USING BONE LEAD AS A BIOMARKER FOR LEAD TOXICITY IN CONDORS:

Accuracy of Portable L-X-ray Fluorescence (LXRF) Device to Quantify Lead (Pb) in Condor Bone In Vivo

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Abstract

Our lab has been working on the development of a portable L-shell x-ray fluorescence (LXRF) device to quantify lead in bone in human in vivo. The purpose of this project is to determine the accuracy of the portable LXRF machine in measuring the lead content of condor bones in vivo. While the K-shell x-ray fluorescence (KXRF) machine is the most accurate machine to measure lead content in vivo because it has sufficient energy to overcome soft tissue attenuation, it is not very practical for use in a research lab with animals or for researchers covering a large migratory territory of condors (from California to Arizona). The portable LXRF machine is lightweight; does not require continuous maintenance, a radioisotope source, or nitrogen cooling; and provides immediate spectra for analysis in a couple of minutes.

I calibrated the system with Pb-doped bone-equivalent phantoms that were covered with 0.54 mm, 1 mm, and 1.5 mm Lucite to mimic the effects of soft tissue attenuation. Seventeen condor cadaver bones were measured twice (for reproducibility) and the spectra were analyzed with our in-house spectral fitting program written with MatLab. Significant correlation was observed between the bone Pb concentrations measured by the portable XRF and ICP-MS ($R^2 = 0.68, 0.63, \text{ and } 0.74$ for 0.54, 1, and 1.54 mm tissue thicknesses, respectively) and the linear regression of the KXRF results versus the 0.54 mm, 1 mm, and 1.54 mm Lucite thickness measurements was $R^2 = 0.89, 0.81, \text{ and } 0.84$. Two measurements of the same set of bones (Lucite thickness at 0.54 mm, 1 mm, and 1.54 mm) gave rise to the strong correlation with an R^2 of 0.95, 0.76, and 0.81 respectively, which shows a great reproducibility of results.

In conclusion, the portable LXRF device has sufficiently proven to provide accurate measurements.

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INTRODUCTION

Since 1997, about half of the wild population of California condors have required treatment for lead (Pb) poisoning, and the primary source of that poisoning has been identified as Pb ammunition in a study done by Dr. Donald Smith's group at the University of California Santa Cruz (Kelly et al., 2014). Despite multiple Pb ammunition bans and reduction bills, such as the Ridley-Tree Condor Preservation Act in 2007 and Assembly Bill 711 signed in 2013 that required the use of nonlead ammunition statewide for the hunting of all mammals, the percentage of the condors with elevated blood lead (PbB) levels stayed the same. In a recent study, researchers investigated whether the implementation of ammunition regulations affected the PbB levels in a condor population. They found that median PbB changed from 14 ug/dL to 13 ug/dL and the annual prevalence of elevated Pb exposure (defined as PbB greater than 10 ug/dL) stayed the same after the ammunition regulation among 150 condors (Kelly et al., 2014).

Pb bullets have remained popular due to their advantages on mass, availability, density, and cost relative to less dense metals. Resistance to switching from Pb to other metals such as copper has continued, despite a consensus statement of scientists released in 2013 by doctors concerned about the risks of lead-based ammunition towards humans and wild animals. Pb has been labeled by the International Agency for Research on Cancer, the National Toxicology Program, and the US Environmental Protection Agency as a carcinogen and endocrine disrupter. The effects on condors are crop paralysis and impending starvation (Cade, 2007), and the effects on human populations are neurological defects, cardiovascular effects, reduced kidney function, and reproductive issues in both sexes (EPA, 2018).

Researchers' understanding of Pb poisoning of condors has been confined to PbB measurements or measurements taken from feathers. Blood level is an adequate biomarker of acute Pb exposure, as the average PbB half-life in humans is around a month (Specht et al., 2016). Bone lead (PbBn) would be a more suitable biomarker to observe the chronic Pb exposure of condors, as the half-life of Pb in human bone is several decades and PbBn contains over 90% of the total Pb body burden (Wilker et al., 2011). Many condors are chronically exposed to harmful levels of lead (Finkelstein et al., 2012). Thus it is logical to assume that PbBn in these birds is a better biomarker than PbB to estimate Pb exposure and hence toxicity associated with this type of long-term exposure.

Our group developed a cadmium-109 (^{109}Cd) based K-shell x-ray fluorescence (KXRF) PbBn measurement system, which has been validated and applied in human health studies (Nie, Chettle, Luo, & O'Meara, 2006; Specht et al., 2016). The KXRF system makes use of the characteristic Pb K x-rays with relatively high energy. The higher energy yields less soft tissue attenuation and so is not as dependent on skin thickness as the portable L-shell x-ray fluorescence (portable LXRF) system. The detection limit for the KXRF PbBn measurement system is 2–3 μg Pb per gram bone (ppm). However, the bulky KXRF system is not suitable for a field study, because it requires a sophisticated device setup including the nitrogen cooling system, licenses for the radioactive source, and 0.5 to 1 hour of measurement per sample.

The portable LXRF device in this study was recently investigated in our lab for measuring PbBn *in vivo* in humans. The advantages of this device are that it is handheld and only takes 3 minutes to complete a measurement, making it ideal for field work. However, the portable XRF is more sensitive to soft tissue attenuation because the energies of the characteristic Pb L X-rays produced by this device are much lower than the characteristic Pb K X-rays produced by the KXRF system. For this reason, the detection limit of PbBn for the portable LXRF device increases significantly with the increase of the soft tissue thickness overlaying the bone. A previous study performed in our lab showed a detection limit of 1.2 ppm to 11 ppm for soft tissue thickness ranging from 0 mm to 5 mm for the portable XRF PbBn measurement system (Specht, Weisskopf, & Nie, 2014).

XRF technology could be used to collect measurements of PbBn *in vivo* to monitor condors' lifetime exposure levels to Pb, which could possibly guide policy and intervention to forestall both human and animal exposure to Pb. This study validated the use of the portable LXRF to measure Pb in condor bone samples and compared the values to those obtained using the KXRF device and inductively coupled plasma mass spectrometry (ICP-MS).

MATERIALS AND METHODS

Portable LXRF PbBn Measurement Device

A customized XL3t GOLDD+ device with improved geometry and detector technology (Fisher Scientific, Incorporated, Billerica, MA) was used to measure both the bone equivalent phantoms as well as the condor bone samples. The device consists of a low-energy x-ray tube with energy up to 50 keV, a thermoelectric-cooled silicon drift detector, and

Figure 1. Schematic configuration of the portable LXRF device.

surrounded shielding. A variety of filter combinations with different thicknesses can be selected to optimize the detection of elements in different applications. In this project, a filter combination with silver and iron, and the X-ray tube setting with 50 keV and 40 μA , were selected for condor PbBn measurement. Figure 1 illustrates a schematic configuration of the portable XRF device for *in vivo* PbBn measurement (Nie et al., 2011). This technique has been calibrated and used previously for *in vivo* human studies in our group (Specht et al., 2014, 2016). The current study aims to determine whether the device can accurately quantify Pb in condor bones.

Different thickness of Lucite plates were placed over each bare condor tibia bone to simulate the *in vivo* situation with soft tissue over bone. Measurements of the bare condor tibia bones (1 measurement per bone) and the bones with different thicknesses of Lucite plates were completed with the device in its stand such that the bones could lay as flat as possible against the beam aperture and detector. Samples were measured at the center of the diaphysis for 3-minute increments and data was collected from the device.

Bones that were significant outliers were remeasured to be sure they lay as flat as possible against the aperture (some were taped to the Lucite itself). Correlation constants improved for all tissue thicknesses and against both the ICP-MS and the KXRF, but the same bones remained similar outliers. See Discussion and Conclusion, below.

KXRF PbBn Measurement System

A KXRF bone Pb measurement system was used in our previous studies to validate the portable LXRF for *in vivo* PbBn measurement in humans (Nie et al., 2006; Specht et al., 2014, 2016). This system contains

four 16 mm high-purified germanium detectors (HpGe) with 10 mm thickness, four feedback resistance preamplifiers, a computer, and four digital signal-processing systems. A 135mCi ^{109}Cd radioisotope source was used to irradiate condor tibia bone or bone equivalent phantoms to produce the characteristic Pb K x-rays. Samples were placed about 1 cm from the ^{109}Cd , and the center for the diaphysis was aligned with the source. Spectra were analyzed with an in-house peak-fitting program, and Pb concentrations were calculated (Nie et al., 2006). Whole body effective dose from this system for human adults with a 30-minute measurement is 0.26 micro Sv (Nie, Chettle, Luo, & O'Meara, 2007).

ICP-MS Analysis

ICP-MS measurements were performed at the University of Idaho's Analytical Sciences Laboratory with an Agilent 4500 ICP-MS (Agilent, Santa Clara, California). Each measurement was made using a 1 cm sample cut 3–4 cm from the diaphysis. Soft tissue was removed, and the bone was dried and grounded. The sample was then digested in trace metal grade nitric acid (69%) using a Tecator open block digestion system. The samples were further digested at 30 degrees Celsius for 6 hours, then 70 degrees Celsius for 1 hour with a 1-hour ramp time, and finally at 120 degrees Celsius for 8 hours with 1-hour ramp time. Each sample weighed 0.25 ± 0.05 g, and the digested samples were diluted to 10 mL using Type 1 water. Calibration verification, reagent blanks, and a standard reference material were used for standard quality control.

Bone Equivalent Phantoms and Soft Tissue Equivalent Phantoms

Bone phantoms were made from plaster of paris and doped with Pb concentrations ranging from 0 to 100 ppm (measurements were taken from 0, 5, 10, 15, 20, 30, 50, 75, and 100 ppm, respectively). Lucite phantoms made of polymethyl were used to mimic the soft tissue thickness over the condor bones that would be used to simulate in vivo measurements. The thickness ranges from 0.54 mm to 1.54 mm. There is no known data on the soft tissue thickness for condors, and we presume that this is the right range.

Condor Bone Samples

Seventeen condor bone samples were obtained from the University of Arizona. They were bare bone samples taken from the avian equivalent of the tibia in

Figure 2. One of the condor tibia bone samples.

condors with unknown amounts of Pb exposure. The bones were 2–5 cm in length and about 1–2 cm in width. Figure 2 shows a sample of one of the condor tibia bones measured in the study.

Data Analysis

For the portable XRF device, calibration method was performed by placing different thickness of Lucite plates (0 to 3.04 mm) over the 100 ppm Pb bone-equivalent phantom to simulate the soft tissue thickness over bone. The net count of Pb L_{β} peak from the 100 ppm phantom is derived as a function of Compton peak counts because more Compton scatters and signal attenuation are induced by increased soft tissue thickness. This function was used together with the Pb net counts obtained from the fitting conducted by Matlab to find Pb concentrations in vivo. Spectra were analyzed using peak fitting method where a Gaussian function is used to describe the Pb L_{β} peak, and an exponential function was used to fit the background. Negative values for PbBn are kept because they represent the point estimate of PbBn with the uncertainty in the measurement. If the concentration is close to zero, the point measurement can be estimated to be negative or positive due to associated uncertainty.

Bones were measured with 0.54 mm, 1 mm, and 1.54 mm Lucite thicknesses by the portable XRF system. Pb concentrations were calculated using the net counts obtained from the fitting and the calibration line for the Pb L_{β} peak (12.6 keV). Microsoft Excel was used for the correlation and linear regression analysis, and the correlation between the PbBn levels measured by the portable XRF, KXRF, and ICP-MS were obtained. Results are shown in sections 3.2–3.3.

Reproducibility was tested by comparing measurements and analysis completed by Dr. Aaron Specht with the current measurements completed in our laboratory. Results are shown in section 3.4.

RESULTS

Calibration Line of the Portable XRF System

The calibration line of the portable XRF system, with a 100 ppm phantom, used to quantify PbBn is shown in Figure 3. Pb L_{β} net count for the phantom decreases with the increase of the Compton peak due to the soft tissue attenuation.

Figure 3. Calibration line of the portable XRF system with a 100 ppm phantom.

Correlation Between the PbBn Measured by the Portable XRF and KXRF

Figures 4 a–c show the correlation between the PbBn level measured by the portable XRF and KXRF methods in different soft tissue thickness. The R^2 value ranged from 0.81 to 0.89.

Figure 4a. Correlation between portable LXRF and KXRF bone Pb measurements in condor tibia bone samples with 0.54 mm Lucite thickness.

Correlation Between the PbBn Measured by the Portable XRF and ICP-MS

Figures 5 a–c show the correlation between the PbBn level measured by the portable XRF and ICP-MS in different soft tissue thickness. The R^2 value ranged from 0.63 to 0.74.

Figure 4b. Correlation between portable LXRF and KXRF bone Pb measurements in condor tibia bone samples with 1 mm Lucite thickness.

Figure 4c. Correlation between portable LXRF and KXRF bone Pb measurements in condor tibia bone samples with 1.54 mm Lucite thickness.

Figure 5a. Correlation between portable LXRF and ICP-MS PbBn measurements in condor tibia bone samples with 0.54 mm Lucite thickness.

Figure 5b. Correlation between portable LXRF and ICP-MS bone Pb measurements in condor tibia bone samples with 1 mm Lucite thickness.

Figure 5c. Correlation between portable LXRF and ICP-MS bone Pb measurements in condor tibia bone samples with 1.54 mm Lucite thickness.

Reproducibility

Figures 6a-c show the correlation between the PbBn level with different Lucite thickness measured by different lab peer in the same laboratory with the portable XRF system. The R^2 value for the Lucite thickness of 0.54, 1.0, and 1.54 mm are 0.95, 0.77, and 0.81, respectively.

DISCUSSION AND CONCLUSION

The effects of the extinction of most scavenging birds have not been widely studied, but an article written by Şekercioğlu, Daily, and Ehrlich (2004) suggests that there could be unanticipated top-down or bottom-up consequences due to nutrient deposition reductions or trophic cascades, and an increase in the spread of diseases in human communities due to carcasses that take a long time to decompose (Şekercioğlu et al., 2004). These possible diseases include rabies, bubonic plague, and anthrax.

Our results indicate that the portable XRF could be used to effectively quantify PbBn for condors and distinguish highly exposed individuals from normal environmental exposures. Lucite has previously been proven to produce almost identical spectra to human skin measured in cadaver tibia bones (Specht et al., 2014), further validating our results for in vivo measurements. The uncertainty remained relatively low with an increase in the soft tissue, and the correlation value did not change significantly as the Lucite thickness increased, nor did the quantification change significantly. One advantage of using this device for the avian species is that the avian species will likely have much thinner soft tissue thickness, and therefore have lower detection limit.

Issues concerning the portable XRF Pb measurement are its geometric sensitivity and lower energy of the characteristic x-rays. The lower energies did not seem to affect the results as much due to the relatively small skin thicknesses comparing to that of humans, but the geometry seems to be causing

Figure 6a. Correlation between portable LXRF and previous portable XRF PbBn measurements in condor tibia bone samples with 0.54 mm Lucite thickness.

Figure 6b. Correlation between portable LXRF and previous portable LXRF bone Pb measurements in condor tibia bone samples with 1 mm Lucite thickness.

Figure 6c. Correlation between portable LXRF and previous portable LXRF bone Pb measurements in condor tibia bone samples with 1.54 mm Lucite thickness.

outliers in similar bones, even after remeasurements. The air space in the bones would not contribute to issues in in vivo measurements because the grooves of the bone would be covered by soft tissue to prevent unaccounted airspace. The worse correlation between ICP-MS and portable XRF comparing to KXRF and portable XRF could partially be explained by the fact that measurements from ICP-MS were done using bone section that was not measured by either portable XRF or KXRF. In addition, a couple of factors may affect the results from all three methods. First, bone composition changes from cortical to trabecular bone, which results in different accumulation and resorption rates with the differences in bone type (Somervaille et al., 1989). Second, the units of the quantification for the three methods are different. More study is needed to investigate the differences between measurement methods, the effect of a unit conversion factor, and the effect from the sample geometry for PbBn measurements. We attempted to overcome geometric issues by taping outliers on the Lucite to minimize air space, which gave us better

correlation constants, but the same bones remained outliers.

In summary, both KXRF and ICP-MS bone Pb measurement techniques demonstrated good correlation with portable XRF results. The detection limit of the portable XRF was low enough to feasibly measure PbBn in vivo. The higher soft tissue thickness typically causes higher uncertainty, but this is unlikely to be a great issue for avian species. Portable XRF would be the optimal device for measurement of PbBn in vivo on avian species in the field given the impracticality of ^{109}Cd -induced KXRF measurements in the same area.

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