SRM 1400 Bone Ash were in good agreement with certified reference values. Using this approach, we quantitatively measured the spatial distribution of lead in a cross-section of goat metacarpal from a lead-dosed animal. The lead content was spatially variable in the range of 2 to $30\text{ }\mu\text{g g}^{-1}$ with a complex distribution. In some sections, lead appeared to be enriched in the center of the bone relative to peripheral areas, indicating preferential accumulation in trabecular (spongy) rather than cortical bone. In addition, there were discrete areas of lead enrichment, or hot spots, of 100 to $200\text{ }\mu\text{m}$ in width.

1. Introduction

Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) is known to be a powerful technique for measurement of the spatial distribution of trace elements at the micrometer scale in a variety of solid matrices, including biological and environmental samples¹. A potentially important and little explored application of LA-ICP-MS is the measurement of the spatial distribution lead or other toxic metals in bone. Such studies are required to develop an understanding the biological fate of ingested toxins following environmental or occupational exposure, or following deliberate administration during drug therapy. In the case of lead, this data would be valuable for the application of bone lead measurements as a biomarker of long-term or prior lead exposure and for understanding the potential for lead remobilization resulting from bone demineralization. LA-ICP-MS studies

are frequently limited, however, by a lack of suitable matrix-matched calibration standards and Certified Reference Materials (CRMs). Whilst qualitative or semi-quantitative data are sufficient for some applications, rigorous quantitative analysis is desirable for clinical and biomedical samples.

Bone has been defined as “a specialized connective tissue composed of a complex microcrystalline or amorphous mineral substance containing calcium, phosphate and carbonate deposited within a soft organic matrix”². The mineral fraction, about 70% by weight, is composed of hydroxyapatite. The simplified formula for hydroxyapatite is written as $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH}_2)$, but the overall composition is better given by³:

Bone accumulates lead over many years or decades, and this compartment typically accounts for 70 to 95 % of the total body lead burden in humans, depending on age.⁴ Estimates of bone lead levels in contemporary humans vary substantially depending on prior exposure and bone type, however, the available data indicate the range is <1 to 50 $\mu\text{g g}^{-1}$.^{5–7} Lead is thought to bind to bone through replacement of Ca^{2+} in the hydroxyapatite matrix, or via adsorption onto crystal surfaces and binding in the hydration shell². Binding to organic components can also occur, and lead can be cycled between the different compartments⁸. A major factor influencing the distribution of lead in bone is the blood supply, given that blood transports absorbed and remobilized lead throughout the body. Bone is a highly vascular material with an extensive system of arteries, veins, and capillaries⁹, allowing penetration of blood and thus lead into the bone matrix.

The skeleton can become an important source of blood lead through bone demineralization, particularly in women during pregnancy, or lactation, or post-menopause, as the body mobilizes stored calcium^{10, 11}. This remobilization of lead has clear potential health consequences for the subject and, in the case of pregnancy, for the fetus, since lead crosses the placenta. Lead remobilization can also occur during chelate administration for the medical management of lead poisoning¹². The specific location of lead in bone, and the binding characteristics of the element, are expected to have a major influence on lead remobilization.

Studies of lead distribution in bone at macro scales^{13–17} have reported variation in lead content among subjects due to age and level of lead exposure, between different bones in a single subject and within individual bones. Early studies of lead distribution in bone at the micrometer scale using particle induced x-ray emission (PIXE) identified heterogeneous distribution of lead in human femur.¹⁸ More recent studies have used synchrotron XRF with a 15- μm diameter beam to show preferential accumulation of lead near the surface of a human hip bone.¹⁹ Arruda-Neto et al.²⁰ used induced neutron-fission analysis to examine the microdistribution of uranium in bone from a dog, and reported discrete ‘hot spots’. The first use of LA-ICP-MS for spatially resolved analysis of bone appears to be the work of Wang and et al.²¹ who recorded elevated Rb/Ca ratios near the surface of cross-sections of pig femur. Emerging applications of LA-ICP-MS for spatial analysis of bone include measurements of lanthanum in rat autopsy and human bone biopsy from subjects administered with the lanthanum carbonate-containing drug Forensol[®],²² and determination of rare earth elements near surgical implants.²³

There have been numerous studies utilizing LA-ICP-MS for the determination of relative (i.e. non-quantitative) spatial distribution of lead and other trace elements in teeth^{24–30}, which contain calcium hydroxyapatite but with a lower organic content compared to bone.

Uryu et al.²⁹ used a 1-point calibration curve based on the Pb/Ca ratio of ground, pelletized NIST SRM 1486 Bone Meal (National Institute of Standards and Technology Gaithersburg, MD)²⁹ to quantitate Pb content in tooth enamel. In that study²⁹, they used the same calibration approach to analyze bulk, pelletized SRM 1400 Bone Ash, and commercial chicken bone as an 'in house' control material. Other published applications of LA-ICP-MS for bone analysis include strontium isotope ratio determination³¹ and U series dating of archaeological bone samples³².

Potential calibration strategies for LA-ICP-MS measurements of lead in bone include (a) preparation of pressed pellets from NIST SRM 1486 and SRM 1400, or from commercially purchased bone²⁹, (b) precipitation of hydroxyapatite from a lead-spiked solution²³, (c) algal growth of hydroxyapatite in a lead-spiked solution, or (d) precipitation of calcium phosphate from a lead-spiked solution.

The New York State Department of Health (NYSDOH) is currently engaged in a program funded by the National Institute for Environmental Health Sciences, USA (NIEHS) to produce multiple-level ground RMs for lead in bone principally for use with methods such as electrothermal atomic absorption spectrometry (ETAAS) and ICP-MS. Clearly, the NYS ground bone candidate RMs offer potential advantages for calibrating LA-ICP-MS instrumentation for measurements of lead in bone. For example, the NYS RMs have an identical matrix to the sample, are physiologically enriched in lead, and consist of four concentration levels. Furthermore, their use would allow NIST SRM 1486 Bone Meal and SRM 1400 Bone Ash to be used for independent calibration verification. The principal objective of the work reported here was to establish a protocol for measuring the spatial distribution of lead in bone at the micrometer scale using LA-ICP-MS calibrated with pelletized NYS candidate RMs for lead in bone (levels 1 through 4). To our knowledge, this is the first report of a multi-point calibration strategy for quantitative analysis of bone for Pb by LA-ICP-MS that has been rigorously validated using NIST SRMs. We report on the use of a new reference material that is now available to other researchers for the calibration of LA-ICP-MS measurements for Pb in bone. We also report quantitative measurements that describe the spatial distribution of Pb in bone based on LA-ICP-MS.

2. Materials and Methods

Sample preparation

Full details regarding the production and characterization of the New York State candidate Reference Materials Lead in Bovine or Caprine Bone (2005) levels 1 through 4 (NYS RMs 05-01 to 04) will be given elsewhere. Briefly, the NYS RMs 05-01 to 04 were produced from a repository of bovine (cow) and caprine (goat) long bones collected *post-mortem* from the Wadsworth Center's farm facility located near Albany, NY, from 1994 to 2004. Some of the animals were orally dosed with lead acetate during their lifetimes, for the primary purpose of creating blood-pools for the NYSDOH Proficiency Testing (PT) program for blood lead. Adult goats were dosed with lead under an active protocol (number 01-096) approved by the Wadsworth Center. Soft tissues were removed using tungsten-based tools, thoroughly cleaned with hydrogen peroxide solution, and defatted with ether.

Bones in the repository were sub-divided into four pools, based on their bovine or caprine origin and on the lifetime cumulative lead dose of the animal. The pooled bones were processed into a fine powder in three stages, using (1) a Retsch SM 2000 knife mill, (2) a Retsch ZM 200 ultra-centrifugal mill, and (3) a WAB Turbula homogenizer (all supplied by Glen Mills, Clifton, NJ). The homogenized bulk materials were sample-scooped into plastic bottles to produce the candidate reference materials provisionally named NYS RM 05-01

Lead in Bovine Bone level 1 (un-dosed), NYS RM 05-02 Lead in Bovine Bone level 2, NYS RM 05-03 Lead in Caprine Bone level 3, and NYS RM 05-04 Lead in Caprine Bone level 4.

The NYS RMs 05-01 to 04 and NIST SRMs 1486 Bone Meal and 1400 Bone Ash were each converted into pressed pellets (briquettes) for LA-ICP-MS analysis as follows; Two grams of bone (or bone ash) were thoroughly mixed with 2 ml Liquid Binder (Chemplex Industries, Palm City, FL) and allowed to dry in a fume hood, in accordance with the manufacturer's directions. Liquid Binder consists of a polymeric binding agent $(C_6H_9ON)_n$ in methylene chloride solvent, which coats individual sample particles, increasing their adhesive properties. The Liquid Binder product is manufactured principally for infra-red (IR) and XRF spectrometric applications. The dried bone-binder mixture was de-coagulated using a mortar and pestle before compaction into discs using a Carver (Wabash, IN) Model 4300 manual pellet press with a 13-mm diameter die, under approximately 6 metric tons of force.

For the spatial distribution studies, we used the right metacarpal bone from a goat having a lifetime lead dose of 10 g. The cleaned bone was reduced to a 3-cm length sample near the mid-point of the shaft, with a high-speed diamond disc saw (Dremel, Racine, WI). A cross-section of the metacarpal was prepared using an Isomet low-speed diamond disc saw (Buehler Ltd, Evanston, IL). The analytical surface was then momentarily etched with 1 mol dm^{-3} hydrochloric acid solution, quenched with high purity water (MilliQ 18.2M Ω cm^{-1} , Millipore, Billerica, MA), dried with acetone and immediately transferred to the laser ablation chamber to minimize the risk of surface contamination.

Analytical measurements

The NYS RMs 05-01 to 04 were distributed to 38 laboratories participating in an international, inter-laboratory comparison exercise that began in August 2005. Since certified target values were not available at the time of our LA-ICP-MS study, we used preliminary Pb values for laser ablation calibration purposes. These values had been obtained using a validated ETAAS method³³, which included microwave-assisted digestion of 0.2 to 0.5 g (dry weight) of bone powder with ultra-pure nitric acid (Veritas Double Distilled, GFS Chemicals, Columbus, OH). Subsequent dilutions were performed using a diluent containing $Ca(NO_3)_2$ (Calcium matrix modifier, Aldrich, St. Louis, MO) and monobasic ammonium phosphate (Ultrex Ultrapure Reagent, J. T. Baker, Phillipsburg, NJ) as the modifier in 1% (v/v) HNO_3 acidified high purity water (MilliQ 18.2M Ω cm^{-1} , Millipore, Billerica, MA). The ETAAS method was validated using NIST SRM 1486 and SRM 1400.

LA-ICP-MS measurements were performed using a CETAC (Omaha, NE) LSX100 LA system coupled to a Perkin Elmer (Shelton, CT) ELAN 6000 ICP-MS. We employed the laser ablation parameters described by Kang and coauthors²⁹ for spatially resolved analysis of teeth. These parameters were a continuously firing 10-Hz frequency laser at energy level 13/20, delivering about 1 mJ of energy, as measured by an Energy Max 400 detector (Molelectron Detector Inc, Portland, OR). The laser was focused on the sample surface, creating an approximate ablation diameter of 20 μm . The sample stage was translocated in a straight line at a speed of 20 $\mu m s^{-1}$ (along the x-axis). Standard ICP-MS operating parameters were optimized on each day of analysis. The isotopes ^{43}Ca , ^{46}Ca , ^{64}Zn , ^{88}Sr , ^{206}Pb , ^{207}Pb , and ^{208}Pb were sequentially detected for 100 ms dwell time per mass. The time required for one cycle of measurements was 700 ms, that provided a spatial resolution of 14 μm based on the stage translocation rate.

3. Results and Discussion

Calibration and validation of LA-ICP-MS of lead in bone

Signal intensities obtained during LA-ICP-MS analysis of NYS RMs 05-01 to 04 and NIST SRM 1486 and SRM 1400 were sufficient to detect all of the selected isotopes above the gas blank. We selected ^{43}Ca as a normalizing element since it had superior signal intensity and a better signal-to-blank ratio than did ^{46}Ca , as well as a lower relative standard deviation (RSD) than ^{88}Sr , suggesting that strontium was not homogeneously distributed in the sample matrix. We chose ^{208}Pb as the analyte isotope, due to its superior signal and better signal-to-blank ratio, and its lower RSD, as compared to the less abundant lead isotopes. The RSD of the ablation lines (Figure 1) varied from 10 to 14% for ^{43}Ca , and from 38 to 132% for ^{208}Pb . This variation can be attributed to (1) variability in the laser ablation process, (2) variability in the ICP-MS measurement and (3) variability in element distribution within the sample. Previous studies based on flame AAS measurements of Ca distribution in bone indicate that it is uniformly distributed, especially when compared to lead¹⁶.

It is apparent from the plots shown in Figure 1 that there is some variation in the lead signal from both the NYS RMs 05-01 to 04 and the NIST SRM 1486 and SRM 1400 that cannot be attributed to instrumental sources, indicating possible micrometer scale heterogeneity in these samples. The RMs can be considered as a composite of discrete micrometer-sized particles with a distribution of lead contents, with the mean of these lead-content values being the calibration of reference value. Each laser shot, of 10-Hz frequency and ICP-MS measurement cycle of seven isotopes (sequentially) in 700 ms, or 14 μm , samples a relatively small collection of particles, as compared to bulk analysis. In other words, only a small part of the distribution of the lead-content values is measured, leading to deviations from the mean value. To provide an accurate estimate of the mean value by LA-ICP-MS, long acquisition times are required, thereby creating a high number of measurements.

Figure 2 shows a plot of the mean $^{208}\text{Pb}/^{43}\text{Ca}$ ratios (y) recorded by LA-ICP-MS, calculated from the individual means of five successive 150-s ablation lines (i.e., 750 s total ablation), against the bulk lead concentration (x) of the RMs as established by ETAAS (Table 1). Linear regression of the data showed that a relatively long ablation time was sufficient to provide a good representation of the bulk mean lead content to form a linear calibration curve ($y = 0.0033x$, $r^2 = 0.998$). A more time-efficient calibration strategy was tested the following day, using duplicate ablation lines and regressing the individual means calculated from over 150 s ablation. The relationship was very similar to that observed using five replicates ($y = 0.0032x$, $r^2 = 0.988$), indicating that the shortened protocol was suitable for a daily calibration strategy.

The limit of detection (LOD), defined as 3 times the standard deviation of the blank, was estimated from the ratio of the ^{208}Pb signal of a gas blank to the subsequent ^{43}Ca signal of NYS RMs 05-01. The LOD was found to be $0.2 \mu\text{g g}^{-1}$. The results of the analysis of pelletized NIST SRM 1486 Bone Ash and SRM 1400 Bone Meal are given in Table 2. The lead content measured by LA-ICP-MS was in good agreement with the certified values, and within the standard deviation of the measurement. The measured value for NIST SRM 1400 required a correction, to account for the effect of the dry ashing process used to produce this SRM. Mineralized bone typically consists of about 70% hydroxyapatite and 25% organic matrix. The organic matrix is removed during dry ashing, leading to relatively higher calcium content in terms of the dry weight. Therefore, we applied a correction by normalizing the composition of NIST SRM 1400 (Bone Ash) to the composition of NIST SRM 1486 (Bone Meal). The correction was calculated in two ways, using either (1) the certified calcium contents or (2) using the loss on ignition at 1000°C values. The two methods gave results that were identical to the first decimal place.

LA-ICP-MS measurements of the spatial distribution of lead in bone

The spatial distribution of lead in a cross-section of goat metacarpal, as recorded by LA-ICP-MS measurement of the $^{208}\text{Pb}/^{43}\text{Ca}$ ratio using a calibration based on duplicate measurements of NYS RMs 05-01 to-04 and validated by NIST SRMs 1486 Bone Meal and 1400 Bone Ash, is shown in Figure 3. To avoid skewing the distribution with short-term noise from the ^{43}Ca signal that did not correspond with variation in lead, we used the best-fitting 2nd order polynomial of the ^{43}Ca data to normalize the lead signal. The lead content of the metacarpal cross-section varied substantially within, and between, each ablation line from about 2 to 30 $\mu\text{g g}^{-1}$. Ablation lines a, c and d of Figure 3 indicate that the central area of this particular metacarpal is enriched compared to peripheral areas. There are also numerous areas of sharply elevated lead deposits of 100 to 200 μm width. In contrast, line b has a lower, and more uniform lead concentration of $2.3 \pm 1.0 \mu\text{g g}^{-1}$. However, the best estimate of intra-line precision ($\pm 1 \mu\text{g g}^{-1}$) reflects contributions from the instrument and from minor physiologic heterogeneity.

The animal from which this bone was obtained had a lifetime, cumulative dose of 10 g lead, administered orally as lead acetate. In long bones such as the metacarpal, blood is supplied directly to the medullar cavity by the longitudinal principal nutrient artery⁹. Blood supply in bone, *via* arteries, veins or capillaries, typically flows from the endosteal (interior) surface to the periosteal (exterior) surface. It is feasible that blood may penetrate the bone at the periosteal surface, but this is considered less likely⁹. Additionally, the epiphyses at each extremity of the bone shaft have a separate blood supply. Trabecular, or 'spongy', bone is typically more vascularized compared to cortical, or 'compact', bone, and may have greater blood capacity.

Further structural analysis of the bone and additional LA-ICP-MS data are required to understand the mechanism(s) that control lead uptake and distribution into bone. We suspect, however, that lead preferentially accumulates in trabecular bone, and forms discrete, highly enriched areas, or hot spots²⁰. Visual examination of the caprine metacarpal bone suggests the central area has a more trabecular appearance than peripheral areas. It is possible that line b probed a more cortical region of bone, as the track is perpendicular to a ridge. This ridge develops into a bone that spans the medullar cavity, and extends towards the epiphyses. Higher lead content in trabecular bone is consistent with lead deposition from the blood compartment, and with data from KXRF studies that show trabecular bones, such as the patella and calcaneus, with higher lead content than more compact bones such as the tibia. In addition, our previous studies of lead deposition in human tibiae, based on analysis by ETAAS, have shown an enrichment of lead in the outer 1–2 mm of the bone surface¹⁵. Similar results have been observed in tibiae from our lead-dosed goats, so further LA-ICP-MS studies are warranted¹⁶.

4. Conclusion

Quantitative analysis of bones for lead using LA-ICP-MS is achieved by monitoring the $^{208}\text{Pb}/^{43}\text{Ca}$ ratio, and by using pelletized NYS candidate RMs Lead in Bovine Bone, level 1 and level 2 (05-01 and 05-02), and Lead in Caprine Bone, level 3 and level 4 (05-03 and 05-04), as calibration standards. Localized differences in lead distribution within the pelletized standards necessitate use of relatively lengthy acquisition times, in order to provide mean $^{208}\text{Pb}/^{43}\text{Ca}$ ratios that are representative of the bulk Pb composition. The calculated LOD was 0.2 $\mu\text{g g}^{-1}$. The calibration strategy adopted was validated by analysis of NIST SRM 1486 Bone Meal and SRM 1400 Bone Ash, following correction for the altered mineral-to-organic content of the latter. This calibration proved suitable for quantifying micrometer-scale spatial distribution of lead in bone, as demonstrated by the analysis of a cross-section of goat metacarpal. The lead content was variable and complex,

and generally higher in the central region of the bone than at the outer edges. There were discrete areas of enriched lead 100 to 200 μm in width. This study demonstrates the value of having of having multiple-level calibration standards of similar matrix to the sample, thereby allowing CRMs to be used for calibration verification purposes. Similar multiple-level calibration or RMs, composed of other biological matrices, would be valuable for future LA-ICP-MS studies of the micrometer scale spatial distribution of toxic elements in biological samples.

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Figure 1.
LA-ICP-MS analysis of pelletized NYS candidate RMs 05-01 to 05-04, and NIST SRM 1486 and SRM 1400. Stage translocation rate was of $20\ \mu\text{ ms}^{-1}$.

Figure 2. Plot of the $^{208}\text{Pb}/^{43}\text{Ca}$ ratio measured by LA-ICP-MS in pelletized NYS RMs 05-01 to 05-04 (data points and error bars are mean $\pm$ standard deviation calculated from 150 s of ablation of 5 successive ablation lines) against preliminary lead target values as determined by ETAAS (Table 1). Line is a linear regression through the origin with equation $y = 0.0033x$ where $r^2 = 0.998$.

Figure 3.

(1) Photographs of cross section of goat metacarpal bone taken at x8 and x 40 magnification. Box shows magnified area and arrows indicate the path and direction of the displayed laser ablation tracks from endosteal (inner) surface to periosteal (outer) surface. (2) Lead content of cross section of goat metacarpal as recorded by LA-ICP-MS measurement of $^{208}\text{Pb}/^{43}\text{Ca}$ calibrated by NYS RMs 05-01 to 05-04. Normalization was achieved using the best-fitting 2nd order polynomial of the ^{43}Ca data.

Table 1

Calibration values established for the candidate reference materials for Lead in Bone (NYS RMs 05-01 to 05-04) by ETAAS. Lead content is given as the mean $\pm$ standard deviation (s) of 21 measurements. The lead content measured for NIST SRM 1486 and that found for SRM 1400 were in good agreement with the certified values (1.335 ± 0.014 , and $9.07 \pm 0.12 \mu\text{g g}^{-1}$ respectively)

RM/SRM id	Pb ($\mu\text{g g}^{-1}$)		
	n	mean	s
NYS RM 05-01	21	1.1	0.3
NYS RM 05-02	21	15.5	1.0
NYS RM 05-03	21	12.8	0.7
NYS RM 05-04	21	30.5	1.4
NIST SRM 1486	21	1.4	0.1
NIST SRM 1400	21	9.1	0.5

Table 2

LA-ICP-MS measurements of the lead content ($\text{mean} \pm s \mu\text{g g}^{-1}$ dry weight of $n=x$ ablation lines of 150 s duration) of NIST SRM 1486 Bone Meal and SRM 1400 Bone Ash pellets after calibration with NYS RMs 05-01 to 05-04 compared to the certified reference values. (a) Calibration based on five replicate ablation lines per pellet and (b) duplicate ablation lines.

Value	NIST SRM 1486	NIST SRM 1400
	$\text{mean} \pm s \mu\text{g g}^{-1}$	$(\text{mean} \pm s \mu\text{g g}^{-1})$
(a) LA-ICP-MS	1.3 ± 0.2	$8.0 \pm 2.0^*$
(b) LA-ICP-MS	1.3 ± 0.1	$9.3 \pm 2.4^*$
Certified Values	1.335 ± 0.014	9.07 ± 0.12

* results for NIST SRM 1400 Bone Ash were corrected for the different mineral-to-organic content resulting from the dry ashing process, through normalization to the composition of NIST SRM 1486 using either the calcium content or loss on ignition values. The result of each method was identical to the first decimal place