QUANTIFICATION OF SODIUM IN BONE AND SOFT TISSUE WITH *IN VIVO* NEUTRON ACTIVATION ANALYSIS

by

Mychaela Coyne

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THE PURDUE UNIVERSITY GRADUATE SCHOOL
STATEMENT OF COMMITTEE APPROVAL

Dr. Linda H. Nie, Chair
School of Health Sciences

Dr. Jim Schweitzer
School of Health Sciences

Dr. Carlos Perez Torres
School of Health Sciences

Dr. Uzay Emir
School of Health Sciences

Approved by:
Dr. Aaron Bowman

To my mom, for always supporting my education and encouraging me to reach for the stars.

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ABSTRACT

Excess sodium (Na) intake is directly related to hypertension and an increased risk of developing many chronic diseases, but there is currently no method to directly quantify Na retained in the body. Because of this, the locations of Na storage and its exchange mechanisms are not well known. This information is critical for understanding the impact of increased Na intake in modern diets. In order to non-invasively quantify Na in bone and soft tissue, a compact deuterium-deuterium (DD) neutron generator-based *in vivo* neutron activation analysis (IVNAA) system was developed. MCNP was used to design a custom irradiation assembly to maximize Na activation in hand bone while minimizing dose. In order to test the system, live pigs were used. Two 100% efficient high purity germanium (HPGe) detectors collected Na-24 counts over 24 hours post irradiation. From the pig studies, a two-compartment model of exchange was developed to quantify Na in bone and in soft tissue. The right legs of four live pigs, two on a low Na diet and two on a high Na diet, both for 14 days, were irradiated inside the customized irradiation cave for 10 minutes (45 mSv dose to the leg) and then measured with the HPGe detectors. The spectra were analyzed to obtain the net Na counts at different time points. Analysis shows exponential decrease of Na in the leg during the first one hour of measurement, while the change was minimal at the second hour, and the counts were stabilized at the second and third 2 hour measurements, taken 7 and 21 hours post irradiation. Bone Na and soft tissue Na concentrations were calculated using calibration lines created with bone and soft tissue equivalent Na phantoms as well as the parameters obtained from the two-compartment model. The results show that the difference in bone and soft tissue Na between the pigs on high vs low Na diets was significant. With these results, we conclude that DD neutron generator-based IVNAA can be used to accurately quantify Na in bone and soft tissue *in vivo* and the system is a potential valuable tool for nutrition studies.

CHAPTER 1. INTRODUCTION

1.1 Sodium Retention and Storage in the Body

Sodium (Na) is an essential element in the human body required for many purposes like blood pressure and volume control in addition to muscle, nerve and motor function. However, too much Na can result in chronic diseases like high blood pressure, congestive heart failure, or diseases of the liver and kidney (Titze, Müller and Luft, 2014). Dietary Na intake is also directly related to osteoporosis risk, with reduced daily intake shown to be as effective as calcium (Ca) supplements in preventing bone loss (Devine *et al.*, 1995). Unfortunately, dietary intake recommendations for Na are typically exceeded by individuals in the US. While the current estimated daily intake is 3400 mg, the recommendation was to reduce it to 2300 mg or less and to an even lower 1500 mg in at risk populations (DGAC, 2010). Recently, a Chronic Disease Risk Reduction Intake (CDRR) for sodium of 1500 mg/day was established using evidence of the beneficial effect of reducing sodium intake on cardiovascular disease risk, hypertension risk, systolic blood pressure, and diastolic blood pressure (National Academies of Sciences, 2019). Reductions in intakes that exceed the CDRR are expected to reduce chronic disease risk within even healthy populations. With the CDRR in mind, all individuals should now aim to reduce their daily sodium intake to 1500 mg/day (National Academies of Sciences, 2019). These recommendations are based in part on the Dietary Approaches to Stop Hypertension (DASH)-Sodium trials (Appel *et al.*, 1997; Sacks *et al.*, 2001; Appel *et al.*, 2005). Metabolic balance studies are needed to understand the influence of the Na intake on Na retention, while understanding the influence of diet on Na distribution, storage, and exchange in the body require new approaches.

This research is timely in that the current standard of Na measurement is challenged by recent data. Typically, Na is measured with spectrophotometry in biological samples like urine,

feces and sweat (Palacios *et al.*, 2013). The retention of Na can be relatively measured by subtracting the amount in the urine and feces from the known dietary input, but this requires strict diet regulation and regular urine collection over at least 24 hours, so it can be an impractical method for human studies. In addition, even in a long term, controlled environment study in young and healthy participants (Mars520), large differences in day-to-day Na excretion concluded that standard 24 hour urine collection measurements are not representative of Na retention and metabolism in the body (Rakova *et al.*, 2013). This may be explained by the deposit of Na in bodily tissues (Machnik *et al.*, 2009). Extra-renal regulation has been observed in muscle and skin with Na-MRI, which is unable to quantitate bone Na (Titze, 2014). Bone is a storage organ for many metals, including Na (Massey and Whiting, 1996). The contribution of the proposed research is expected to be a novel method to noninvasively quantify Na in bone using *in vivo* neutron activation analysis *(IVNAA)*. This contribution will be significant because there is a current need to measure Na in the body in order to better understand the effects of dietary Na intake on Na regulation. Excessive dietary Na intake continues despite public awareness of negative health outcomes from high Na diets. This will be a persistent public health concern requiring innovation in research to support studies seeking to understand Na in the body and prevent related diseases. Investigating the deposit of Na in bone and its exchange in the body will increase knowledge about how Na retention influences diseases like blood pressure and osteoporosis. This research will directly enhance understanding in the field of nutrition science which stands to positively benefit public health.

1.2 Methods to Study Sodium in the Body

Typically, sodium is measured with biologic samples like urine, feces and sweat. Samples can be measured using spectrophotometry methods, most commonly atomic absorption

spectrophotometry (Palacios *et al.*, 2013). The retention of sodium can be relatively measured by subtracting the amount in the urine and feces from the known dietary input. Since this requires strict diet regulation and regular urine collection over at least 24 hours, it can be an impractical method for human studies. Additionally, the validity of current methods are under question since new data shows that urinary sodium excretion does not mirror sodium intake on a day-to-day basis (Weaver *et al.*, 2016). This may be explained by the deposit of sodium in bodily tissues (Machnik *et al.*, 2009).

Bone is a reliable biomarker that does not require a physical sample. Observations of the effects on sodium in bone are consistent with animal data suggesting long term accumulation in the bone (Massey and Whiting, 1996). There is some agreement to support two different types of sodium storage in the body, with both fast and slow exchange compartments, but the nature of those compartments is still debated. Skeletal analysis has previously identified excess sodium stored in bone that cannot be extracted physiologically to supply serum levels and is directly proportional to calcium (Harrison, 1937). *Ex vivo* analysis of rat bones found sodium half-lives of 4.36 and 167 hours for the fast and slow exchange compartments, which are hypothesized to be soft tissue and bone, respectively (Pilecka, 1992).

In vivo neutron activation analysis with Californium-252 found similar results in human hand but with a fast exchange half-life of 1 hour and slow exchange half-lives of 79 hours in normal bone and 35 hours in bone with low calcium concentration (Comar, 1981). The direct relationship in bone sodium and calcium concentration suggests that the slowly exchangeable compartment of sodium behaves the same as bone calcium, although more analysis is needed to show the mechanisms of sodium deposition and exchange over time. Further, the ratio of sodium

to calcium is consistent throughout the skeleton, indicating the slow exchange compartment may be independent of the fast exchange compartments. (Comar, 1981)

The slow exchange compartments are hypothesized to be soft tissue, muscle, and/or skin. Skin has been observed as an osmotically inactive storage site for excess salt from dietary sources (Titze *et al.*, 2003). Sodium MRI studies show sodium storage in the epidermis with a hypothesized countercurrent system for electrolyte exchange (Hofmeister, Perisic and Titze, 2015). Understanding the storage and exchange of sodium will be useful for many applications. Neutron Activation Analysis (NAA) is a unique method for quantifying sodium in the body that will advance research in sodium retention studies.

1.3 Neutron Activation Analysis

In elemental analysis utilizing neutron activation, neutrons interact with elements in the sample to produce characteristic γ rays, as shown in equation 1.

$$AX + n \rightarrow (A + 1)X + \gamma$$

Equation 1

These γ rays can be detected and their spectrum analyzed to determine what elements are present in the irradiated sample. The concentration of the element is determined by the total counts under the corresponding energy peak. The probability of interaction is based on the cross section of an elemental isotope. The higher the cross section, the more likely a characteristic γ ray will be produced.

The neutron source can come from a nuclear reactor, accelerator, generator or radionuclide. For standard neutron activation analysis, a reactor is desirable due to the high flux produced. However, both reactors and accelerators require large dedicated spaces and professional operation.

While radionuclides are compact, they require constant shielding, special licensing, and replacement based on their half-life. Generators, therefore, are a preferable solution when space is limited and both durability and portability are required.

Neutron generators are accelerators capable of producing neutrons with energies of 2.45 MeV, for deuterium-deuterium (DD) generators, or 14.1 MeV, for deuterium-tritium (DT) generators. The lower energy of DD produced neutrons is preferable for decreasing the background in the spectrum produced for neutron activation analysis, since the lower energy neutron has a lower inelastic scatter cross section in essential elements like carbon and oxygen (Liu, 2015).

1.4 *In Vivo* Neutron Activation Analysis (IVNAA)

The main difference for *in vivo* neutron activation analysis, meaning the neutron activation is performed in a living organism, is the limitation imposed by the living subject. The most important limitation is the amount of dose to the living subject, which determines maximum parameters for irradiation time and allowable flux. While there is not an applicable regulatory dose limit for such a procedure, 500 mSv (50 Rem) to the extremities is the annual limit for US radiation workers since it is below known levels of consequence and is considered an acceptable risk for the benefit of livelihood. Considering the benefit of the only current method for *in vivo* bone sodium quantification, 10% of the annual occupational dose limit was determined to be an acceptable upper limit for this study.

The living subject also imposes a limit on the irradiation geometry, so an assembly is designed and constructed to facilitate the easiest positioning of the desired area while shielding the rest of the body. This needs to be modifiable for variation in subjects, like with children of varying height or in animal studies. The length of the procedure is also limited by how long a person can reasonably be expected to remain still, since a change in hand position will decrease data precision.

The hand is selected for irradiation due to the ability to extend it away from the body and easily position it. Additionally, physiologically it is more acceptable to have a hand experimented on than the head or torso. The hand also does not contain active bone marrow in persons above age 10 (ICRP, 1996), so the risk from irradiation is minimized.

Since the system will have a more significant impact on human studies if it is able to reach different populations, it was designed to be compact and transportable, which is unique for a high flux neutron generator. The modular design allows for separate shipping, repair and upgrade of components which, permits transportation and use in remote areas.

The IVNAA system previously developed at Purdue University with the older model DD-109M neutron generator was successfully used for detecting manganese (Liu *et al.*, 2014) and aluminum (Byrne *et al.*, 2016) in bone. The higher flux and optimized design of the new generator make it better suited for higher sensitivity for certain elements with lower radiation dose to the subject. This research investigates the effect of short period dietary intervention on bone sodium concentration. The aim is to show the effectiveness of *in vivo* neutron activation analysis as a non-invasive method for accurate determination of sodium composition in human bone.

1.5 Using IVNAA for Sodium Detection in Bone

Sodium is activated by the IVNAA system through the Na-23 (n, γ) Na-24 reaction. As illustrated in Figure 1, radioactive Na-24 decays by β^- emission to excited levels of Mg-24, whereby characteristic γ rays are emitted through transition to stable Mg-24. Two primary intensive γ rays at 1368 and 2754 keV are observed during this process. (Chechev, 2014)

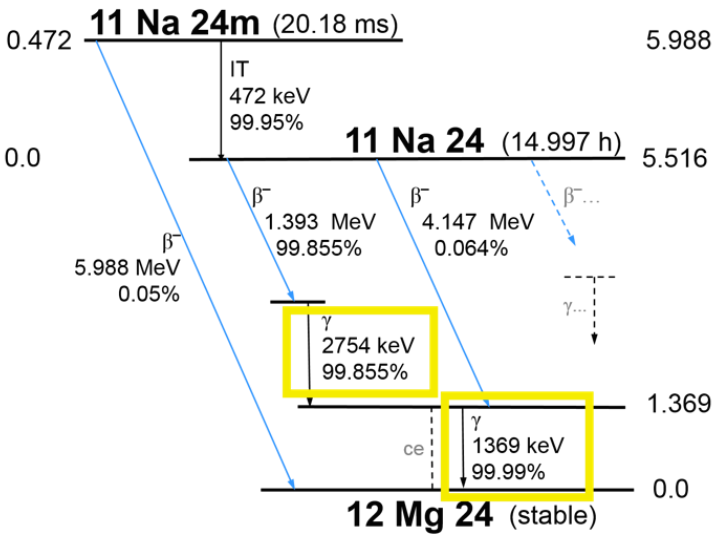


Figure 1 Na-24 decay scheme (Nucleonica)

Sodium's inherent isotopic properties make it favorable for IVNAA. The 14.9 hour half-life of Na-24 is long enough to detect the exchange of sodium in the hand post irradiation. The 100% abundance of Na-24, along with the high branch ratios of 90% for 1368 keV and 100% for 2754 keV, accommodate sensitivity in the detection of activated sodium therefore enabling the quantification of sodium in the irradiated sample.

The concentration of bone sodium is best represented as a ratio to the amount of calcium detected by the system. Since experimental reproducibility can be challenging with *in vivo* studies, normalizing the amount of sodium detected by the system to the amount of calcium detected corrects for differences in sample geometry and position. Bone is mainly composed of calcium, which is activated in IVNAA through the Ca-48 (n, γ) Ca-49 reaction. Radioactive Ca-49 has a relatively short half-life of 8.7 minutes, so only an initial count post irradiation can be used for calcium quantification. Figure 2 shows the decay scheme of Ca-49 to stable Sc-49 through β^- emission and the subsequent characteristic γ ray emission at 3084 keV.

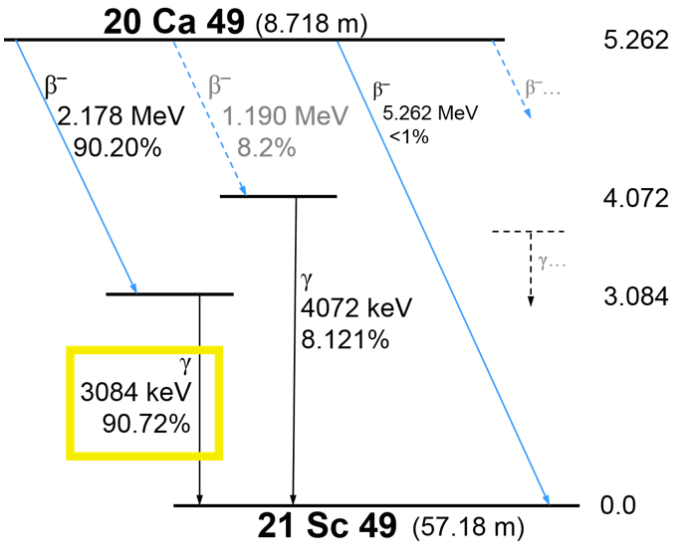


Figure 2 Ca-49 decay scheme (Nucleonica)

Bone is composed of cortical (compact) and trabecular (cancellous) bone, as shown in Figure 3. Cortical bone is solid, without spaces visible unless looking under a microscope, while trabecular bone is a spongy matrix with many spaces visible. They both have the same density *in vivo*, although differences are seen *in situ* due to bone marrow. There are two main types of bone marrow: red and yellow marrow. Red marrow is the fraction of spaces in the bone containing blood, while yellow marrow is the fraction containing adipose tissue. When considering activation of Na in bone, we are focused on the cortical matrix. However, the hard outer layer of bones composed of compact bone tissue is covered in periarticular tissue, which is connective tissue that is closely integrated with the skeleton. Distinguishing bone Na concentration and soft tissue Na concentration is thus an important consideration for IVNAA.

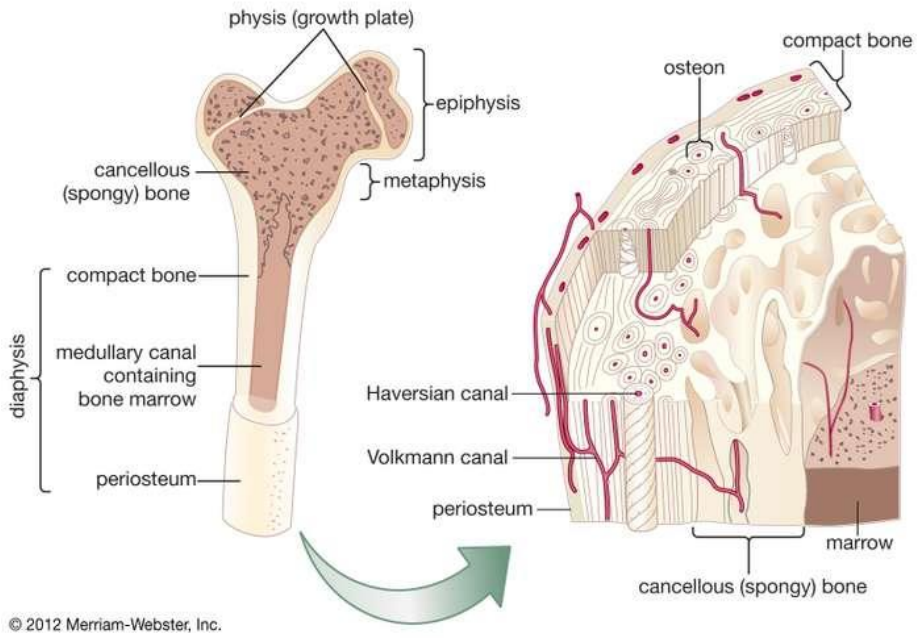


Figure 3 Internal structure of bone (Britannica, 2012)

The absolute amount of Na has been estimated with many different methods in different parts of the body, mostly *in situ*. The total amount of Na in a 70 kg man is estimated to be anywhere from 70 g (Forbes, Cooper and Mitchell, 1953) to 100 g (ICRP, 1975), with whole body neutron activation analysis finding 80 g Na in the body (Chamberlain *et al.*, 1968).

Table 1 below summarizes the total weight of tissues in the body and the average mass of Na expected in a reference 70 kg man (ICRP, 1975). These values are highly variable, with results dependent on a variety of factors including age, diet, environment, and disease. For example, skeleton Na levels are reported in the table for the reference man as 3200 ppm, but other studies report bone Na concentrations from 2000-7000 ppm (Harrison, 1937; Bourne, 2014; Richmond, 1985; ICRP, 1975).

Table 1 Sodium mass in tissues of reference man (ICRP, 1975)

	Weight (g)	Percent of total body (%)	Na Mass (g)	PPM
Total Body	70000	100	100	1429
Muscle (skeletal)	28000	40	21	750
Skeleton	10000	14	32	3200
Soft tissue (total)	60000	85	68	1133
Bone	5000	7.2	-	
Cortical	4000	5.7	-	
Trabecular	1000	1.4	-	
Cartilage	1100	1.6	4.9	4455
Periarticular tissue	900	1.3	4.7	5222
Connective tissue	3400	4.8	19	5588
Cartilage (included with skeleton)	1100	1.6	6	5455
Yellow marrow	1500	2.1	6	4000
Adipose Tissue	15000	21	7.6	507
Subcutaneous	7500	11	3.8	507
Skin	2600	3.7	0.18	69
Teeth	46	0.06	-	
Nails	3	0.004	-	
Heart	330	0.47	4	12121
Blood	500	0.71	0.74	1480
Blood (total)	5500	7.8	10	1818

Previous experiments have successfully used *in vivo* neutron activation analysis to study Na in the body using Californium-252 (Cohen-Boulakia, Maziere and Comar, 1981) and an accelerator (Prestwich, 2016) as neutron sources. This research is unique in its compact neutron source and optimized irradiation assembly for Na activation.

CHAPTER 2. COMPACT DD GENERATOR-BASED IN VIVO NEUTRON ACTIVATION ANALYSIS (IVNAA) SYSTEM TO DETERMINE SODIUM CONCENTRATIONS IN HUMAN BONE

This chapter contains the published article *Compact DD generator-based in vivo neutron activation analysis (IVNAA) system to determine sodium concentrations in human bone* by Coyne M D, Neumann C, Zhang X, Byrne P, Liu Y, Weaver C M and Nie L H in 2018, Physiological measurement 39 055004.

2.1 Background

Sodium (Na) is an essential element found in the human body required for proper bodily functions. It is a naturally occurring substance in most foods and even water. Na is important for blood pressure and volume control in the body. Proper muscle, nerve and motor function also rely on adequate levels of Na. However, too much Na can result in chronic diseases like high blood pressure, congestive heart failure, or diseases of the liver and kidney (Titze, Müller and Luft, 2014). Dietary Na intake is also directly related to osteoporosis risk, with reduced daily intake shown to be as effective as calcium (Ca) supplements in preventing bone loss (Devine *et al.*, 1995). Reducing daily dietary Na is also known to improve kidney function (Aviv, 2001; Wright and Cavanaugh, 2010) and reduce long term risk of cardiovascular events (Cook *et al.*, 2007). There is a positive relationship between Na intake and blood pressure, even among children (DGAC, 2010).

Unfortunately, dietary intake recommendations for Na are typically exceeded by individuals in the US. While the current estimated daily intake is 3400 mg, the recommendation is to reduce it to 2300 mg or less (DGAC, 2010). The current recommendations are based in part on the Dietary Approaches to Stop Hypertension (DASH)-Sodium trials (Appel *et al.*, 1997; Sacks *et al.*, 2001; Appel *et al.*, 2005). Metabolic balance studies are needed to understand the influence of

the DASH diet on Na retention, while understanding the influence of diet on Na distribution, storage, and exchange in the body require new approaches.

Typically, Na is measured with biologic samples like urine, feces and sweat. Samples can be measured using spectrophotometry methods, most commonly atomic absorption spectrophotometry (Palacios *et al.*, 2013). The retention of Na can be relatively measured by subtracting the amount in the urine and feces from the known dietary input. Since this requires strict diet regulation and regular urine collection over at least 24 hours, it can be an impractical method for human studies. Additionally, the validity of current methods used to estimate Na intake from free-living individuals are under question since new data shows that urinary Na excretion does not mirror Na intake on a day-to-day basis (Weaver *et al.*, 2016). This may be explained by the deposit of Na in bodily tissues (Machnik *et al.*, 2009). Extra-renal regulation has been observed in muscle and skin with Na-MRI, which is unable to quantitate bone Na (Titze, 2014). Bone is a storage organ for many metals, including Na (Massey and Whiting, 1996). In this study, a novel neutron activation analysis technology was considered to noninvasively quantify Na in bone in vivo.

In elemental analysis utilizing neutron activation, neutrons interact with elements in the sample to produce characteristic γ-rays. These γ-rays can be detected and their spectrum analyzed to determine what elements are present in the irradiated sample. The neutron source can come from a reactor, an accelerator, a neutron generator, or a radioisotope. For standard neutron activation analysis, a reactor is desirable due to the high flux produced. However, both reactors and accelerators require large dedicated spaces and complicated licensing issues. While radioisotopes are compact, they require complex licensing, constant shielding, and replacement

based on their half-life. Neutron generators, therefore, are a preferable solution when space is limited and both durability and portability are required (Liu, 2015).

Neutron generators are mini accelerators capable of producing neutrons with energies of 2.45 MeV, for deuterium-deuterium (DD) generators, or 14.1 MeV, for deuterium-tritium (DT) generators. In this project, a DD neutron generator based in vivo neutron activation analysis (IVNAA) system is explored for the quantification of Na in bone. Previous experiments have successfully used IVNAA to study Na kinetics in the body using Californium-252 (Cohen-Boulakia, Maziere and Comar, 1981) and an accelerator (Mohseni *et al.*, 2016) as neutron sources. This study is unique in its compact neutron source and optimized irradiation assembly for Na activation. A similar IVNAA system utilizing a compact DD generator has been used successfully to determine manganese (Liu *et al.*, 2017) and aluminum (Byrne *et al.*, 2016) concentrations in human hand bone. This study utilized a newly developed DD generator with a much higher neutron flux. The goal of this study was to optimize the DD neutron generator based IVNAA system for the quantification of Na in bone, to calibrate the system and determine the detection limit of the system, and to show the feasibility of determining small Na changes in bone using this system. Radiation dose to the human subject was also estimated.

Since the current methods used to estimate Na retention in the human body have limitations, a new direct method of measurement would be clinically significant in Na metabolism and retention studies. The potential clinical impacts of the IVNAA system include the ability to directly quantify Na concentration in bone; investigation of Na storage in the human body; better understanding of Na metabolism in the body; examining the relation between Na and Ca in bone; and insight into Na retention and related diseases, such as hypertension and osteoporosis.

2.2 Methods and Materials

2.2.1 Monte Carlo Simulation

The Las Alamos National Laboratory particle transport code Monte Carlo N-Particle (MCNP) 6.1 (Goorley *et al.*, 2012) and ENDF 7 (Chadwick *et al.*, 2011) continuous energy cross-section library were used to simulate IVNAA with a neutron generator and bone equivalent phantom.

2.2.1.1 System Geometry

In order to determine the feasibility of using IVNAA to detect the concentration of Na in hand bone, a model was developed to represent an updated version of the system previously utilized at Purdue University. The model includes a neutron generator, moderator, reflectors, shielding, and irradiation cave as shown in Figure 4. The hand was selected for irradiation due to the ability to extend it away from the body and easily position it. The hand also does not contain active bone marrow in persons above age 10 (ICRP, 1996), so the risk from irradiation can be minimized.

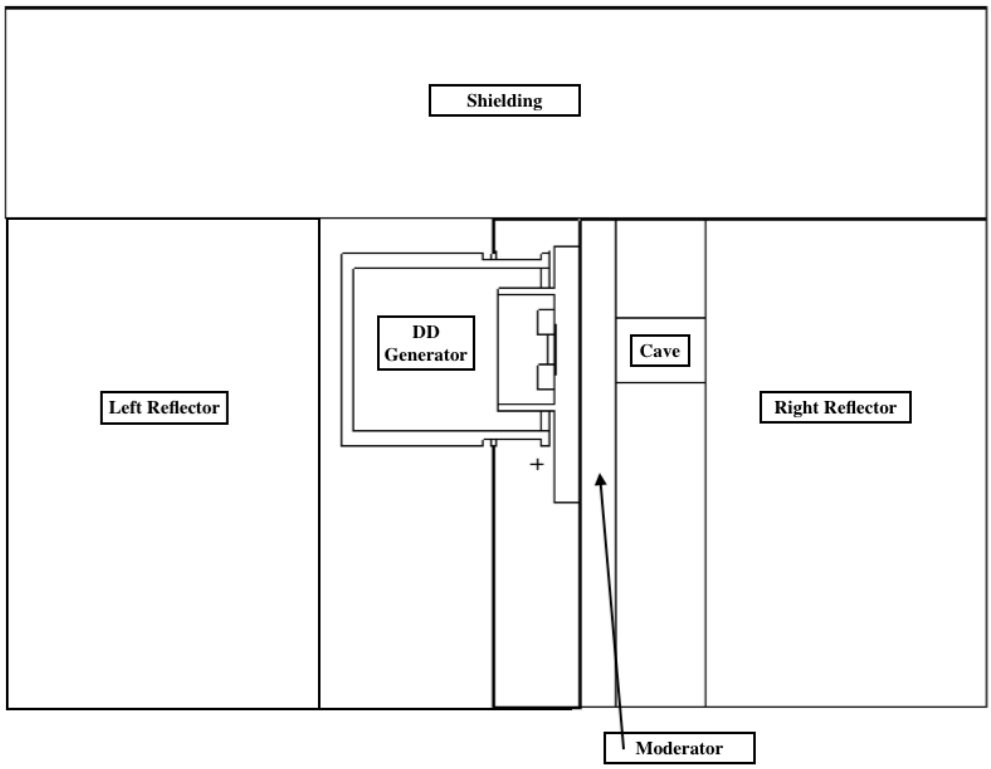


Figure 4 Geometry of MCNP input

For this study, the neutron generator was modeled based on the geometry and configurations of a new generator which is different from the one used in our previous studies (Liu *et al.*, 2014). Polyethylene blocks, with density 0.93 g/cm^3, surround the generator, in order to moderate the neutrons, reflect neutrons towards the irradiation chamber, and provide shielding. The irradiation chamber contains a representative bone phantom, of density 1.85 g/cm^3. Optimization of the irradiation assembly was performed on the moderator, the left and right side reflectors, and the shielding, in order to maximize the thermal neutron flux while keeping the dose to the hand below 50 mSv, 10% of the occupational dose limit to extremities of the body.

2.2.1.2 Neutron Activation

In neutron activation, low energy neutrons interact with Na-23 and produce Na-24 with two major γ-lines with energies of 1368 keV and 2754 keV and a decay half-life of 15 hours. The number of activated nuclide per neutron source at saturation (without considering the irradiation time, decay time, and counting time) inside a sample volume (unit: number/cm^3 per neutron) from a neutron source was simulated in MCNP as RN_0, where R is a response function which corresponds to the normalized integrative cross-section (unit: (number/cm^2) * barn), N_0 is the normalized atom number of the targeted nuclide (unit: atoms/(cm*barn)), and barn is a unit of area equal to 10^{-28} m^2. The normalized atom number N_0 for Na-23 is calculated as:

$$\frac{6.022x10^{23}\ atoms}{mole} \times \frac{mole}{23\ grams} \times \frac{1.293 \times 10^{-6} grams}{cm^3} \times \frac{1x10^{-24} cm^2}{barn} = 3.385x10^{-8} \frac{atoms}{(cm * barn)}$$

Equation 2

Where 1.293×10^{-6} is the density of Na in the bone phantom at the concentration of 1 μg Na/g dry bone (ppm) with a unit of grams/cm^3 and 23 is the atomic weight of 1 mol Na in grams.

Similarly, N_0 for Ca-48 can be calculated as:

$$\frac{6.022x10^{23}\ atoms}{mole} \times \frac{mole}{48\ grams} \times \frac{0.26\ grams}{cm^3} \times \frac{1x10^{-24} cm^2}{barn} = 3.244x10^{-3} \frac{atoms}{(cm * barn)}$$

Equation 3

Where 0.26 g/cm^3 is the density of Ca in the bone phantom at the reference value of 200,000 ppm. These numbers were used in the MCNP input file as tallies to simulate the activation

of Na and Ca in the samples. The tally accounts for the volume of the sample and uses the simulated energy dependent neutron fluence to recall cross-section data with the response function R.

2.2.2 Na Phantoms and System Calibration

Ten bone phantoms doped with different amounts of Na from 0-2000 ppm were constructed to create a calibration line. The phantoms were made following the standardized bone phantom recipe in our previous work (Liu *et al.*, 2014). The phantoms include corresponding amounts of Na according to their concentration, with additional elements found in bone that contribute to the collected spectra, like calcium, chlorine, and magnesium.

All of the phantoms were irradiated for 10 minutes, allowed to decay for 10 minutes, and then measured for 60 minutes to collect a γ-ray spectrum. The net counts from Na and Ca characteristic γ-rays were calculated by an in-house spectral analysis program. The ratios of Na/Ca were calculated and plotted against the Na concentration for system calibration. Since the activated Na and Ca counts came from the same neutron flux and Ca concentration is the same in each phantom and presumably in the bone in *in vivo* measurements, the normalization of Na signal to Ca signal is expected to correct for the variation of several factors such as neutron flux, hand palm attenuation, and counting geometry factors.

2.2.3 DD Neutron Generator and γ-ray Detector

The DD109M neutron generator (Adelphi Technology, Inc., Redwood, CA) is a customized compact deuterium-deuterium generator that produces a close-to-isotropic source of 2.45 MeV neutrons. The flux can be adjusted with current and voltage to produce a flux of up to $5x10^9$ n/s. Neutrons are produced as shown in the equation below.

$$2D + 2D \rightarrow 4He \rightarrow 3He + n$$

Equation 4

One advantage of this generator for *in vivo* quantification is its transportability and accessibility to be used in large populations.

The γ-ray detector used for this study was a 100% high efficiency ORTEK GEM100P4-95 HPGe (AMETEK, Oak Ridge, TN). The software used to collect the γ-ray spectra is MAESTRO by ORTEK (AMETEK, Oak Ridge, TN). The efficiencies of the detector at different energies were previously determined by simulation of a validated MCNP model of the HPGe detector for cylindrical bone phantoms containing multiple radionuclides (Liu, 2015).

2.2.4 Radiation Dose

For IVNAA elemental quantification system, one important restriction is the amount of dose to the living subject. The radiation dose to a human subject in an *in vivo* bone Na measurement was estimated by simulation and experiments. Both neutron and photon doses were simulated and/or measured. In the MCNP simulation, in order to determine the neutron dose to the hand, the dose-to-flux (DF) tally is used. During the measurements, a Fuji electronic personal dosimeter NRF31 was used to measure the neutron and photon dose inside the irradiation cave while a Fuji NSN3 neutron survey meter, a Victoreen 190N portable neutron survey meter, and a Bicron Micro-Rem radiation monitor were used to measure the neutron and photon doses outside the irradiation and shielding assembly.

2.3 Results

2.3.1 System Optimization using MC Simulations

2.3.1.1 Dimension Optimization

The dimensions of the moderator and reflectors were considered independently with high density polyethylene for initial optimization. The ultimate goal of the optimization is to maximize the activated Na atoms in the bone while keeping the radiation dose to the bone at an acceptable level. To achieve that, the ratio of the activation tally, in units of activated Na atoms per neutron, to the dose tally, in units of mSv in 10 minutes, was graphed. A phantom with 1 ppm Na was used for all optimization simulations. The results of the moderator optimization are shown in Figure 5. To maximize the Na activation in bone while keeping the radiation dose under 50 mSv, a moderator thickness of 6 cm was chosen which gives rise to a dose of 46 mSv to the hand bone with a neutron flux of 1×10^9 neutrons/sec for 10 minutes.

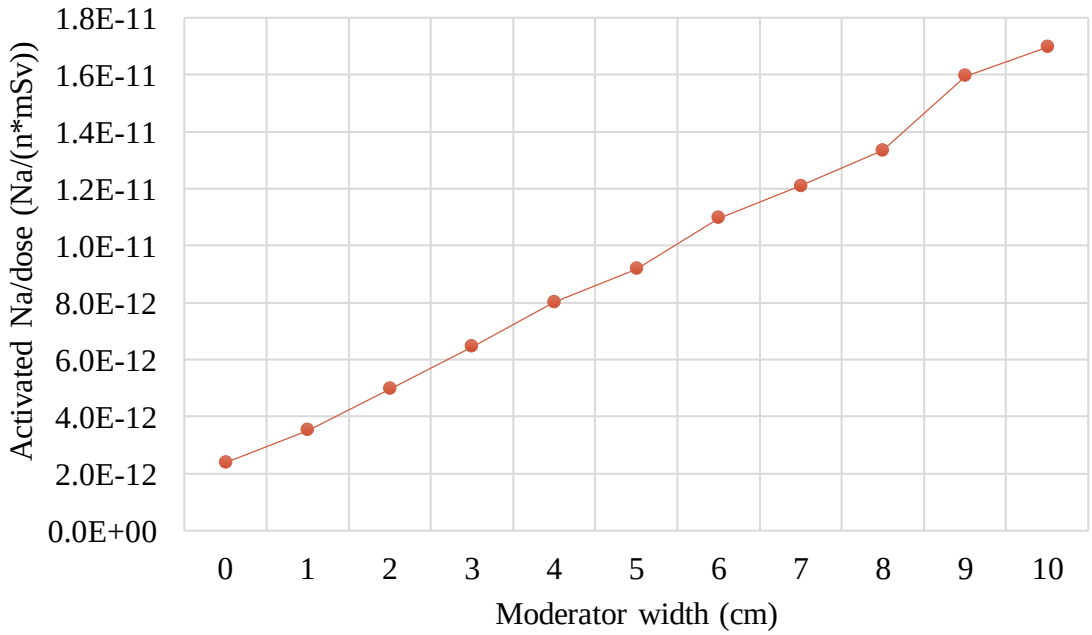


Figure 5 Moderator optimization

The reflector optimization was performed with the optimized moderator in place by finding the point of activation saturation, where an increase in thickness did not result in an increase in activation. For the left reflector, the Na activation tally stayed the same from 4-10 cm, so optimization was not necessary. Thus 10 cm was chosen for modeling the left reflector width based on past irradiation assembly setups. For the right reflector, the output tally was graphed against reflector thickness as shown in Figure 6.

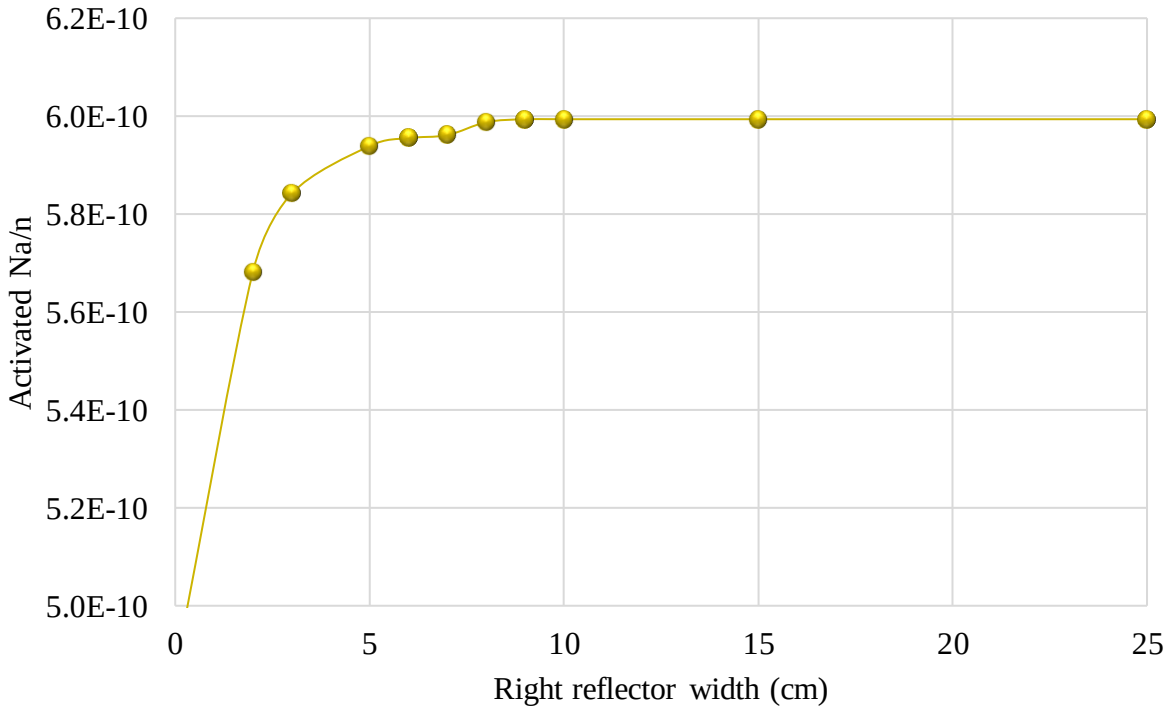


Figure 6 Right reflector optimization

The onset of saturation curve is visible at 9 cm for Na. Thus 9 cm was selected as the optimized right reflector width. In summary, for the final optimized dimensions, the moderator is 6 cm, the left reflector is 10 cm and the right reflector is 9 cm.

2.3.1.2 Material Selection

For the material optimization, four materials were considered, including high density polyethylene (HDPE), graphite, aluminum, and water. For the moderator modeled with different materials, the range of activated Na per neutron was from $6.2x10^{-9}$ to $8.6x10^{-9}$, with graphite yielding the highest activation. Both reflectors were also modeled with all four materials, with results ranging from $5.37x10^{-9}$ to $6.35x10^{-9}$ activated Na per neutron. Both reflectors showed that graphite resulted in the highest activation. This is explained by the high scatter neutron cross section for graphite. However, the selection of the final materials was based on materials readily available and the ratio of activation to dose, which showed that high density polyethylene was the best selection for the right reflector and moderator, while graphite was best for the left reflector. High density polyethylene is readily available, so it was selected for the moderator and reflectors in the final optimized assembly in this study, as shown in Figure 7.

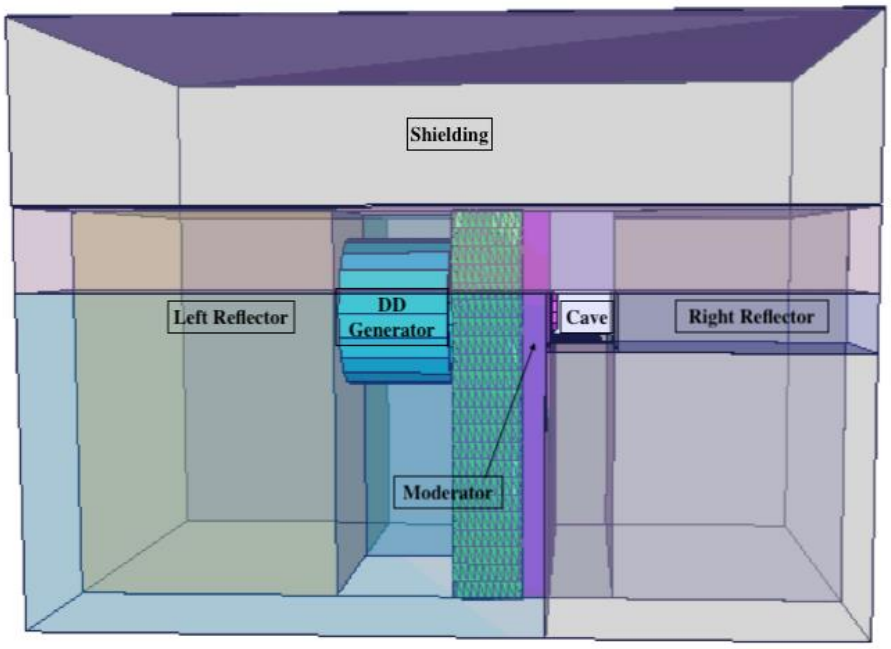


Figure 7 Optimized simulated assembly

2.3.1.3. Optimized Activation Results for 1 ppm Na in Bone

The final optimized input file was run with $1x10^5$ particle histories to give a relative error of <5% for all tallies. The Na activation tally is in units of activated Na atom per neutron source. In order to convert this to units of counts/ppm, the equation below was used.

$$C[\frac{\text{Counts}}{\text{ppm}}] = RN_0 \times \varepsilon \times \theta \times a \times F_n \times s \times d \times c/\lambda$$

Equation 5

RN_0 is from the MCNP tally, as explained previously. The detector efficiency, ε, is dependent on the energy and is estimated based on previous simulation for cylindrical bone phantoms (Liu, 2015). The branching ratio, θ, is the yield for a specific Na-24 γ ray and the abundance, a, is the isotopic abundance of Na-23. The flux, F_n, is $1x10^9$ n/s. The saturation factor s, decay factor d, and counting factor c, were calculated to account for the decay coefficient (λ) with irradiation time, 10 minutes, decay time, 10 minutes, and counting time, 60 minutes.

The final optimized RN_0 was $4.99X10^{-10}$ ± 1.4% activated Na atom per neutron. Using equation 4, the simulated counts were 0.41 counts/ppm Na at 1368 keV and 0.39 counts/ppm Na at 2754 keV.

2.3.2 Experimental Set up

Figure 8 shows the generator with the moderator/reflector/shielding assembly in place.

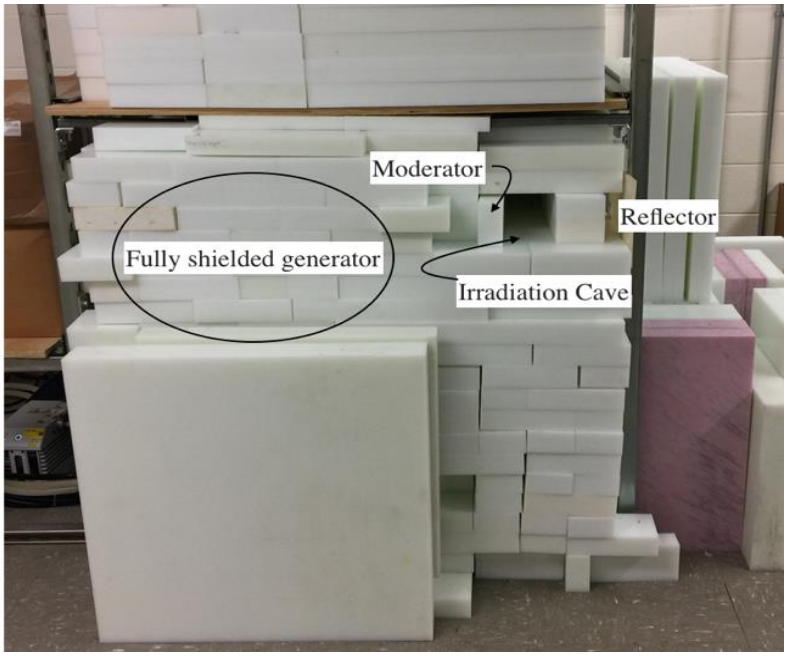


Figure 8 Experimental setup

The protocol for experimental measurements was the same as simulation, with a 10 minute irradiation, 10 minute decay, and 60 minute count time. An electronic personal dosimeter was placed inside the irradiation cavity during experiments to verify dose consistency among experiments. The irradiation cave was also fully closed with additional HDPE during these experiments, while a human arm would normally fill the cavity during the *in vivo* measurements. The detector and detection cave are shown in Figure 9 with phantom in place for measurement. The phantom was placed closely to the detector window to maximize the detection efficiency.

Figure 9 Detection setup

2.3.3 System Calibration

2.3.3.1 Phantom Calibration Lines

The Na bone phantoms were measured and analyzed to determine total Na counts. Figure 10 shows the spectrum of the 1500 ppm sodium phantom after 10 minute irradiation and 60 minute measurement. Using spectral analysis, the experimental counts for Na 1368 keV and Na 2754 keV were 1896 ± 32 and 1074 ± 23, respectively.

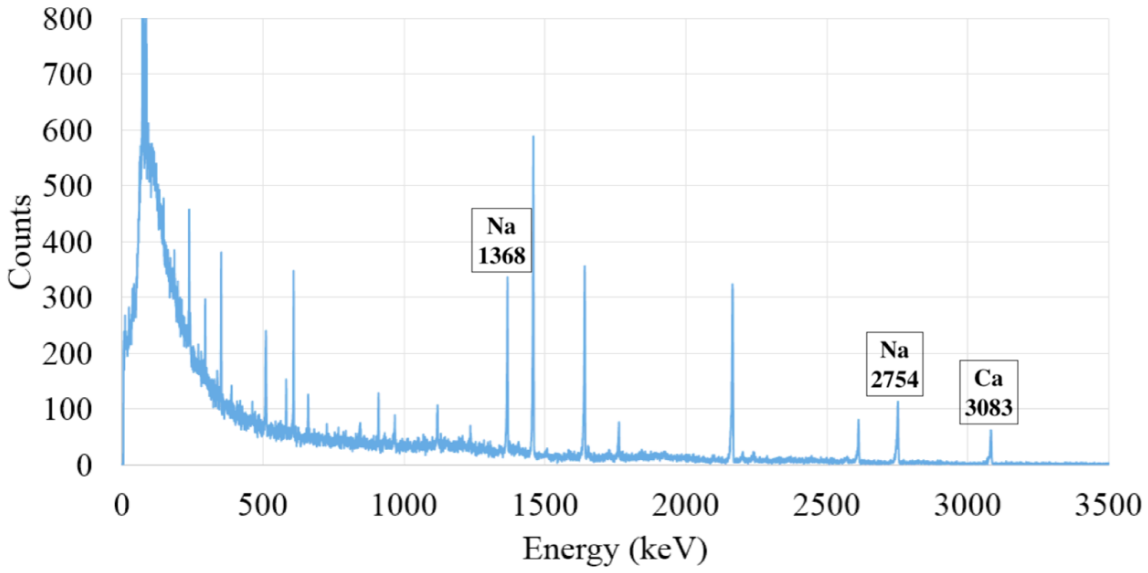


Figure 10 1500 ppm Na phantom 60 minute spectrum

Calibration lines were established using the Na doped phantoms as shown in Figure 11 for 1368 keV and 2754 keV γ-rays. As mentioned before, Na counts were normalized to Ca counts to correct for the variation of several factors such as hand shape and counting geometry factors. The calibration lines will be used to estimate the bone Na concentration in *in vivo* studies.

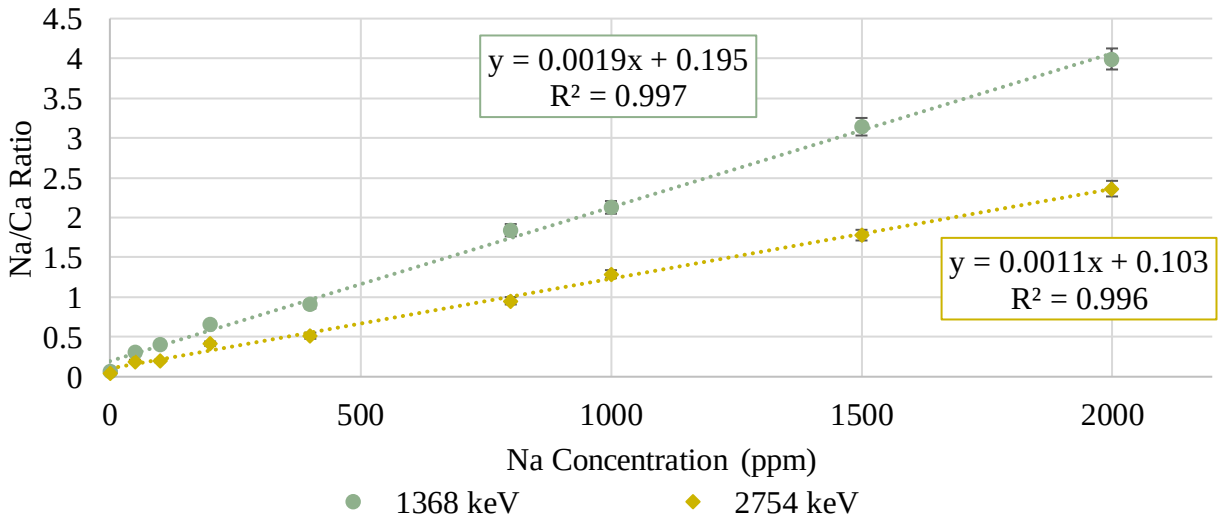


Figure 11 Calibration line for Na

2.3.3.2 Detection Limit

The detection limit was calculated using the equation below, where $\sigma_{\frac{Na}{Ca}}$ is the uncertainty for the Na/Ca ratio. For the experimental detection limit, this was determined using the 0 ppm Na phantom.

$$DL = \frac{2\sigma_{\frac{Na}{Ca}}}{C}$$

Equation 6

In the equation, C is the experimental calibration slope in (Na/Ca)/ppm. The experimental detection limits are thus 26 ppm for Na 1368 keV and 20 ppm for Na 2754 keV. A weighted detection limit was calculated to be 16 ppm, equivalent to 16 μg Na/g dry bone.

2.3.4 Radiation Dose

Using the flux to dose tally conversion, the simulated neutron dose for 10 minutes of irradiation to the hand is 47 mSv. Using a Fuji electronic personal dosimeter NRF31 placed during a 10 minute irradiation, the experimental neutron dose was 46 mSv and the experimental photon dose was 3.6 mSv. Using measurements of about 0.02 mSv/hr neutron dose and 0.1 mSv/hr photon dose to the body outside the assembly during irradiation, the total effective dose as calculated from past *in vivo* experiments (Liu *et al.*, 2014) is estimated to be 27 μSv during a 10 minute irradiation.

2.4 Discussion

Considering bone Na levels in the general population of about 2000-7000 ppm (Harrison, 1937; Bourne, 2014; Richmond, 1985), this system is suitable for detection of Na in the bone with

a detection limit of 16 ppm. The low detection limit also indicates that the system is well suited for the detection of small changes of Na concentration in bone.

The average percent error between simulated and experimental counts is 60%. The difference can be explained by several factors, with the most likely being the uncertainty of the detector efficiency for an arbitrary phantom. A new simulation will be created for the detector with updated phantom geometry.

The hand dose is 10% of the 500 mSv extremity dose limit to occupational workers, but still modifications may be considered to lower the dose before implementation with human subjects. The dose was calculated as discussed in previous *in vivo* feasibility studies (Liu *et al.*, 2014), but the new generator has a higher photon dose. This dose can be lowered with more lead shielding on the assembly in addition to a lead apron for the subject. The benefit of the study to the individual will be considered, as will the age of the subject. Past age 10, there is no active bone marrow in the hand or wrist (ICRP, 1996), but extra external shielding would further reduce dose to the body.

Past Purdue University studies using a compact DD generator have been successful for determining elemental concentrations in bone for several elements including manganese (Liu *et al.*, 2014) and aluminum (Byrne *et al.*, 2016). However, with the investigation of essential instead of potentially toxic trace elements, the focus shifts from measuring low levels of toxicity to detecting small changes, like from a change in diet. The newly developed neutron generator, customized specifically for human hand irradiation, is able to detect elemental concentrations with lower detection limits than the previous system used in our group due to optimized generator geometry and increased neutron flux. This study showed that the detection limit for Na is sufficiently low to feasibly detect small changes in concentrations in human hand bone. Further improvement of the system could be obtained by more optimization parameters including the

exploration of new materials. Optimization of the generator's operation parameters is also being investigated. A second HPGe detector would also lower the detection limit by a factor of 1.4.

The application of the system for Na retention studies is feasible based on the simulation and experimental results. Future studies with both animal and human subjects are in preparation and will be beneficial for determining the effects of Na retention with noninvasive measurements. The current protocol of 10 minute irradiation, 10 minute decay, and 60 minute measurement may need to change in order to separate the Na amount in the soft tissue and in bone. Additional Na γ-ray measurements are required to reveal slow and fast exchange compartments in the hand. Sodium concentration in the bone can be measured as Na in the slow exchange compartment, since Na in the blood will circulate throughout the body and Na in soft tissue will leave with a determinable exchange time after radiation decay is corrected for. The Na/Ca ratio will still be used to correct for the geometry of the hand and can estimate the kinetics of Na exchange among different subjects. Due to the short half-life of Ca-49, the amount of Ca is fixed to the total Ca counts in the first hour of measurement. The radiation decay corrected Na/Ca ratio will initially vary with time, but stabilize in later measurements when Na in the fast compartment leaves the irradiated hand. The stabilized Na concentration can be used to quantify Na in bone, while the changing Na/Ca ratio can be used to estimate Na exchange. Challenges with this model include the difficulty of system calibration and the different tissues exchanging Na over time.

Another possible application of the system is to investigate the effect of low and high Na diets on human health such as its association with hypertension. Through dietary intervention, the system will be able to quantify the effects of dietary Na intake non-invasively, with more accuracy and less time than current collection methods. More importantly, this method yields information

about long term effects of diet since the detection limit is low enough to see small accumulations or deteriorations of Na in bone.

2.5 Conclusion

A feasibility study using Monte Carlo simulations and experiments was performed on the potential use of a customized compact DD neutron generator system for the determination of Na concentration in bone. With the moderator and reflectors optimized to provide maximum flux to the hand while limiting the dose to 50 mSv, the system is able to detect concentrations of Na with a sufficiently low detection limit. Future studies will be performed to investigate the sites of sodium storage in the body and the exchange of sodium in different tissues.

CHAPTER 3. DETERMINATION OF BONE SODIUM (NA) AND NA EXCHANGE IN PIG LEG USING IN VIVO NEUTRON ACTIVATION ANALYSIS (IVNAA)

This chapter contains the published article *Determination of bone sodium (Na) and Na exchange in pig leg using in vivo neutron activation analysis (IVNAA)* by Coyne, M D, Lobene A J, Neumann C R, Lachcik P, Weaver C M and Nie L H in 2019, Physiological measurement 40 075009.

3.1 Introduction

The purpose of this study was to investigate the application of a customized compact deuterium-deuterium (DD) generator for *in vivo* neutron activation analysis (IVNAA) in sodium (Na) nutrition studies. The system was previously optimized and calibrated for human hand irradiation (Coyne *et al.*, 2018) and was adapted for use with live pigs. The two primary objectives of the study were to investigate the use of the system for detecting changes in bone Na from dietary intervention and to investigate the influence of dietary sodium on tissue Na storage and exchange in the body.

Na is an essential element in the body. However, too much Na in the exchangeable fluid can result in high blood pressure which in turn can increase incidence of congestive heart failure or diseases of the liver and kidney (Titze, Müller and Luft, 2014). Na Dietary Reference Intakes are typically exceeded by individuals in the US. The storage site of Na in tissue is likely to influence its ability to influence blood pressure, depending on whether fluid retention is affected. Thus, there is a pressing need to better understand how Na is metabolized and stored in the body and the influence of perturbing factors such as dietary Na intake on tissue Na storage and, ultimately, disease progression.

Typically, Na is measured within biologic samples such as urine. However, the validity of current methods used to estimate Na intake from free-living individuals is under question since

new data show that urinary Na excretion does not mirror Na intake on a day-to-day basis (Weaver *et al.*, 2016). This may be explained by the deposit of Na in bodily tissues (Machnik *et al.*, 2009). Indeed, bone is a storage organ for many metals, including Na (Massey and Whiting, 1996). Na is primarily stored in the matrix of cortical bone. The effect of bone Na stores on urinary Na excretion and estimates of Na intake, as well as the effect of these stores on human health, remain to be elucidated. In order to do this, a non-invasive method for quantifying bone sodium content *in vivo* is needed. In this study, a novel *in vivo* neutron activation analysis (IVNAA) technology was applied to noninvasively quantify Na in pig bone. Because the produced radionuclide Na-24 has a physical half-life of 15 hours, its exchange between the pig leg and the rest of the pig body was also studied.

3.2 Materials and Methods

3.2.1 *In Vivo* NAA (IVNAA) System Setup and Measurement of Na

The neutron source for irradiation was a DD109M neutron generator (Adelphi Technology, Inc., Redwood, CA). The DD109M is a customized compact deuterium-deuterium generator that produces a close-to-isotropic source of 2.45 MeV neutrons. The flux can be adjusted with current and voltage to produce a flux of up to $5x10^9$ n/s. The generator has a modifiable moderator/reflector/shielding assembly previously optimized for human hand irradiation (Coyne *et al.*, 2018). This study used a larger irradiation cave with adjusted reflector/shielding assembly to accommodate a young pig.

With neutron activation of Na, low energy neutrons interact with Na-23 and produce Na-24 with two major γ-lines with energies of 1368 keV and 2754 keV. The 100% abundance of Na-24, along with the high branch ratios of 90% for 1368 keV and 100% for 2754 keV, accommodate

high sensitivity in the detection of activated sodium. Similarly with calcium (Ca), Ca-48 is activated to produce Ca-49 with a distinct γ-line of energy 3083 keV. Since the activated Na and Ca counts are from the same neutron flux, the normalization of Na signal to Ca signal is expected to correct for the variation of several factors including neutron flux, leg attenuation, and counting geometry factors.

The net counts from Na and Ca characteristic γ-rays are calculated by an in-house spectral analysis program. The γ-ray detector used for this study was a 100% high efficiency ORTEC GEM100P4-95 HPGe (AMETEK, Oak Ridge, TN). The software used to collect the γ-ray spectra is MAESTRO by ORTEC (AMETEK, Oak Ridge, TN).

3.2.2 Pig Characteristics and Dietary Intervention

All study personnel who interacted with the pigs completed the appropriate trainings, and all study procedures were approved by the Animal Care and Use Committee at Purdue University. This study was completed on two male domestic commercial pigs. Pigs were acquired shortly after weaning and housed together in the large animal facility at Purdue University. Pigs acclimated to the facility and gradually transitioned to the study diet for 4 days. On day 5 pigs were fully transitioned to diet. Pig 1 was fed a low Na diet (0.2% Na w/w) and pig 2 was fed a high Na diet (0.6% Na w/w) (Research Diets, Inc). Both diets were a pelleted casein-based growing swine feed, and Na was provided in the form of NaCl. Both pigs consumed their diet for a full 14 days before IVNAA measurements were conducted. At the time of the IVNAA measurements, the low and high Na pig weighed 16.8 kg and 17 kg respectively and both had a leg circumference of 15 cm at the irradiation site.

3.2.3 IVNAA for Na Measurement in Pig Leg and Leg Bone

The right posterior leg of a live pig was irradiated for 10 minutes with the center of irradiation aimed as shown in Figure 12. During irradiation and subsequent detections, pigs were anesthetized to prevent movement and intubated to maintain a clear airway while under anesthetic. Vital signs were monitored every 10 minutes to ensure the safety of the animals.

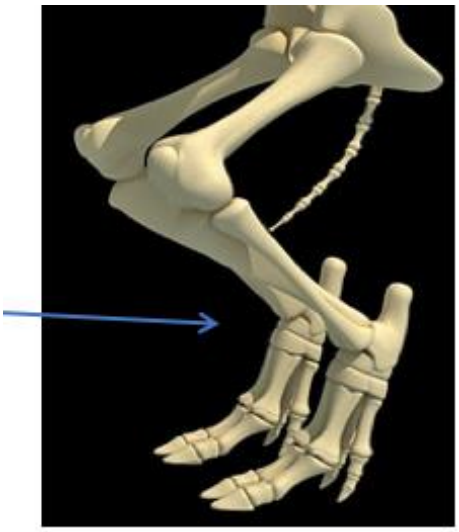


Figure 12 Location of irradiation on pig leg

The irradiation cave was modified for this study in order to comfortably accommodate the pig, as shown in Figure 13. The non irradiated left leg was shielded and an electronic dosimeter was placed to record the given dose. Both pigs received an extremity dose of 45 mSv to the right hind leg, which agrees with the anticipated dose from simulation and calibration (Coyne *et al.*, 2018). Accounting for a tissue weighting factor of 0.01 for the leg skin and bone surfaces, and with the irradiated area estimated to be 1.25% of the total body weight, the effective dose to each pig was 5.63 μSv.

Figure 13 Modified irradiation cave for pig study

Following irradiation, the pigs were moved to the detector as shown in Figure 14, where consistent leg placement was visually confirmed for each measurement.

Figure 14 Detection of irradiated pig leg

The pig legs were measured with the High Purity Germanium detector (HPGe) for up to 2 hours (with 5 minute counting intervals) at decay times of 4 minutes, 7.5 hours, and 21.5 hours.

The pigs were anesthetized during the measurements, but were awake and able to move between measurements. The anesthesia process contributed to slight differences in detection start and end times between pigs. Following the final measurement, the pigs were euthanized, and both hind legs were removed and dissected.

3.2.4 *Ex vivo* NAA for Na Measurement in Pig Leg and Leg Bone

All four hind legs were irradiated and detected following pig dissection. The legs were first irradiated as a whole piece and then dissected into muscle, skin and bone groups.

3.3 Results

3.3.1 Na Concentration

3.3.1.1 In Vivo Spectrum and First 2-hr Measurement

Figure 15 below shows an *in vivo* spectrum of the low Na diet pig during the first 60 minutes of detection.

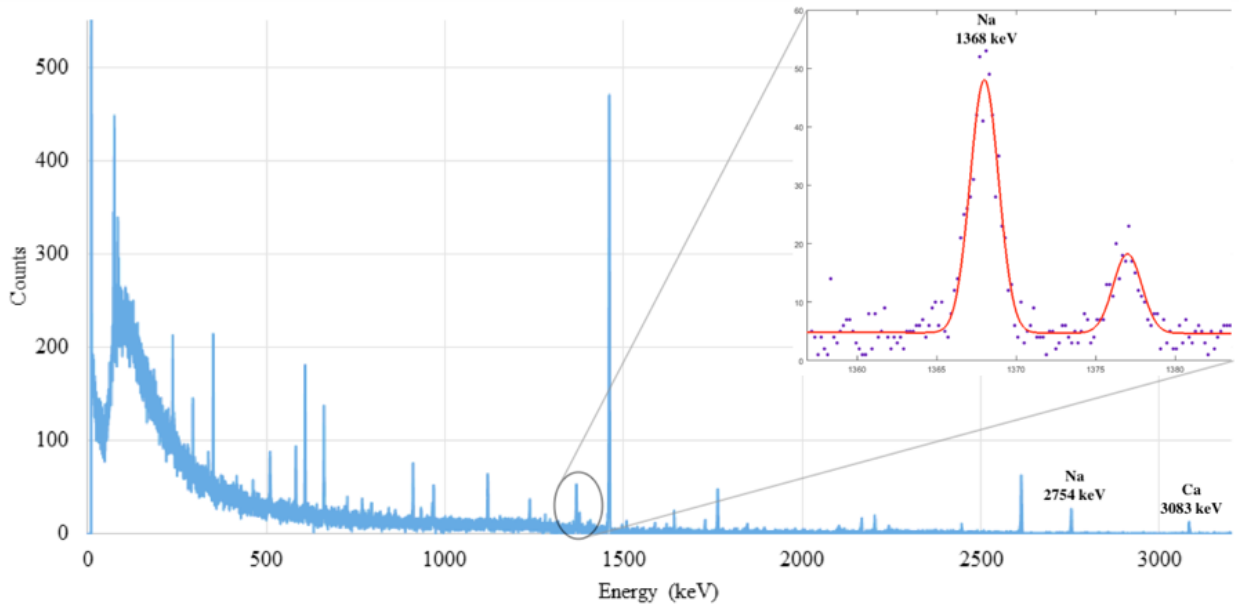


Figure 15 Low Na pig *in vivo* spectrum

Spectral analysis was performed to determine the net Na and Ca counts for each pig during the first 60 minutes of measurement. The ratio of Na to Ca counts is used in order to normalize the Na counts to bone Ca for each pig, as shown in Figure 16. Since Ca has a short half-life of 8.7 minutes, the total Ca counts during the first 10 minutes of detection were used to normalize each Na count interval. All Na counts were decay corrected to represent Na counts at time 0, immediately following irradiation. The decay corrected intervals show higher Na counts per Ca counts for the high Na diet pig, which is also observed with the 2754 keV Na counts.

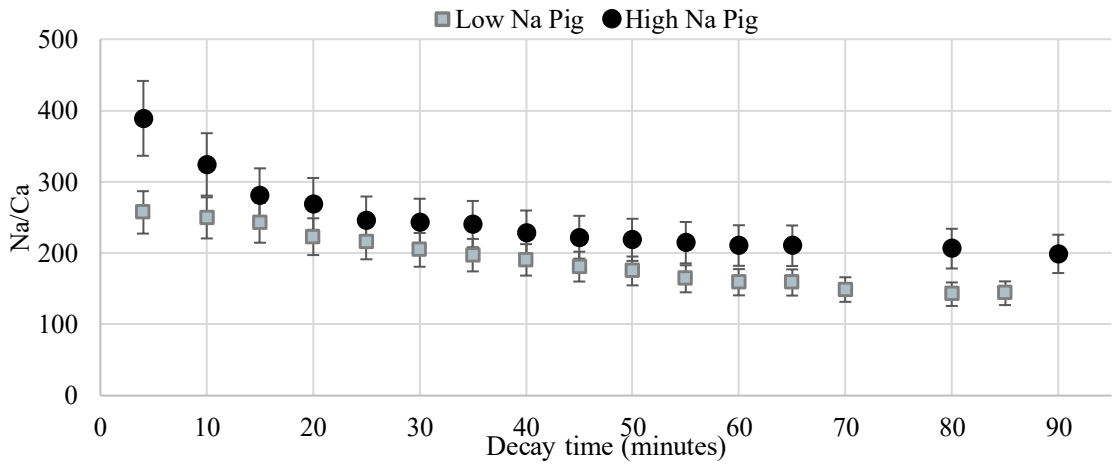


Figure 16 First measurement, Low Na vs High Na Diet Pig Na/Ca counts at 1368 keV

The linear trend lines from the previously obtained bone Na concentration calibration curves (Coyne *et al.*, 2018; Coyne, 2018) were used to approximate Na concentration of the pig leg. Using the first 1 hour *in vivo* sum of Na and Ca counts, the low Na pig is estimated to have a Na/Ca concentration of 2441 +/- 186 ppm while the high Na pig is estimated to have a Na/Ca concentration of 2812 +/- 232 ppm. This results in a concentration difference of 372 +/- 297 ppm ($p=0.22$).

3.3.1.2 Comparison among Low and High Na Diet Pig at Second 2-hr Measurement and Bone Na

The initial measurement reflects Na signal from the entire leg, including highly exchangeable blood. It is hypothesized that bone Na concentration is better reflected in later measurements, when the activated Na in blood has left the leg and the stable Na signal reflects Na in slowly exchanging compartments, like bone. Figure 17 shows the comparison in Na/Ca ratio during the second 2 hour measurement, 7 hours post irradiation. The figure is corrected for radioactive decay and counting time and shows the high Na diet pig has a higher normalized ratio

than the low Na diet pig. A similar trend is seen during the third 2 hour measurement, 14 hours post irradiation, and both energies of Na show the same pattern.

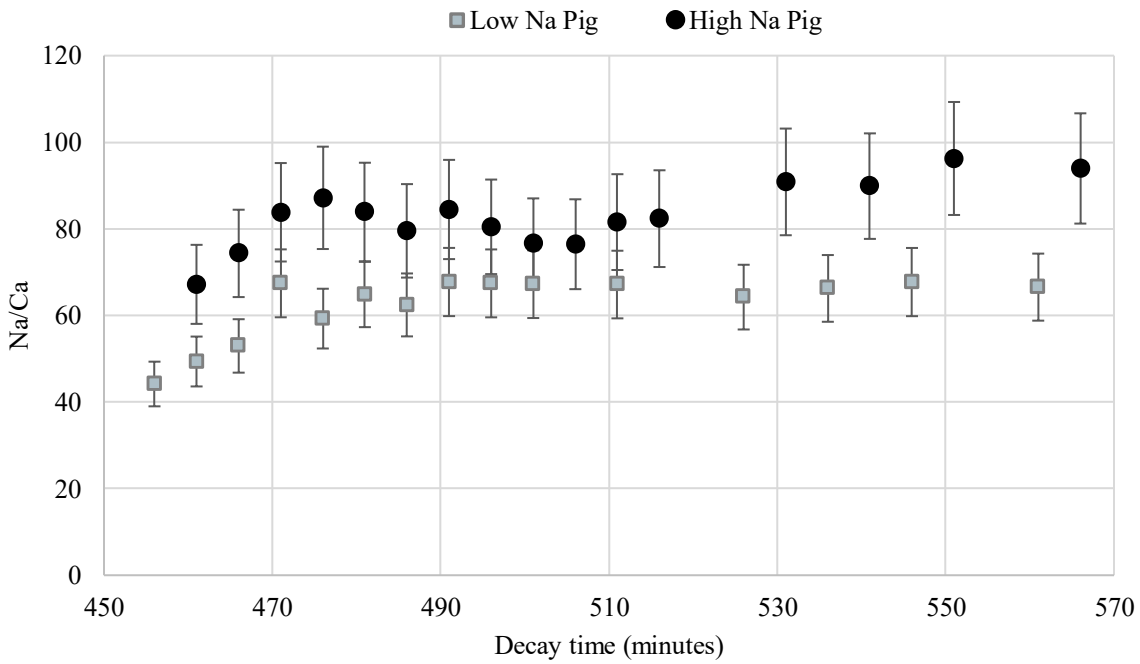


Figure 17 Second measurement, Low vs High Na Diet Pig Na/Ca counts at 2754 keV

The linear trend lines from the previously obtained bone Na/Ca calibration curves (Coyne *et al.*, 2018) are used to approximate Na/Ca concentration of the pigs. Using the calibration curve adjusted for 7 hours of decay and the *in vivo* measurements averaged during the second measurement, the low Na diet pig is estimated to have a Na/Ca bone concentration of 1165 +/- 80 ppm while the high Na diet pig is estimated to have a Na/Ca concentration of 1631 +/- 111 ppm. This results in a concentration difference of 466 +/-137 ppm ($p=0.041$). Since the amount of activated Na is relatively stable during the second measurement, these concentrations most likely reflect bone content. These results exhibit a clear separation in bone Na concentration over a short dietary intervention time, for the two pigs studied.

3.3.1.3 Ex Vivo Comparison

Following the completion of the *in vivo* study, the removed pig legs were first irradiated whole as *ex vivo* samples, with the same irradiation protocol as the *in vivo* study. Using the bone Na phantom calibration, the low Na diet pig had an *ex vivo* Na concentration of 5547 +/- 331 ppm and the high diet Na pig had a concentration of 6836 +/- 433 ppm, resulting in a difference of 1288 +/- 545 ppm. However, the calibration was not intended for *ex vivo* analysis, so these estimated concentrations are provided to further demonstrate the difference in Na concentration between the low and high Na diet pigs, as seen in the *in vivo* study. The *ex vivo* concentrations are higher due to the lack of circulation and exchange of Na in the leg, and shows the significant contribution of Na from the fast exchange compartment that decreases during *in vivo* measurements. Additionally, the *ex vivo* concentration is higher due to the difference in irradiation and detection geometry between a live pig and a dissected leg. Despite the differences, these data can be used for designing future *in vivo* studies.

In order to determine where Na is stored in the body, the legs were dissected and irradiated under the same conditions as the *in vivo* study. The results show Na is present in all categories of dissected leg- bone, muscle, skin and connective tissue. Ca was only found in bone. As shown in Figure 18 for 2754 keV, there is significant storage of Na in the short bones, which are the primary target of irradiation. These results were mirrored at 1368 keV.

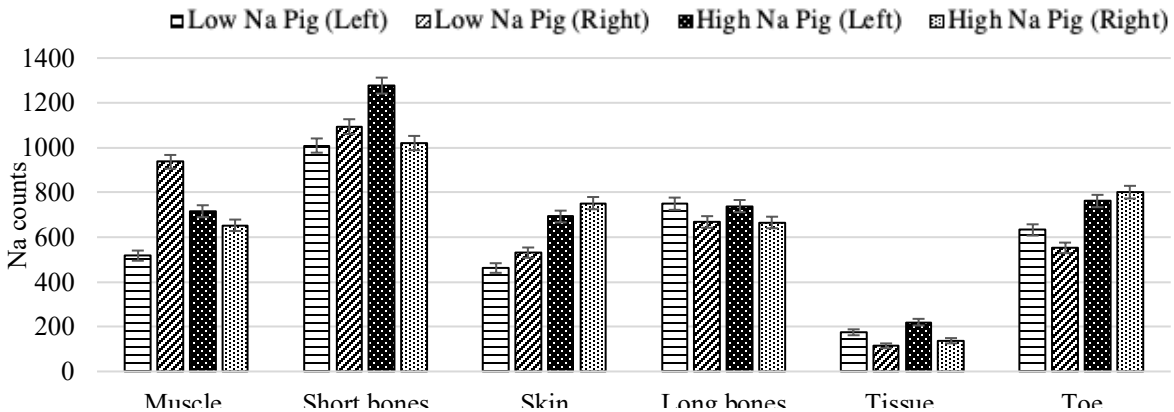


Figure 18 Ex vivo net Na counts at 2754 keV

The Na counts were normalized to total weight of disected pieces to yield Na per gram as shown in Figure 19.

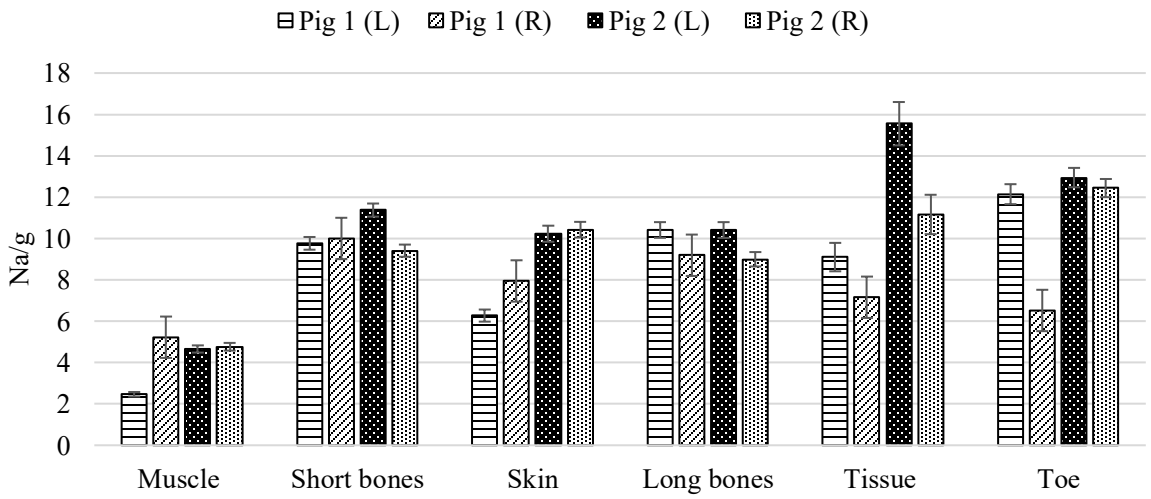


Figure 19 Na counts at 2754 keV normalized to weight

The two hour bone Na counts normalized to 1 hour Ca counts are shown for all 4 legs in Figure 20 for 2754 keV. Accounting for error propagation, the *ex vivo* bone results do not separate as distinctly as for *in vivo* results due to dietary Na levels. Variance was performed on the *ex vivo*

Na/g data to determine if there is a significant difference between the groups studied. These results are shown in Table 2. As expected, there is not a significant difference between the data sets from the two energies of Na, which matches the trend shown in the *in vivo* data. There is also not a difference between Na counts measured in the right hind leg compared to the left. The difference in Na/g between the high and low Na diet pig is marginally significant, based on the *ex vivo* data. When looking at just the results from 1368 keV, which has a higher detection efficiency, there is a significant difference (p=0.028, n=12) between the dissected samples from the high and low Na diet pigs. More pigs are needed to confirm these results, but the *ex vivo* data confirms the *in vivo* conclusion for the two pigs studied.

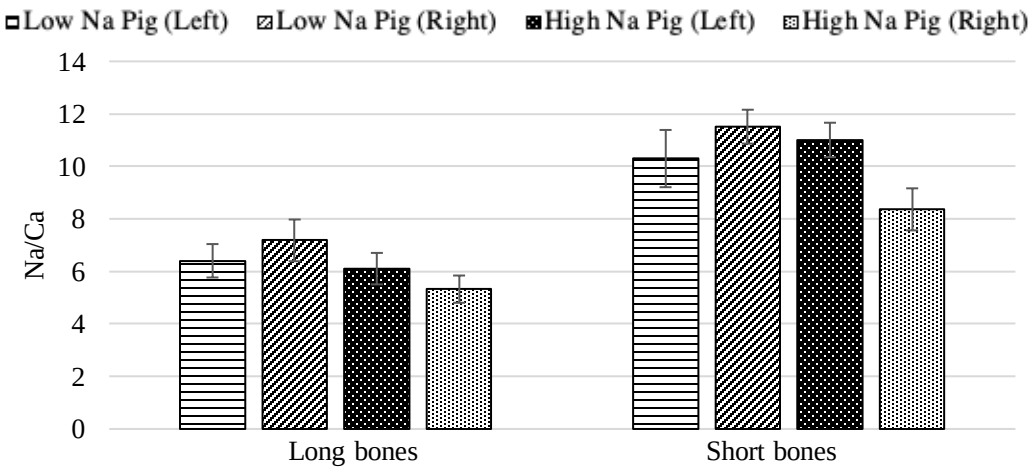


Figure 20 Na/Ca ratio at 2754 keV

Table 2 *Ex vivo* variance with n=24

Comparison	P-value	Significant?
Energies (1368 & 2754 keV)	0.85	No
Legs (left & right)	0.49	No
Pigs (high & low Na diet)	0.06	Marginally

3.3.2 Na Exchange and Storage

In order to examine the exchange of Na in the leg bone, radioactive decay is corrected with the use of saturation, decay and counting factors specific to Na-24 (Coyne *et al.*, 2018). In this study, the time of irradiation was 10 minutes, while the decay and count time varied throughout the measurements. The corrected activity over time is plotted in order to find the exchange half-life, t_e, from the equation $m=\ln 2/t_e$, where m is the slope from the fitted equation $y=Ae^{-mx}$. In order to determine Na exchange times, decay corrected interval Na counts were normalized to a one hour Ca count.

For the first measurement post irradiation, the radioactive decay corrected exchange of Na in the low Na diet pig is shown in Figure 21. The low Na diet pig only completed 80 minutes of the first 2 hour detection session due to issues with anesthesia. The radioactive decay corrected exchange of Na/Ca during the first measurement in the high Na diet pig is shown in Figure 22.

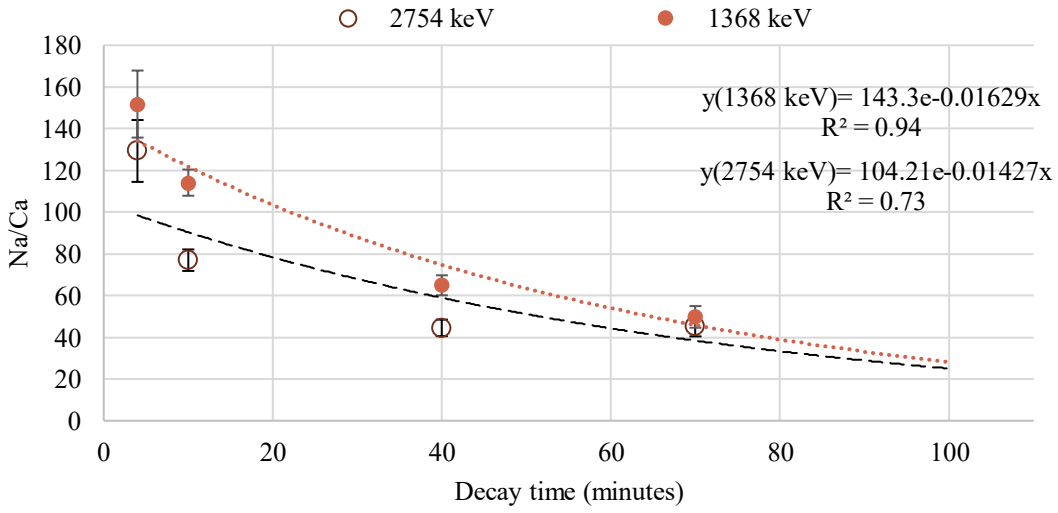


Figure 21 Low Na Diet Pig fast Na exchange

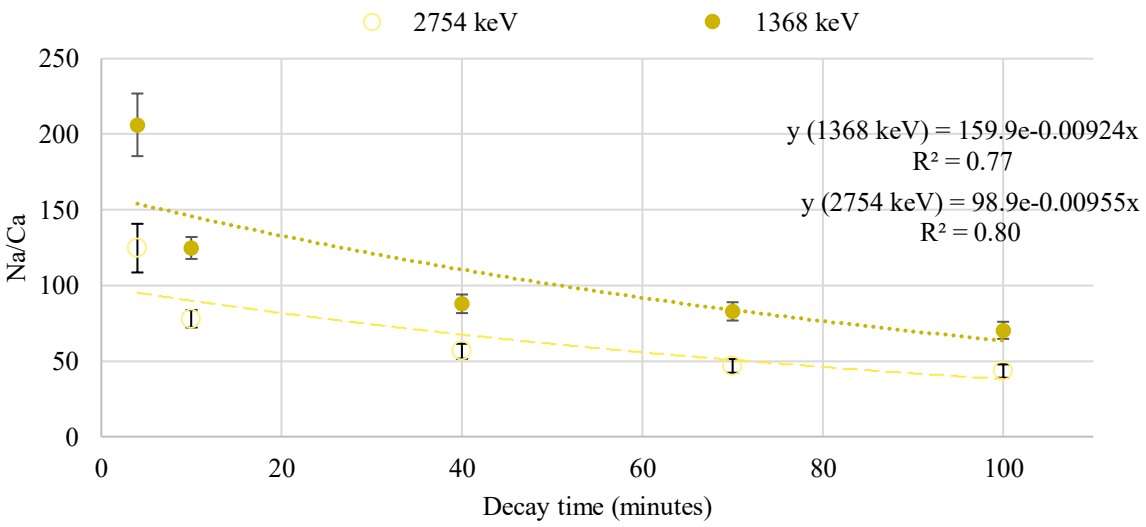


Figure 22 High Na Diet Pig fast Na exchange

To investigate slow exchange of Na, the 2 hour measurements taken at 7 and 21 hours post irradiation are used. For the second and third measurements, the radioactive decay corrected exchange of Na/Ca in the low Na diet pig is shown in Figure 23 and in the high Na diet pig in Figure 24.

For both pigs, the last measurement point from the first 2 hour measurement is relatively stable with the second and third hour measurements. This indicates that the fast exchange half-life can be roughly approximated from the first 90 minutes of measurement for both pigs. Using the 80 minute measurement, the low Na diet pig has an approximate fast exchange half-life of 45 minutes. The fast exchange half-life of the high Na diet pig based on the first 90 minutes of measurement is estimated to be 56 minutes. This results in an average Na fast exchange half-life of 51 minutes for this study. Analysis shows rapid exchange of Na in the leg during the first 2 hour measurements, while the exchange was minimal at the second and third 2 hour measurements, taken 7 and 21 hours post irradiation. There was no significant change of Na in the leg between 2-21 hours.

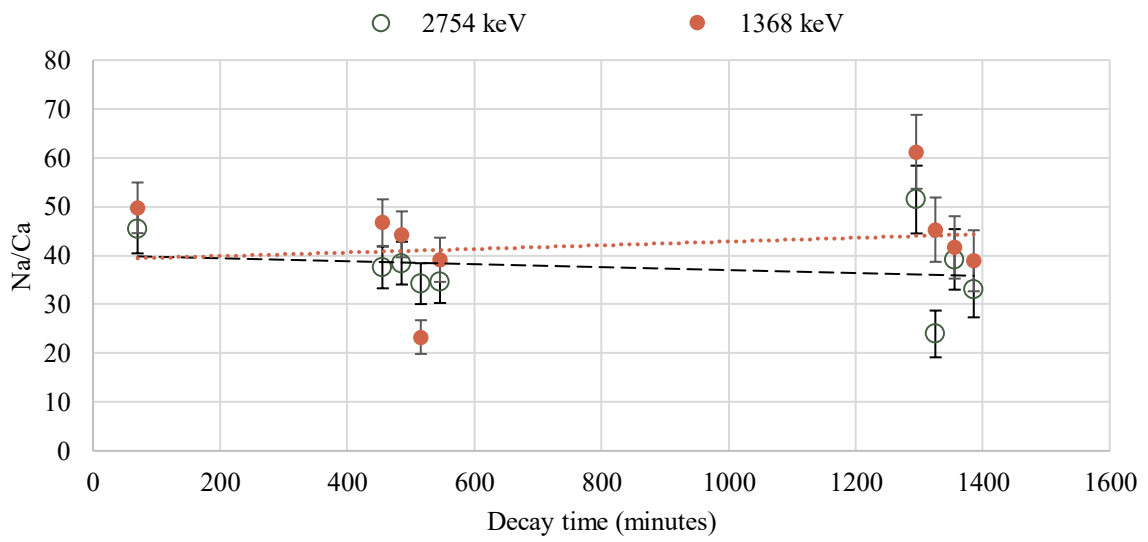


Figure 23 Low Na Diet Pig slow exchange of Na

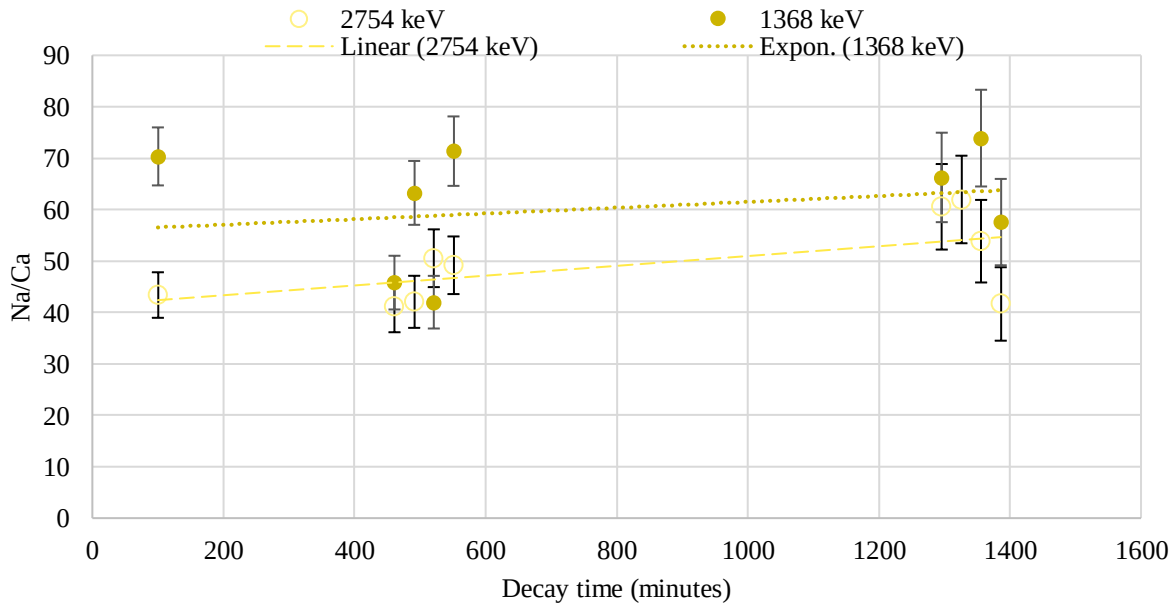


Figure 24 High Na Diet Pig slow exchange of Na

3.4 Discussion and Conclusion

The IVNAA system is able to non-invasively detect differences in tissue Na in pigs consuming low vs high Na diets for a relatively short period of time (14 days). The *in vivo* measurement showed a significantly higher Na/Ca ratio at the second 2-hr measurements. The difference is also observable at the first and third 2-hr measurements. The *ex vivo* analysis shows Na stored in all measured tissues including bone, muscle, skin and soft tissue, as well as significantly higher concentrations of Na/g in bone, soft tissue, and skin. The *ex vivo* results confirm a significant difference in Na between the high and low Na diet pigs studied.

The estimated fast exchange half-life of 51 minutes agrees with a past *in vivo* neutron activation analysis study performed with Californium-252 in human hand that found a fast exchange half-life of 1 hour (Comar, 1981). The Cf-252 study found exchange half-lives of 79 hours in normal bone and 35 hours in bone with low Ca concentration. The direct relationship in bone Na and Ca concentration suggests that the slowly exchangeable compartment behaves the same as bone Ca, although more analysis is needed to show the mechanisms of Na deposition and exchange over time. Further, the ratio of Na to Ca is consistent throughout the skeleton, indicating the slow exchange compartment may be independent of the fast exchange compartments. (Comar, 1981)

The fast exchange compartments are hypothesized to be soft tissue, muscle, and/or skin. Skin has been observed as an osmotically inactive storage site for excess salt from dietary sources (Titze *et al.,* 2003). Na MRI studies show Na storage in the epidermis with a hypothesized countercurrent system for electrolyte exchange (Hofmeister, Perisic and Titze, 2015). During the next pig study, the kinetics of Na exchange and storage in soft tissue will be studied with a Na coil MRI and direct chemical Na analysis of tissues to complement the IVNAA bone measurements.

These findings suggest that dietary Na intake affects tissue Na storage, and that a higher Na diet increases these tissue Na stores. Understanding the storage and exchange of Na will have important implications for human health, especially for chronic diseases such as cardiovascular disease and osteoporosis. With the *in vivo* and *ex vivo* pig study results, we conclude there is a non or low exchangeable compartment (likely to be bone) for Na storage and that the DD neutron generator-based IVNAA is a useful method in Na nutrition studies.

There are important limitations to this study, however. More pigs are needed to verify our results with true statistical significance. More *in vivo* measurements with longer count times would help clarify the exchange time and reduce radiation counting errors. Additionally, 14 days may not be enough time for excess Na to deposit in bone. Future studies will be conducted to address these limitations and further investigate the potential of the system with refined methodology. A larger sample size will be used to clarify the variation in response to dietary Na. A precision study is planned to determine the reproducibility of the measurement. Baseline IVNAA measurements will be added before the dietary intervention starts to better observe the effects of the diet on Na concentration and exchange. The addition of MRI measurements will also help understand the storage and exchange of Na in soft tissues, but not bone.

The system is also anticipated for use in human studies with planned applications in dietary Na interventions. The use of IVNAA will help quantify the long-term effects of dietary change on bone Na and the relation to Ca retention, since research shows bone Ca and Na are deposited and stored similarly. IVNAA has the potential to be instrumental in understanding Na mechanisms in the body and in the prevention of Na related diseases.

CHAPTER 4. CHAPTER 4 DETERMINATION OF TISSUE SODIUM (NA) AND NA METABOLISM IN PIG LEG USING IN VIVO NEUTRON ACTIVATION ANALYSIS (IVNAA)

This chapter contains the unpublished article *Determination of Tissue Sodium (Na) and Na metabolism in Pig Leg using In Vivo Neutron Activation Analysis (IVNAA)* by Coyne, M D, Lobene A J, Lachcik P, Weaver C M and Nie L H, expected to be submitted in 2020.

4.1 Introduction

The purpose of this study was to further investigate the application of a customized compact deuterium-deuterium (DD) generator for *in vivo* neutron activation analysis (IVNAA) in sodium (Na) nutrition studies. The system was previously optimized and calibrated for human hand irradiation (Coyne *et al.*, 2018) and was adapted for use with live pigs (Coyne *et al.*, 2019). The primary objective of this study was to investigate the use of the system for detecting changes in both bone and soft tissue Na during dietary intervention. The simultaneous *in vivo* measurement of both bone and tissue Na would be valuable in nutrition studies, but is currently not possible with any other methods.

Our previous work indicates the customized IVNAA Na system is able to detect changes in bone Na in pigs on controlled Na diets. This study used 2 additional pigs with a few key changes to further investigate the use of IVNAA. First, the pigs were measured with the IVNAA system to determine initial tissue and bone Na concentration before starting the diet. Second, the pigs also had concurrent MRI (Magnetic Resonance Imaging) Na studies to determine soft tissue Na both before and after the diet. Third, adjustments and improvements to the IVNAA system were implemented, including the addition of a second HPGe detector. These changes contributed to a better understanding of the IVNAA results, which contributed to establishing new methodology for estimating soft tissue Na concentration with IVNAA.

Soft tissue Na has been previously estimated in human hand with IVNAA using Cf-252 (Cohen-Boulakia, Maziere and Comar, 1981). Building on this work, soft tissue Na was estimated in pig leg using IVNAA and compared to MR and ICP values.

4.2 Materials and Methods

4.2.1 *In Vivo* NAA (IVNAA) System Setup and Measurement of Na

The neutron source for irradiation was a DD109M neutron generator (Adelphi Technology, Inc., Redwood, CA). The DD109M is a customized compact deuterium-deuterium generator that produces a close-to-isotropic source of 2.45 MeV neutrons and flux of up to $5x10^9$ n/s. The generator has a modifiable moderator/reflector/shielding assembly previously optimized for human hand irradiation (Coyne *et al.*, 2018). This study used a larger irradiation cave with adjusted reflector/shielding assembly to accommodate a young pig, as past studies discussed.

With neutron activation of Na, low energy neutrons interact with Na-23 and produce Na-24 with two major γ-lines with energies of 1368 keV and 2754 keV. The 100% abundance of Na-24, along with the high branch ratios of 90% for 1368 keV and 100% for 2754 keV, accommodate high sensitivity in the detection of activated sodium. The long half-life of Na-24, 14.9 hours, allows for detection long after neutron irradiation. This means Na can be tracked throughout the body for multiple days. Similarly, with calcium (Ca), Ca-48 is activated to produce Ca-49 with a distinct γ-line of energy 3083 keV. Since the activated Na and Ca counts are from the same neutron flux, the normalization of Na signal to Ca signal is expected to correct for the variation of several factors including neutron flux, leg attenuation, and counting geometry factors. However, since the half-life of Ca-49 (8.7 minutes) is much shorter than Na-24 (14.9 hours), only the Ca counts during the first measurement period, immediately following irradiation, can be used.

The two γ-ray detectors used for this study were both 100% high efficiency ORTEC High Purity Germanium (HPGe) Detectors (AMETEK, Oak Ridge, TN). This study added a n-type detector opposite the original p-type detector used in past studies. This allows for increased detection capability and lowers the detection limit, providing increased sensitivity. More lead shielding was also added to decrease background during detection. The software used to collect the γ-ray spectra is MAESTRO by ORTEC (AMETEK, Oak Ridge, TN). An in-house peak fitting program was used to perform spectral analysis.

4.2.2 Pig Characteristics and Dietary Intervention

All study personnel who interacted with the pigs completed the appropriate trainings, and all study procedures were approved by the Animal Care and Use Committee at Purdue University. This study was completed on two male domestic commercial pigs. Pigs were acquired shortly after weaning and housed together in the large animal facility at Purdue University. Pigs acclimated to the facility for 1 week and received a baseline IVNAA measurement in addition to a MR Na scan. At the time of the baseline IVNAA measurements, the low and high Na pig weighed 12.2 kg and 11.1 kg respectively and both had a leg circumference of about 14 cm at the irradiation site.

Following baseline measurements, the pigs were fully transitioned to the intervention diet. Pig 3 was fed a low Na diet (0.2% Na w/w) and pig 4 was fed a high Na diet (0.6% Na w/w) (Research Diets, Inc). Both diets were a pelleted casein-based growing swine feed, and Na was provided in the form of NaCl. Both pigs consumed their diet for a full 14 days before final IVNAA measurements were conducted. At the time of the final IVNAA measurements, the low and high Na pig weighed 24.7 kg and 22.7 kg respectively and had a leg circumference of 22.6 cm and 20.6 cm at the irradiation site.

4.2.3 IVNAA for Na Measurement in Pig Leg

4.2.3.1 Experimental Setup and Protocol

The right posterior leg of a live pig was irradiated for 10 minutes. During irradiation and subsequent detections, pigs were anesthetized to prevent movement and intubated to maintain a clear airway while under anesthetic. Vital signs were monitored every 10 minutes to ensure the safety of the animals.

The irradiation cave was modified for this study in order to comfortably accommodate the pig, as shown in Figure 25. The non-irradiated left leg was shielded and an electronic dosimeter was placed to record the given dose. Both pigs received an extremity dose of 45 mSv to the right hind leg during both baseline and final measurements, which agrees with the anticipated dose from simulation and calibration and the dose during the previous pig study.

Figure 25 Modified irradiation cave for pig study shown at a) baseline and b) post diet, with open views from c) top and d) side

Following irradiation, the pigs were moved to the detectors as shown in Figure 26, with the leg placed as close to the n-type (left) and p-type (right) detectors as anatomically possible, while also still allowing for constant vital monitoring.

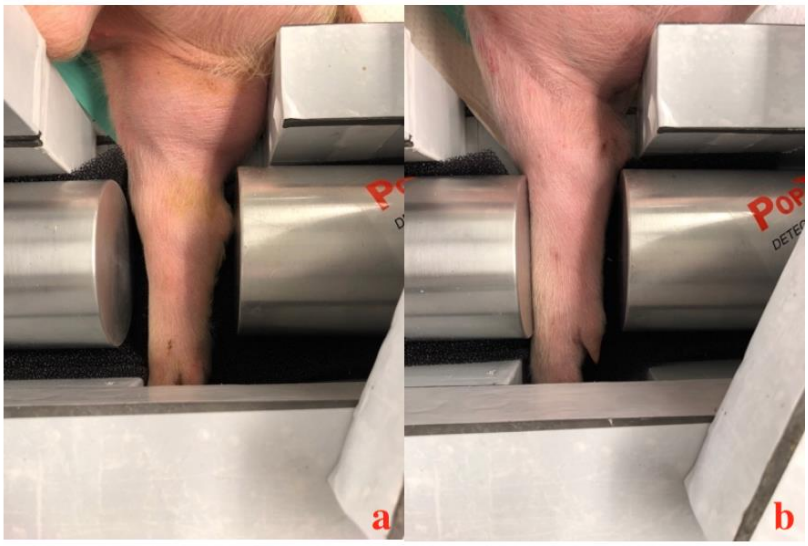


Figure 26 Detection of irradiated pig leg at a) baseline and b) post diet

The pig legs were measured with the detectors for 2 hours (with 10 minute counting intervals) at decay times of 5 minutes and 7.5 hours post 10 minute irradiation. A planned third measurement post-diet was not possible due to a city power outage, and at baseline the third measurement posed difficulty with anesthesia and movement of the leg. Because the physical decay corrected Na count rate at the end of the first measurement is about the same as that for the second measurement, we concluded that the third measurement would not provide much useful information except to verify that there is a slow exchangeable compartment. The pigs were anesthetized during the measurements, but were awake and able to move between measurements. The anesthesia process contributed to slight differences in detection start and end times between

pigs. Following the final measurement, the pigs were euthanized, and both hind legs were removed and dissected.

4.2.3.2 In Vivo NAA for Sodium Measurement in Pig Leg Tissue

a. Bone Calibration

The Na bone phantoms described in past studies were used for bone Na estimation in this study, with a few modifications. The phantoms were moved to vacuum sealed plastic bags in order to reduce the size and air gaps present in the previous storage cups. The phantoms were used to make a new calibration with the updated irradiation and detection set up, including the new n-type detector.

b. Soft Tissue Calibration

In order to determine the soft tissue concentration of Na with IVNAA, soft tissue Na phantoms were constructed. The phantoms were designed to represent the soft tissue in human hand, and consisted of water with a known concentration of Na in the form of NaNO3. Figure 27 shows the 3D printed model and the final simplified model used for the soft tissue phantom calibration. The 3D printed model was designed to represent the geometry of a human hand, but experienced leakage following radiation. Further modifications of the 3D printed model are anticipated. The simplified model was used to create the soft tissue calibration presented in this paper, and geometry differences were accounted for with an updated MCNP input comparing the ratio of simulated counts for different geometries of an irradiated sample.

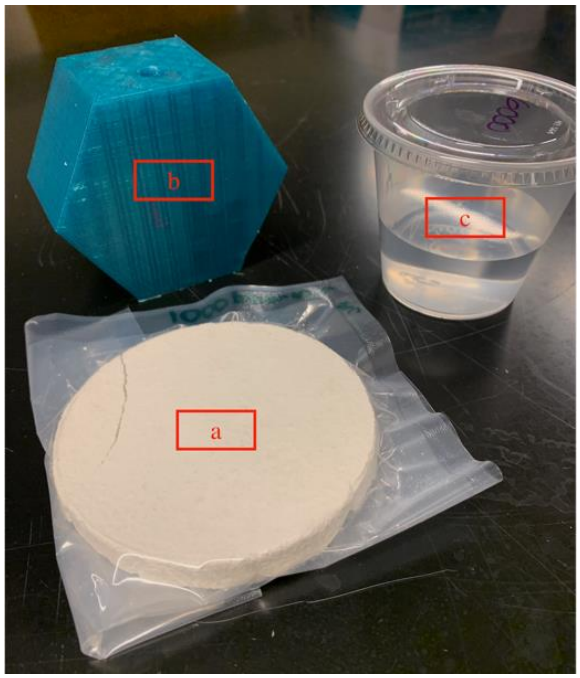


Figure 27 Phantoms used for calibration of Na in a) bone b) soft tissue (3D printed) c) soft tissue (simplified model)

Figure 28 Irradiation and Detection of Na soft tissue phantom

The soft tissue phantoms were irradiated and detected with the same IVNAA setup and protocol used for the animal study, shown in Figure 28. The first 25 minutes of detection following a 10 minute irradiation and 5 minute decay were used for analysis.

c. Analytical method for estimating soft tissue Na with IVNAA

The previous pig study identified a two-compartment model of Na tissue exchange in pig leg. In order to examine the exchange of Na in the leg bone, radioactive decay is corrected with the use of saturation, decay and counting factors specific to Na-24 (Coyne *et al.*, 2018). In this study, the time of irradiation was 10 minutes, while the decay and count time varied throughout the measurements. The corrected activity over time is plotted in order to find the exchange half-life, t_e, from the equation $m=\ln 2/t_e$, where m is the slope from the fitted equation $y=Ae^{-mx}$. In order to determine Na exchange times, decay corrected interval Na counts were normalized to a one hour Ca count.

The biological exchange times are used to find the corrected count rate of Na in soft tissue, which relates to a known amount of sodium from the phantom calibration. A similar methodology was previously used in human hands (Cohen-Boulakia, Maziere and Comar, 1981). This new methodology seeks to improve the estimation of Na by using Na phantoms calibrated for bone and soft tissue, with the customized IVNAA assembly and detection protocol.

Using the new soft tissue Na calibration, soft tissue Na was determined using the first 25 minutes of the first two-hour measurement. This time period was chosen since the first 25-minute measurement, following the 5 minute decay period after the 10 minute irradiation, best represents the fast exchange compartment, assuming a two compartment model. The curve during the first 25 minutes shows both the sharp drop off in counts during the first few minutes of detection, and the gradual fall off in counts before the counts remain relatively stable. The 25-minute count rate for

each pig was used to determine the soft tissue mass using the equations below. In Equation 7, $Na_E(g)$ is the mass of Na in soft tissue of the measured pig leg extrapolated from the calibration curve, which has slope *m* and intercept *b*.

The count rate of soft tissue Na in counts/min, X_E, is the total count rate in the first 25 minutes of measurement, minus the estimated decay corrected bone count rate in the first 25 minutes. X_E' is the decay corrected soft tissue Na count rate in the first 25 minutes. XE' is calculated as shown in Equation 8. In Equation 9 and Equation 10, the correction factors C_1 and C_2 are defined, which includes the radiation decay constant of physical Na decay, λ, and biological Na decay in the fast exchange compartment, λ_E, which is based on the fast exchange compartment half-time previously calculated for each pig. The timing factors of irradiation time, t_i, 10 minutes, and counting time, t_c, 25 minutes, are also used to correct for radiation decay. The correction factor C2 is derived from equation 11 and equation 12. Equation 11 calculates the total count without decay for counting time of tc, and equation 12 calculates the total count with decay for counting time of tc.

$$Na_E(g) = mX'_E + b$$

Equation 7

$$X'_E = X_E C_1 C_2$$

Equation 8

$$C_1 = \frac{\lambda_E}{\lambda}(1 - e^{-\lambda t_i})/(1 - e^{-\lambda_E t_i})$$

Equation 9

$$C_2 = \lambda_E t_c/(1 - e^{-\lambda_E t_c})$$

Equation 10

$$C' = A_0 t_c$$

Equation 11

$$C = A_0(1 - e^{-\lambda_E t_c})/\lambda_E$$

Equation 12

Figure 29 shows a visual representation of the soft tissue count rate, X_E, determined from the total count rate, X_T, and subtracting the decay corrected slow exchange count rate, X_{SE}. The curve represents the biological decay of Na in counts/minute throughout the measurements starting at count time t= 0 and time t= 7 hours. The red part of the curve represents the slow exchange biological count rate that is assumed to be biologically constant throughout the measurements, and which is back calculated to time t=0, which is when counting started after the 10 minute irradiation and 5 minute decay.

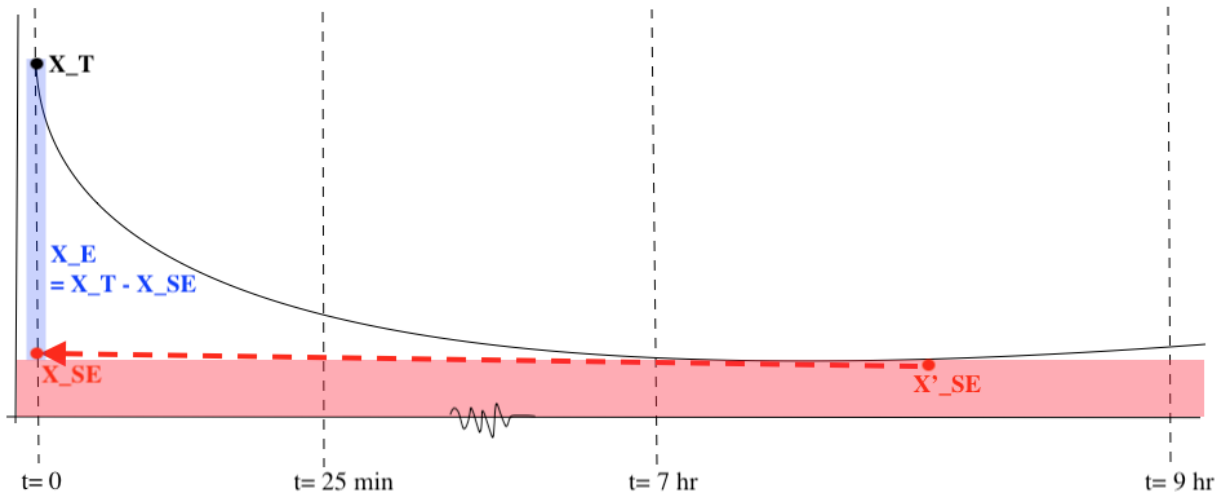


Figure 29 Soft Tissue (X_E) Count Rate Estimation from Total (X_T) and Slow Exchange (X_SE) Count Rate during measurements

4.2.3.3 Ex Vivo NAA for Sodium Measurement in Pig Leg Tissue and Bone

All four hind legs were irradiated and detected following pig dissection. The legs were first irradiated as a whole piece and then dissected into groups of muscle, skin and bone. The dissected pieces were then irradiated by individual group to determine the different Na and Ca concentrations present in skin, muscle, and bone.

4.2.4 Comparative methods for measuring Sodium in Pig Leg

Sodium MRI measurements were taken on the pigs the day prior to both IVNAA measurements. Sodium MRI (23Na-MRI) is a specialized technique that uses strong magnetic fields, gradients, and radio waves to determine the distribution of Na in the body and create an image of soft tissues. Typically, MRI uses protons present in water in the body, but Na MRI uses sodium atoms.

The MRI measurement used the muscle in the upper thigh of the rear hind leg, while the IVNAA measurement used the lower portion of the rear hind leg. Following the studies and euthanasia of the pigs, ICP was performed on all dissected sections of the pigs to determine Na concentration and validate both methods.

However, there are notable differences in tissues measured by each of the three methods. The ICP and MRI sample used an approximately 2 cm cross section from the upper thigh, while IVNAA was aimed at the foot. The bone analyzed in ICP and MRI was a cross section of the femur, which is a long bone comprised of less spongy bone than the short bones of the tarsals used in IVNAA. The MRI scan reported bone marrow, not bone, and used a 3 mm thickness of muscle. The difference in soft tissue between thigh muscle and foot tissue is anticipated to be investigated with ICP, but difficulties with the available ICP facility have delayed sample processing. Validation of IVNAA with ICP is therefore not yet possible.

4.2.4.1 ICP Tissue Sample Processing

Shortly after pigs were sacrificed, both hind legs removed at the hip joint using a scalpel and about 1 ¼ to 1 ½ inch cross-section of the leg were removed. A scalpel and de-ionized water were used to clean out the marrow cavity and remove outer connective tissue.

Individual tissue samples were freeze dried to remove the moisture and then ashed in the muffle furnace to remove all organic material. After ashing, 1 mL of concentrated nitric acid was added to the crucibles to dissolve the mineral ash. Acid-mineral solutions were transferred from crucibles to 25 mL volumetric flasks, and the total volume was brought to 25 mL with ultrapure water. One mL of each stock solution was transferred to a labeled 15 mL Falcon tube using a calibrated pipette, and 10 mL of 2% nitric acid was added to each tube.

Diluted samples were analyzed for Ca, K, Mg, Na, and P concentration by inductively coupled plasma optical emission spectroscopy (ICP-OES). Raw data, along with the dilution factors and weights of samples, were used to calculate the following:

- mg mineral per g of dry weight of tissue (i.e. weight of tissue after freeze-drying)
- mg mineral per g of sample after dissecting (i.e. weight of tissue immediately after dissecting and cleaning, prior to any further processing)
- mg mineral per g of water in the tissue (i.e. weight of the water removed from the tissue by the freeze-dryer).

4.3 Results

4.3.1 Phantom Calibrations

Figure 30 shows the updated bone calibration lines.

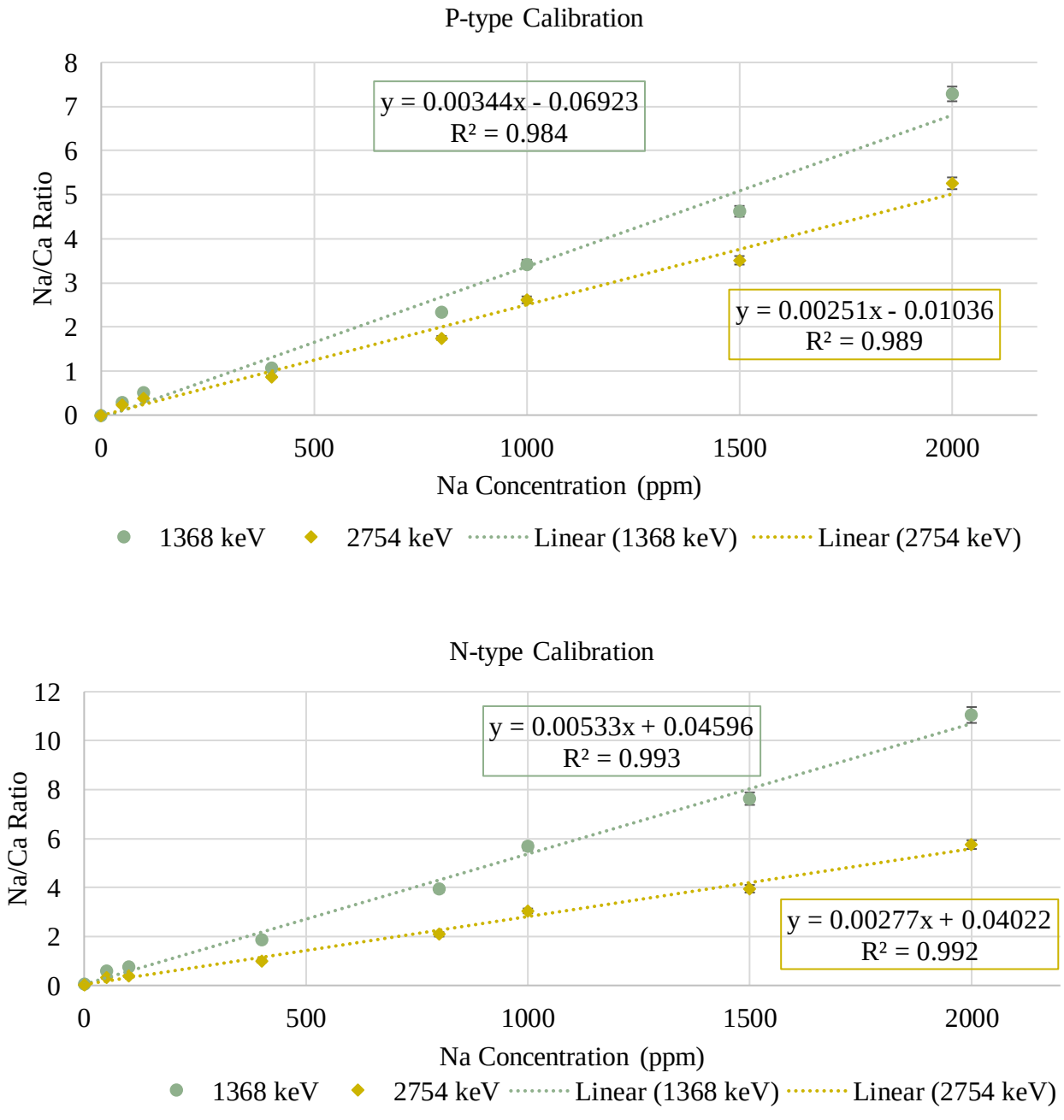


Figure 30 Updated bone calibration with both detectors

MCNP (Monte Carlo N- Particle radiation transport code) was used to determine the effect of attenuation from tissue on the bone phantom calibration. The activation of Na at both energies and the activation of Ca were computed by first simulating the bone phantom in calibration geometry, against the detector. A thin layer of tissue was then simulated in MCNP between the detector and phantom, to determine if tissue has the same impact on attenuating the Ca signal as it does the Na signal. As shown in Table 3, the attenuation from tissue affects both elements equally. This means normalizing the Na counts to Ca should correct for attenuation from tissue in the body. Figure 31 shows the final Na soft tissue calibration with the two detectors.

Table 3 Tissue Attenuation Effect

Energy	Bone Phantom	With Tissue	Ratio
1368	1.979E-02	1.595E-02	1.24
2754	1.197E-02	9.839E-03	1.22
3083	1.093E-02	8.762E-03	1.25

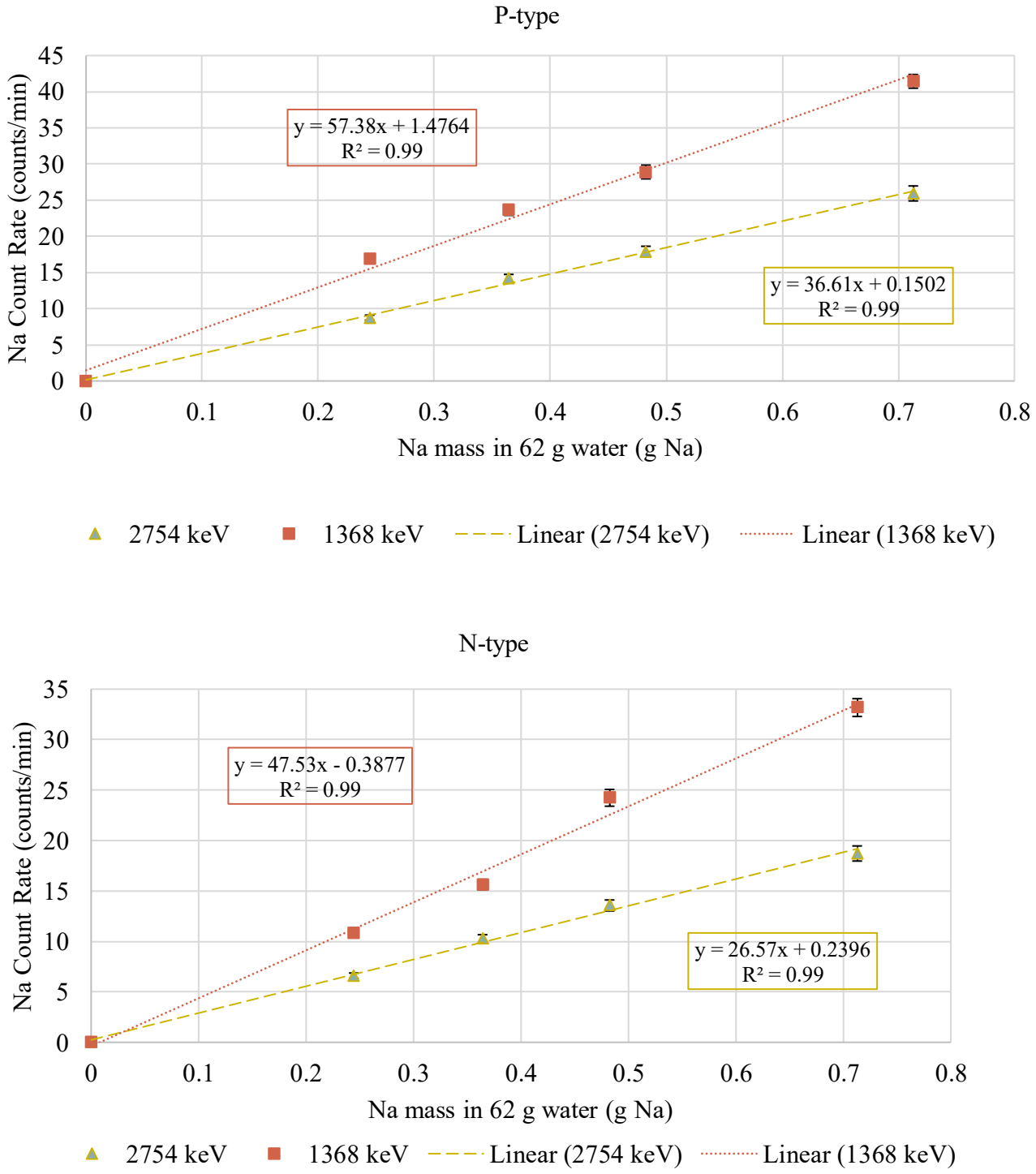


Figure 31 Na Soft Tissue Calibration with P-type and N-type HPGe detectors

4.3.2 In Vivo Spectra

The post diet spectra for the high Na diet pig is shown in Figure 32, with the difference in the two detectors visible. This difference is due in part to the difference in geometry between irradiation and detection for the two detectors, but also due to an established difference in capabilities between the two detectors, with the n-type producing tailed peaks and lower resolution. However, as shown zoomed in Figure 33, Na has such a large and clear peak, especially at 2754 keV, that the difference between the two detectors is minimal. The in-house peak fitting program has two versions to account for the small differences between the detectors, but with the same Gaussian fitting method and analysis parameters.

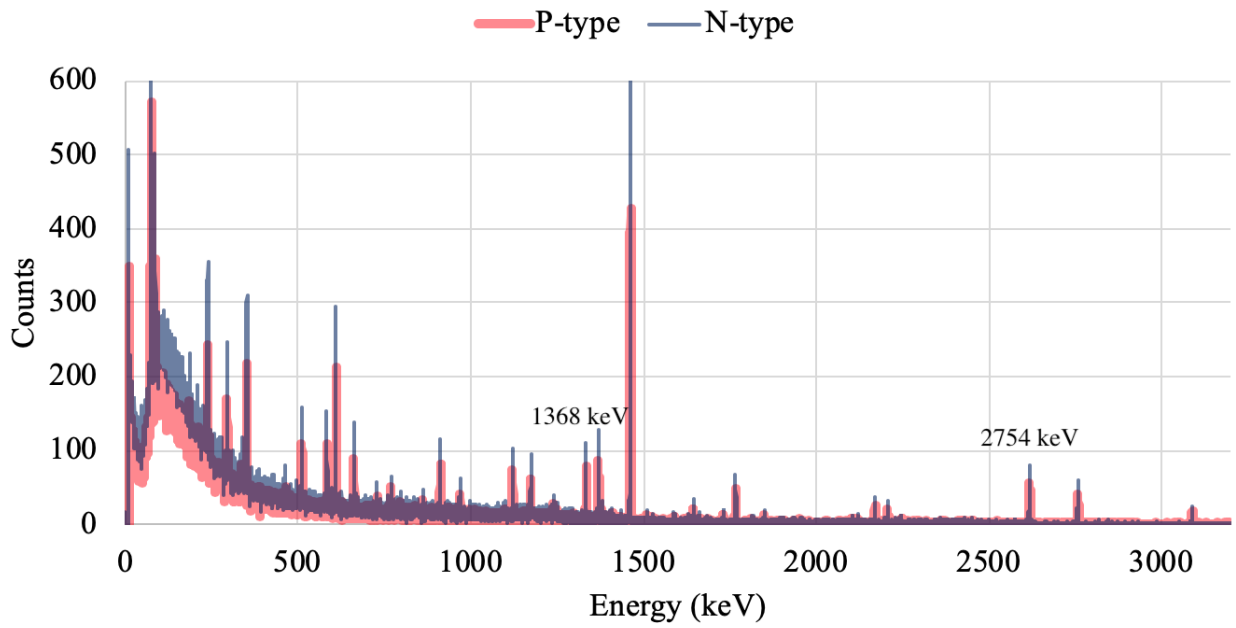


Figure 32 In Vivo Spectra of High Na Pig, Post-Diet, during first 2 hour measurement

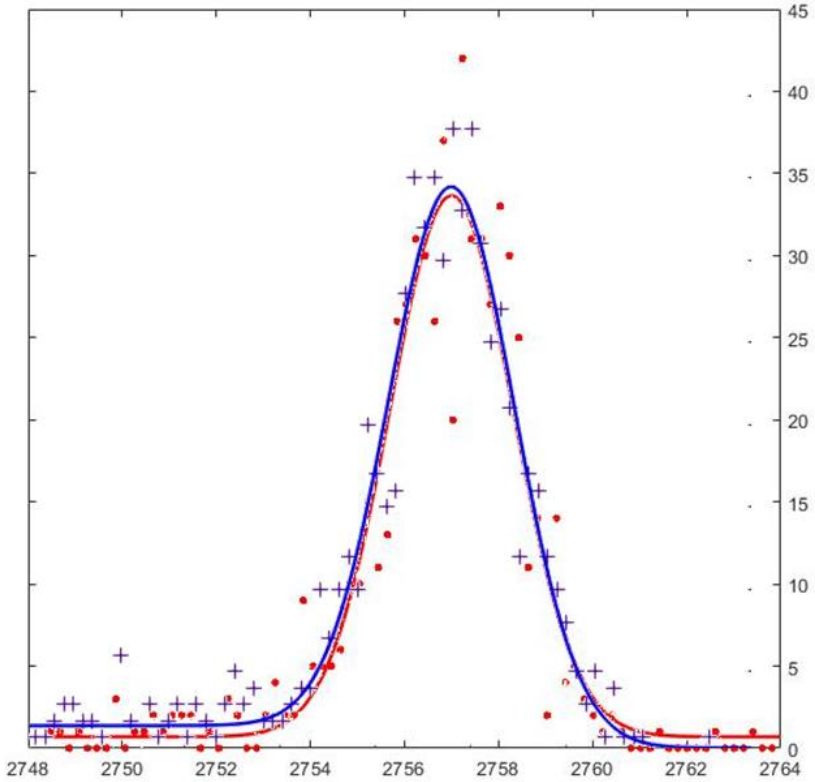


Figure 33 Peak at 2754 keV fit on p-type data (red dots) and n-type data (blue crosses)

4.3.3 Sodium Exchange

In order to determine the biological half-life of Na in the body, the net counts of Na at time periods throughout the first and second measurement were corrected for radiation decay and counting factors. Figure 34 and Figure 35 show the biological decay of Na during the first measurement, with the p-type detector, in the low and high sodium diet pigs, during baseline and post-diet measurements, respectively. Analysis of the n-type detector data yields similar results.

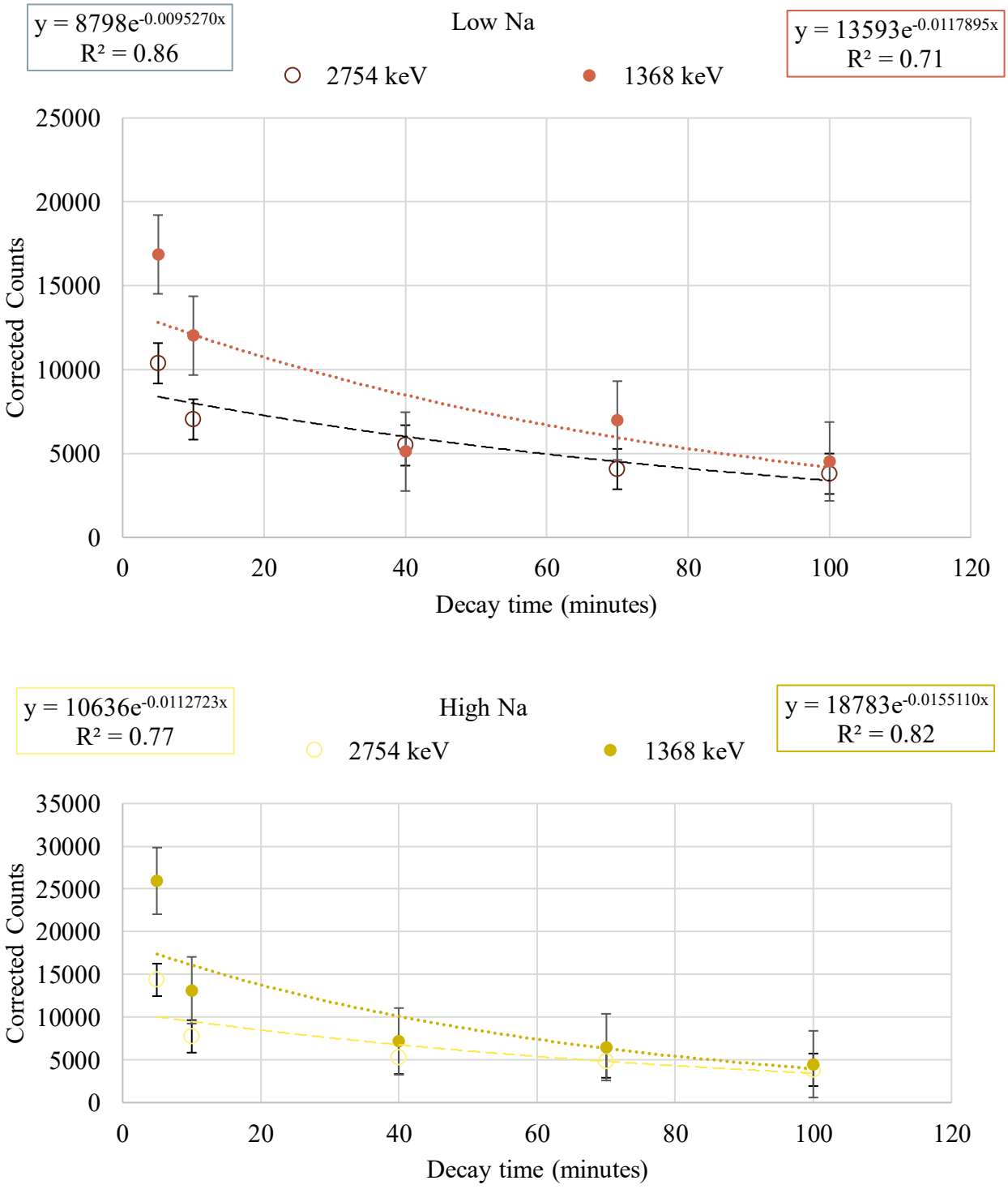
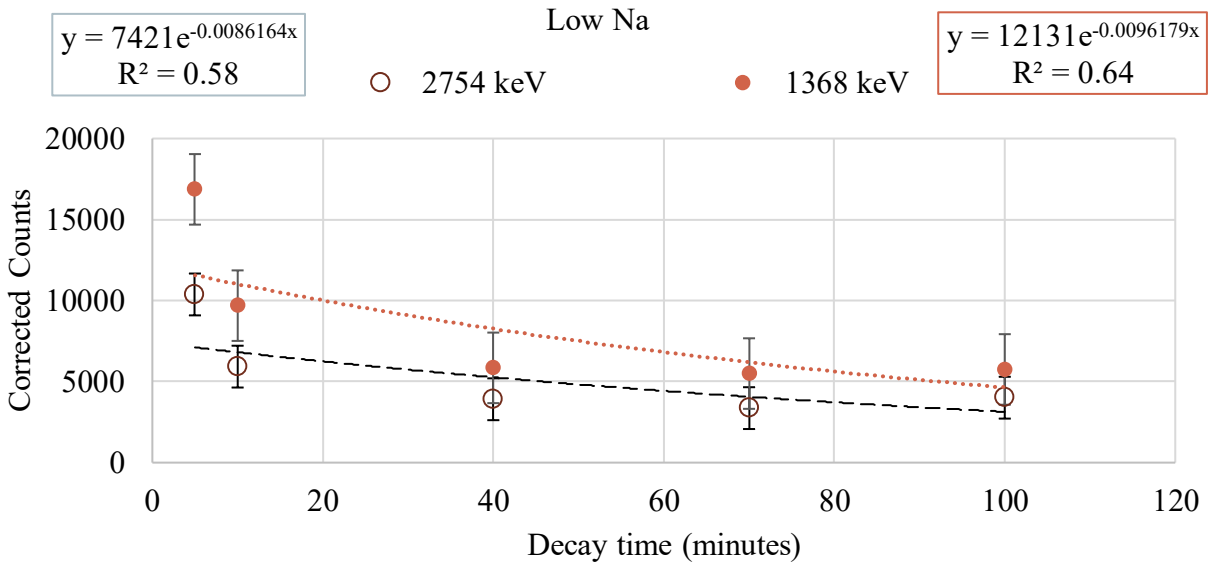


Figure 34 Baseline Fast Exchange time curves



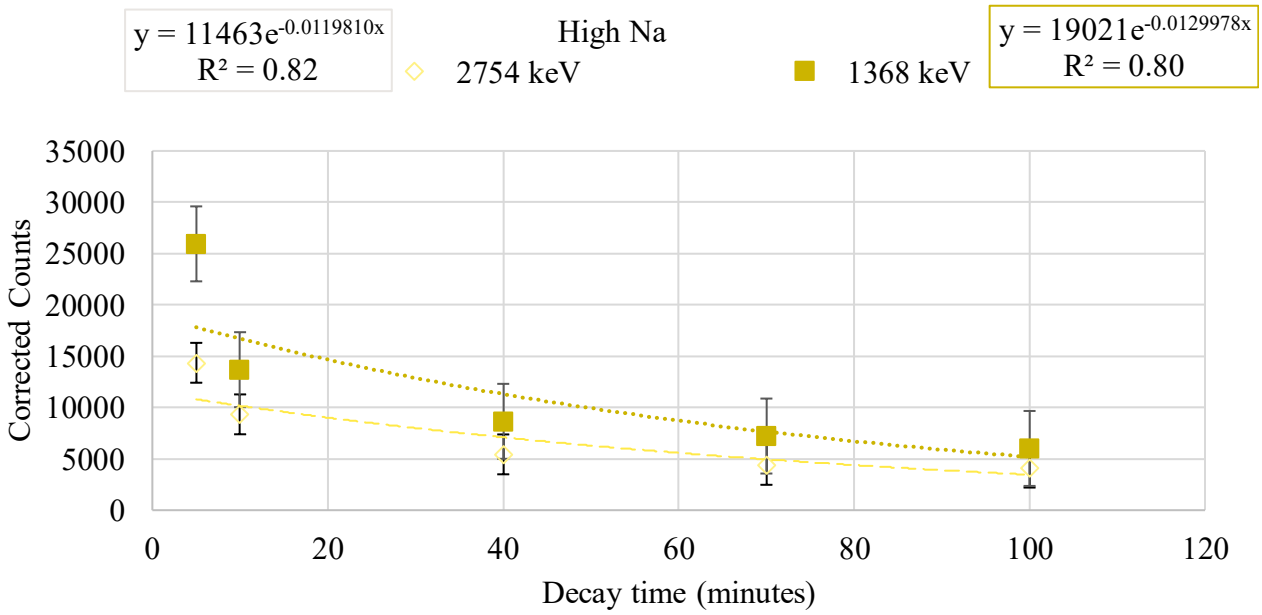


Figure 35 Post-Diet Fast Exchange time curves

Fitting and averaging the slope of the exponential curves from both detectors for both energies of Na yields a fast exchange time of 59 +/- 9 minutes during baseline measurements, and 70 +/- 17 minutes during post-diet measurements, as shown in Table 4.

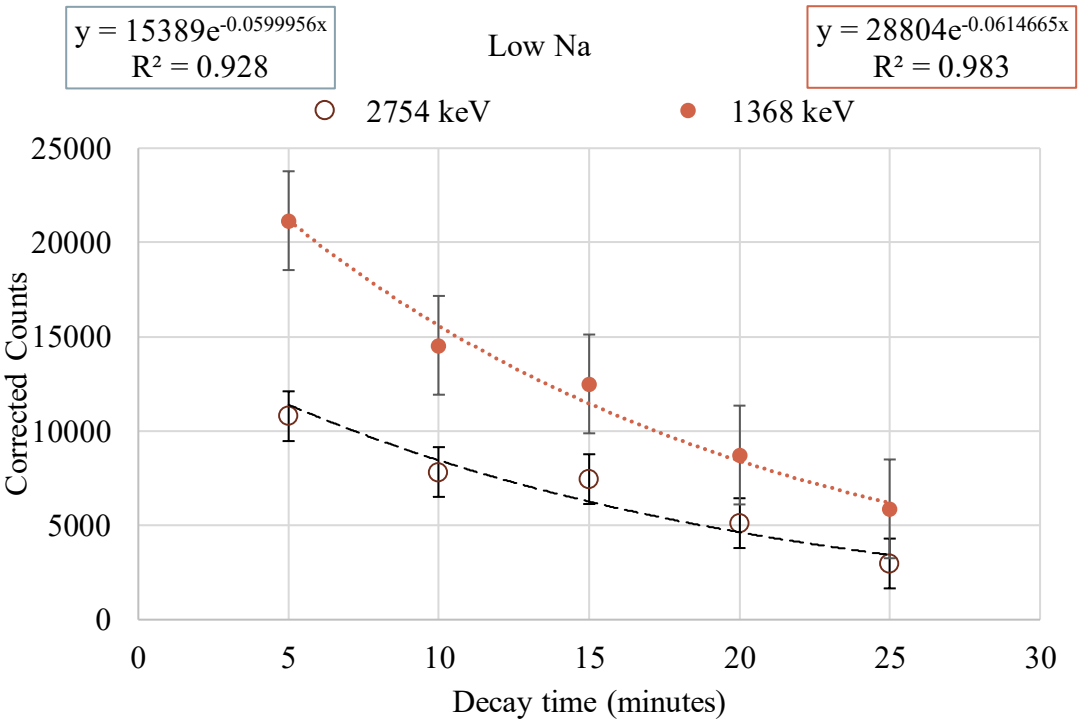
Table 4 Fast Exchange Time Determination

P-type	Pig	Energy (keV)	Slope, *m*	Time, T_E (min)	Average Time (min)
Baseline	Low Na	1368	0.0117890	58.8	59 +/- 12
		2754	0.0095270	72.8	
	High Na	1368	0.0155110	44.7	
		2754	0.0112723	61.5	
Post Diet	Low Na	1368	0.0096179	72.1	66 +/- 12
		2754	0.0086164	80.4	
	High Na	1368	0.0129978	53.3	
		2754	0.0119010	58.2	

N-type	Pig	Energy (keV)	Slope, *m*	Time, T_E (min)	Average Time (min)
Baseline	Low Na	1368	0.0109147	63.5	58 +/- 6
		2754	0.0120697	57.4	
	High Na	1368	0.0140355	49.4	
		2754	0.0113354	61.1	
Post Diet	Low Na	1368	0.0075897	91.3	74 +/- 22
		2754	0.0073773	94.0	
	High Na	1368	0.0120813	57.4	
		2754	0.013193	52.5	

The curves were fit using multiple different methods, with the use of 5 points (1 5 minute decay and 4 30 minute periods during the first measurement) determined to be the most accurate for the two-compartment model based on literature data that cites a fast exchange compartment of about 1 hour (Cohen-Boulakia, Maziere and Comar, 1981). However, further investigation of the first measurement reveals a steep slope that is best fit using only the counts from the first 25

minutes of counting, as shown for both pigs in Figure 36. Using the averaged slopes from baseline, this compartment shows an exchange time of 12 +/- 1.5 minutes.



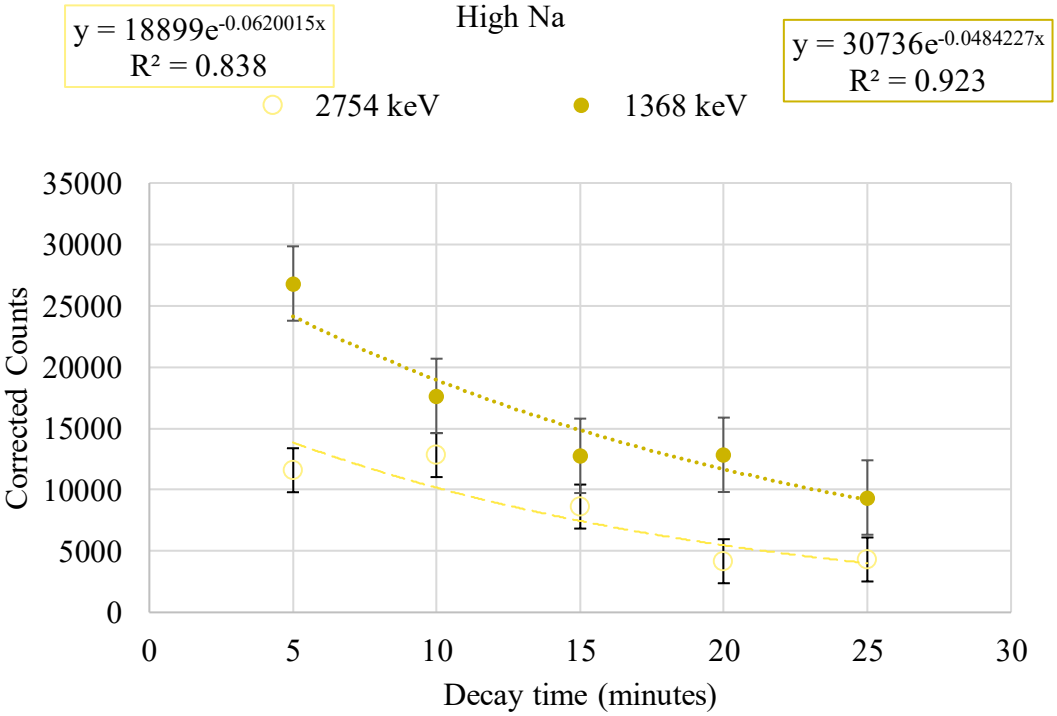


Figure 36 Faster Exchange (baseline, n-type)

To further visualize the exchange and demonstrate the variability of the fast exchange compartment, which may be due to a third compartment, the 10 minute counts from the first 45 minutes of measurement were graphed, as shown in Figure 37. This analysis results in a fast exchange time average of 21 +/- 2 minutes.

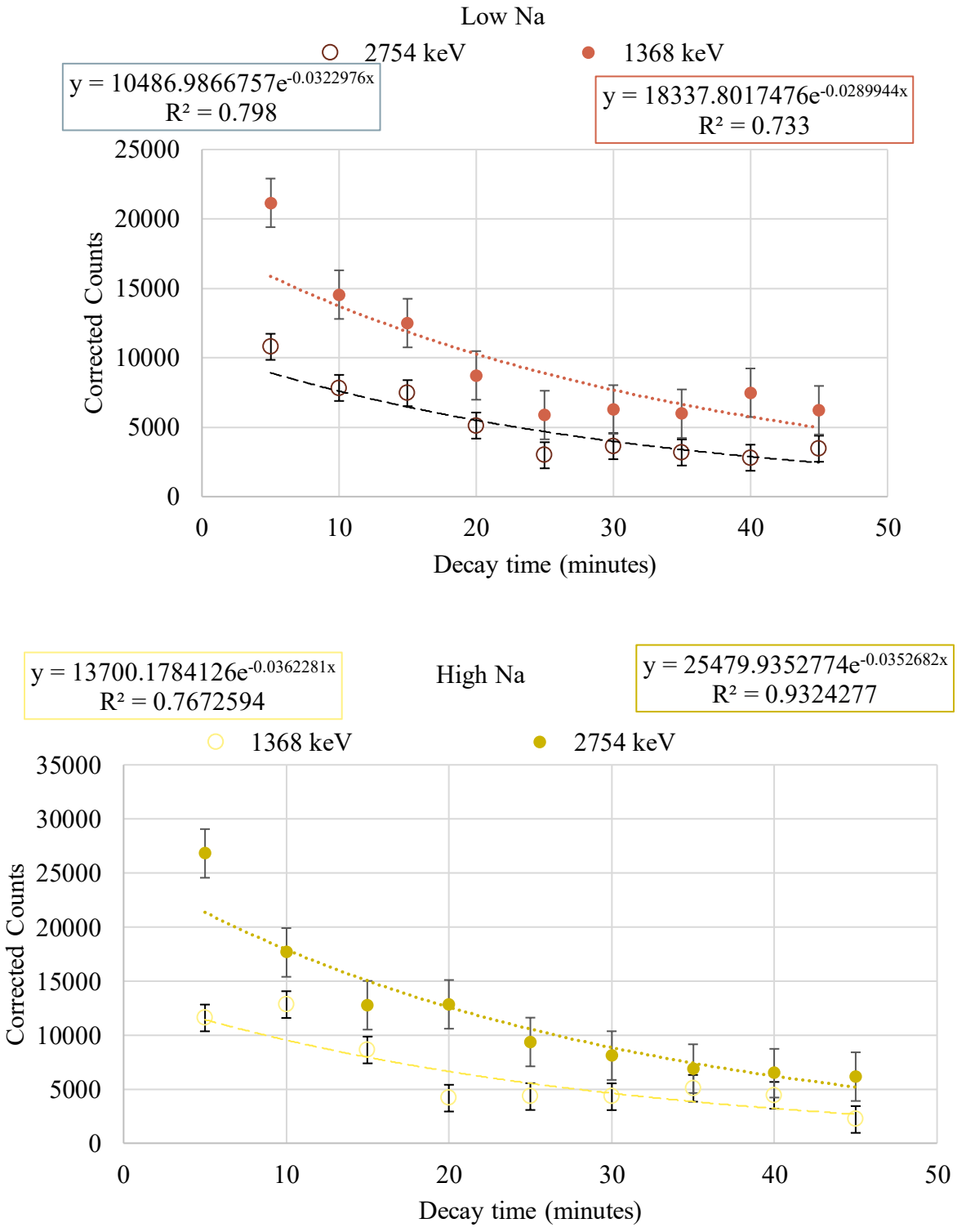


Figure 37 Fast Exchange compartment variability (baseline, n-type)

Fitting and averaging the slope of the curves during the first to second measurement yields the slopes shown in Table 5. Figure 38 and 39 show the corrected counts with decay time for the baseline and post diet measurements, respectively, with the p-type detector. With these results, we conclude there is a slow exchange compartment present with minimal Na exchange. The slow exchange time is not able to be quantified, since it likely exceeds the experimental measurement time.

Table 5 Slow Exchange slope determination

P-type	Pig	Energy (keV)	Slope, *m*
Baseline	Low Na	1368	0.000908
		2754	0.000663
	High Na	1368	0.001088
		2754	0.000152
Post Diet	Low Na	1368	0.000880
		2754	0.000423
	High Na	1368	0.000240
		2754	0.000373

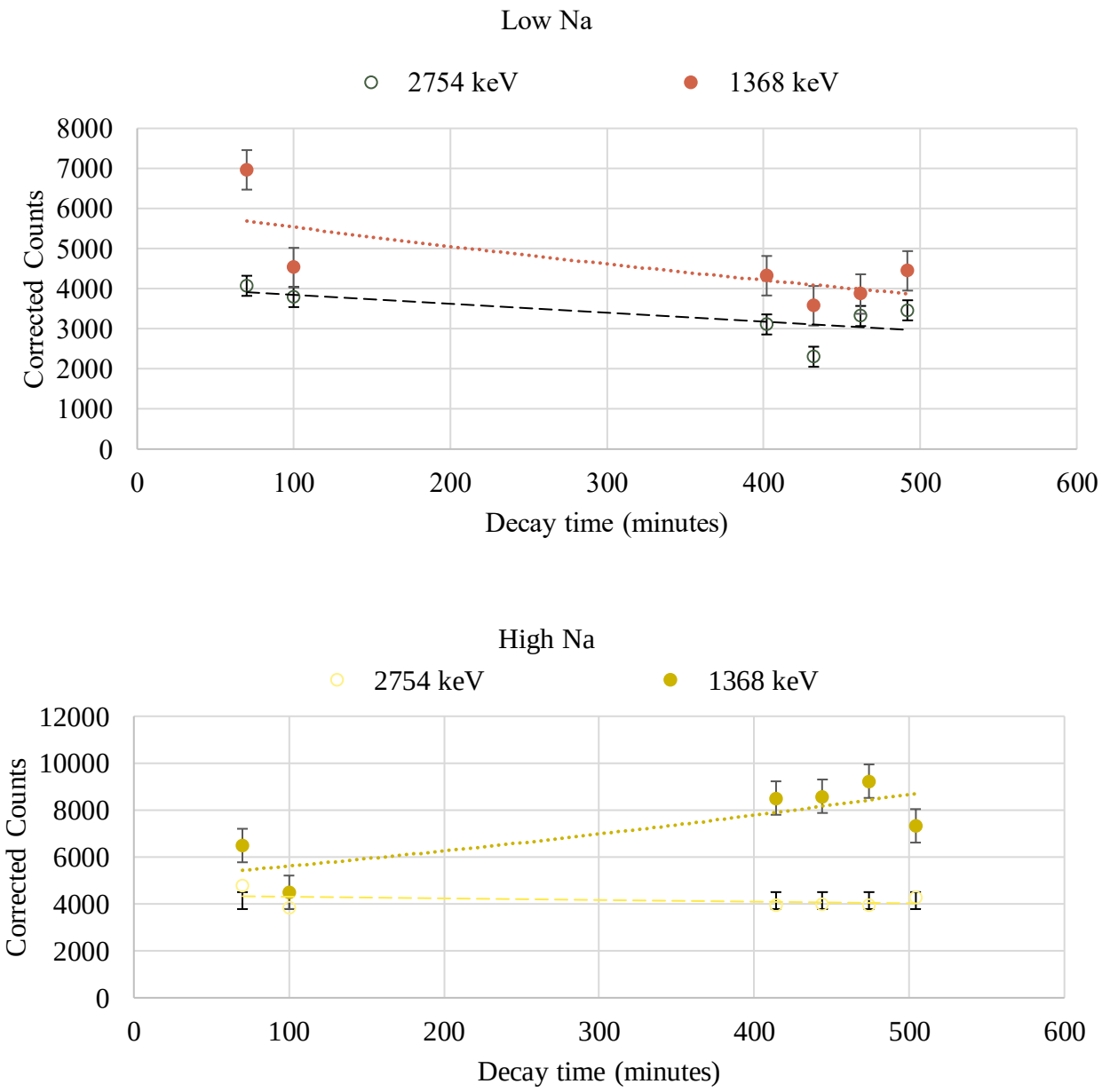


Figure 38 Slow exchange determination during baseline measurements

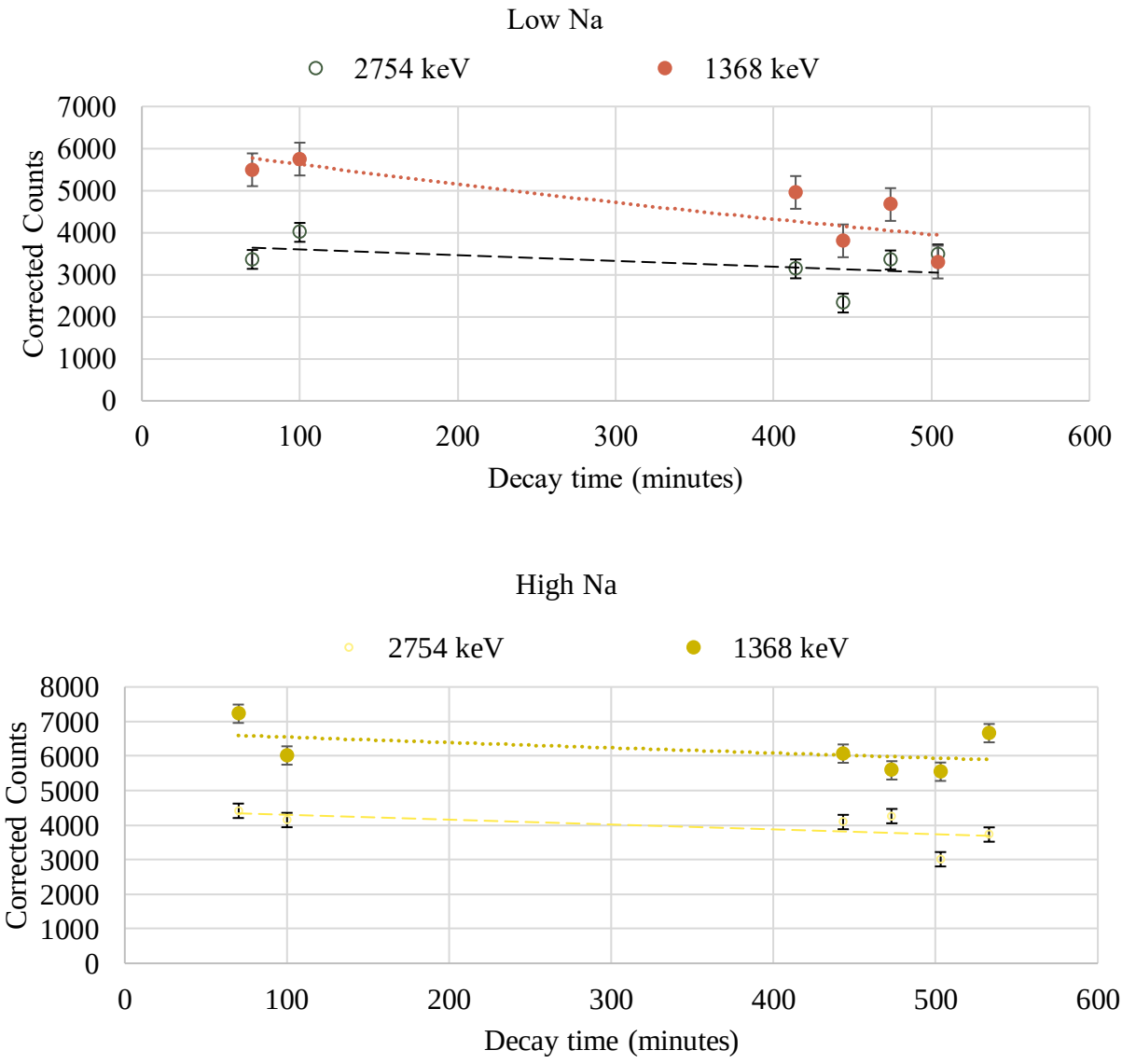


Figure 39 Slow exchange determination during post-diet measurements

4.3.4 Sodium Concentration

4.3.4.1. Bone Sodium

Using the updated bone Na calibration, bone Na was determined using the second two-hour measurement, since the amount of Na is stable and assumed to represent the slow exchange compartment. The net Na counts in two hours during the second measurement were normalized to the first one-hour Ca net count from the first measurement. The values at both Na energies were weighted. Table 6 shows the summary of estimated concentrations. The difference between the low and high Na diet pig post diet was 124 +/- 94 ppm post dietary intervention, which is more meaningful considering the baseline difference between the two pigs was -248 +/- 109 ppm. This result shows a change in baseline for the low Na diet pig of -365 +/- 197 ppm during the dietary intervention, while the high Na diet pig retained a constant Na concentration.

Table 6 Bone Na Concentration

Measurement	Pig	Bone Na (ppm)	Error (+/- ppm)
Baseline	Low Na	1395	86
	High Na	1147	66
	Difference	*-248*	109
Post- Diet	Low Na	1030	67
	High Na	1153	66
	Difference	*+124*	*94*

4.3.4.2. Tissue Sodium

Table 7 summarizes the estimated mass of Na in the soft tissue of pig leg during each measurement for both detectors. Post-Diet, the high Na pig has a higher estimated Na mass in soft tissue. This estimation is dependent on the soft tissue Na phantom calibration, which assumes a 62 g mass of soft tissue in specific geometry, and does not account for the experimental mass of the

pig leg. The difference between the N-type and P-type estimation is due to different detection settings in the n-type during the experiment and later soft tissue calibration.

Table 7 Tissue Na Mass and Concentration

P-type	Pig	Tissue Na (g)	Error (+/- g)	Tissue Na (PPM)	Error (+/- PPM)
Baseline	Low Na	0.15	0.01	2202	168
	High Na	0.17	0.01	2538	183
	Difference	*0.02*	*0.02*	*336*	*248*
Post- Diet	Low Na	0.11	0.01	1552	133
	High Na	0.17	0.01	2414	176
	Difference	*0.06*	*0.02*	*862*	*221*

N-type	Pig	Tissue Na (g)	Error (+/- g)	Tissue Na (PPM)	Error (+/- PPM)
Baseline	Low Na	0.19	0.01	2656	203
	High Na	0.25	0.02	3688	239
	Difference	*0.06*	*0.02*	1033	313
Post- Diet	Low Na	0.14	0.01	2241	205
	High Na	0.27	0.02	3927	247
	Difference	*0.13*	*0.02*	1686	321

4.3.4.3. Ex Vivo Comparison

The whole pig legs were measured with ex vivo NAA. The ratio of Na/Ca is displayed in the table below, with an average of 15.2 +/- 2.8 for the high Na diet pig and 15.6 +/- 2.8 for the low Na diet pig.

Table 8 Ex Vivo NAA of whole pig leg

			N-type				P-type			
Pig	**Side**	**Energy**	**Na/Ca**	**+/-**	**Avg**	**+/-**	**Na/Ca**	**+/-**	**Avg**	**+/-**
High Na	Left	1368	22.0	4.7	15.8	2.8	21.6	4.6	15.5	2.8
		2754	12.4	3.5			12.1	3.5		
	Right	1368	23.1	4.8	16.0	2.8	19.2	4.4	13.7	2.6
		2754	12.3	3.5			10.7	3.3		
Low Na	Left	1368	23.5	4.8	15.9	2.8	19.6	4.4	14.5	2.7
		2754	12.1	3.5			11.5	3.4		
	Right	1368	25.5	5.0	17.7	3.0	20.3	4.5	14.5	2.7
		2754	13.6	3.7			11.3	3.4		

4.3.4.4. MR, ICP comparison

The Na concentration data for the pigs is reported with all three methods studied in Table 9.

Table 9 ICP and MRI Comparison

Sample	**Pig**	**Side**	**ICP**	**MRI**	**IVNAA**
			(ppm)	**(ppm)**	**(ppm)**
Bone*	Low Na	Left	3256	1037	-
		Right	3516	1870	**1030**
		Average	**3386**	**1454**	**-**
	High Na	Left	3345	840	-
		Right	3249	943	**1153**
		Average	**3297**	**892**	**-**
		Difference	*-89*	*-562*	***124***
Soft Tissue	Low Na	Left	97	710	-
		Right	135	630	**1552**
		Average	**116**	**670**	**-**
	High Na	Left	106	627	-
		Right	123	648	**2414**
		Average	**115**	**638**	**-**
		Difference	*-1*	*-33*	*862*
Skin	Low Na	Average	**1342**	**657**	-
	High Na	Average	**1287**	**560**	
		Difference	*55*	*96*	

***Bone ppm measured with MRI is bone marrow**

4.4 Discussion and Conclusion

The IVNAA system is able to non-invasively detect small differences in bone and soft tissue Na in pigs on dietary intervention. This second animal study utilized baseline IVNAA measurements before the dietary intervention to observe the effects of the diet on Na concentration and exchange. The addition of MRI measurements also helped increase understanding of the storage and exchange of Na in soft tissues. New methodology was implemented to estimate soft tissue Na, showing the capability of IVNAA as a non-invasive measurement tool in nutrition studies.

The estimated fast exchange half-life of 58 minutes at baseline and 74 minutes post- diet agrees with the times found in our past pig study. However, as shown, there is evidence of faster exchange occurring during the first few minutes post irradiation. This may indicate a third compartment. Unfortunately, this study does not have sufficient data to further explore this faster exchange, since 10-minute counting intervals were used. In future studies, it would be worthwhile to have shorter counting intervals immediately following irradiation so that the faster exchange compartment can be accurately modeled.

It is notable that the low Na diet pig exhibited slower exchange in the fast compartment than the high Na diet pig. Both pigs also showed slower exchange at post-diet measurements than baseline, which may be due to increased leg size or the lack of a normalization factor in muscle similar to normalization with Ca in the bone measurements. The slow exchange compartment time estimation is not necessarily the decay half-life for the slow compartment since results from the end of the first measurement were included. Using the results from the third measurement, which was only available for the low Na diet pig, showed the decay half-life for the slow compartment is likely much longer. The times given are an approximation with the conclusion being that the exchange time is much longer than the measurement period.

The high Na diet pig did show both higher bone and soft tissue Na post diet than the low Na diet pig. For bone, the high Na diet pig was 124 +/- 94 ppm higher than the low Na diet pig at the end of the study, representing a bone Na change of 6 +/- 93 from baseline The added baseline measurement in this study helped demonstrate the impact of the diet on the growing pig. The diet at baseline, before dietary intervention, was a low Na diet with similar Na concentration to the low Na diet during dietary intervention. While the high Na diet pig kept a stable bone Na concentration, the low Na diet pig lost 365 +/- 109 ppm bone Na from baseline. For soft tissue, the high Na diet pig showed a soft tissue Na change in baseline of -124 +/- 253 ppm, while the low Na diet pig showed a soft tissue Na change in baseline of -650 +/- 214 ppm. This difference is likely due to the dissolution of Na in the low Na diet pig rather than an increase in Na in the high Na diet pig. These estimations also do not account for the drastic size difference between the pigs at baseline to post-diet measurement, and some differences may be explained by the growth of the adolescent pigs. While improvements to the methodology of the soft tissue estimation are anticipated, it is apparent that the IVNAA system is able to distinguish between pigs on a high and low Na diet *in vivo*. Anticipated improvements to the soft tissue estimation methodology include a new experimental soft tissue calibration that uses a phantom more similar to the irradiated subject and shorter counting intervals during the first measurement so that biological exchange can be better determined. The addition of the second detector was anticipated to lower the detection limit, but significant differences in results for the soft tissue estimation between the two detectors raise concerns. The n-type estimated higher Na content than the p-type, which is the opposite of what was expected based on the irradiation and detection geometry, since the N-type faced the non-irradiated side of the leg. The difference, then, may largely be due to difficulty fitting the N-type data, which has a significant low energy tail. There were also changes made to the N-type

detector's settings after the pig study and before the soft tissue phantoms were measured, so a new calibration should be taken.

The *ex vivo* NAA results did not show a significant difference in sodium between the pigs on high and low Na diets by any method. The MRI and ICP studies were anticipated to further clarify and potentially validate the IVNAA findings. However, this part of the experiment experienced numerous setbacks. For MRI, the soft tissue used (the upper thigh muscle) is not comparable to the soft tissue studied with IVNAA (lower leg soft tissue). MRI is able to analyze bone marrow Na content, but not bone Na content. For ICP, problems with the freeze drier, furnace, ICP machine, and processing methods, resulted in significant delays in results. Samples are still awaiting processing at this time. The reported ICP results are similarly difficult to compare to the IVNAA results for the same reasons as MRI, since the sample is from the upper leg. While the skeletal content of Na is consistent throughout the body, the difference in cortical and spongy bone is important when comparing the values from ICP to IVNAA, since Na may deposit more readily in spongy bone, which is more prevalent in the leg than the cortical long bone of the femur used for ICP and MRI. Further exploration of how ICP samples are prepared and processed is essential to directly comparing the two methodologies. This will be investigated in future work, so that ICP can be used to validate the IVNAA results.

The IVNAA data suggest that dietary Na intake affects tissue Na storage, and that a higher Na diet increases these tissue Na stores. Understanding the storage and exchange of Na will have important implications for human health, especially for chronic diseases such as cardiovascular disease and osteoporosis. With the *in vivo* pig study results, we conclude that the DD neutron generator-based IVNAA is a useful method in Na nutrition studies to study both bone and soft tissue Na concentration and exchange.

While more studies are desired to verify our results with statistical significance, the confirmation of many important findings during the previous pig study, in addition to improved methodology and supplemental soft tissue concentration, points towards the usefulness of this system in nutrition studies. Future implementation of our IVNAA system is expected in human nutrition studies soon.

CHAPTER 5. CONCLUSIONS AND FUTURE WORK

5.1 Conclusions

The IVNAA system is able to non-invasively detect small differences in bone and soft tissue Na in pigs. From the initial proposed work, the following aims were achieved, as summarized below.

Specific Aim 1: Design and optimize a neutron generator reflector/moderator/shielding assembly for i*n vivo* neutron activation analysis (IVNAA) of Na in bone and in soft tissue.
This was accomplished by using Monte Carlo (MC) simulations and mirrored experimental design. Bone-equivalent and soft-tissue equivalent Na phantoms were used to calibrate the system.

Specific Aim 2: To validate the IVNAA technology and apply it in the study of Na retention and metabolism using pigs.
We fed 4 pigs a high or low Na diet and perform baseline and post diet IVNAA. We collected incremental Na and Ca counts for at least 24 hours and analyzed the data to estimate bone and tissue Na concentration and model Na exchange. Following IVNAA, the pigs were euthanized and dissected to quantify Na storage in different tissues. The results from the high and low Na diet pigs were compared.

The accomplishment of the first two aims is an IVNAA assembly calibrated for Na quantification to model Na retention. Table 10 shows the estimated bone Na concentrations in the four pigs studied. Figure 40 shows the Na decay and exchange in the four pigs. Unfortunately,

grouping the high and low Na diet pigs from the two studies is not possible, due to weight differences of the pigs. The added acclimation time for a baseline measurement in the second study resulted in the pigs being older and larger at the post diet measurement than the pigs from the first study.

This is the first system utilized to study bone and soft tissue Na under dietary intervention and can be used to help document how dietary Na intake controls blood pressure. The continued application of this research has the potential to yield a novel and valuable measurement tool for use in nutrition studies as well as identify a potential biomarker for disease risk. The insight on Na biokinetics gained from this study can positively impact public health.

Table 10 Bone Na in Pigs 1-4

Measurement	#	Pig	PPM	Error
Baseline	3	Low Na Diet	1395	87
	4	High Na Diet	1147	66
		Difference	*-248*	*109*
Final	3	Low Na Diet	1030	67
	4	High Na Diet	1153	67
		Difference	*124*	*95*
Final	1	Low Na Diet	1222	103
	2	High Na Diet	1414	127
		Difference	*192*	*164*

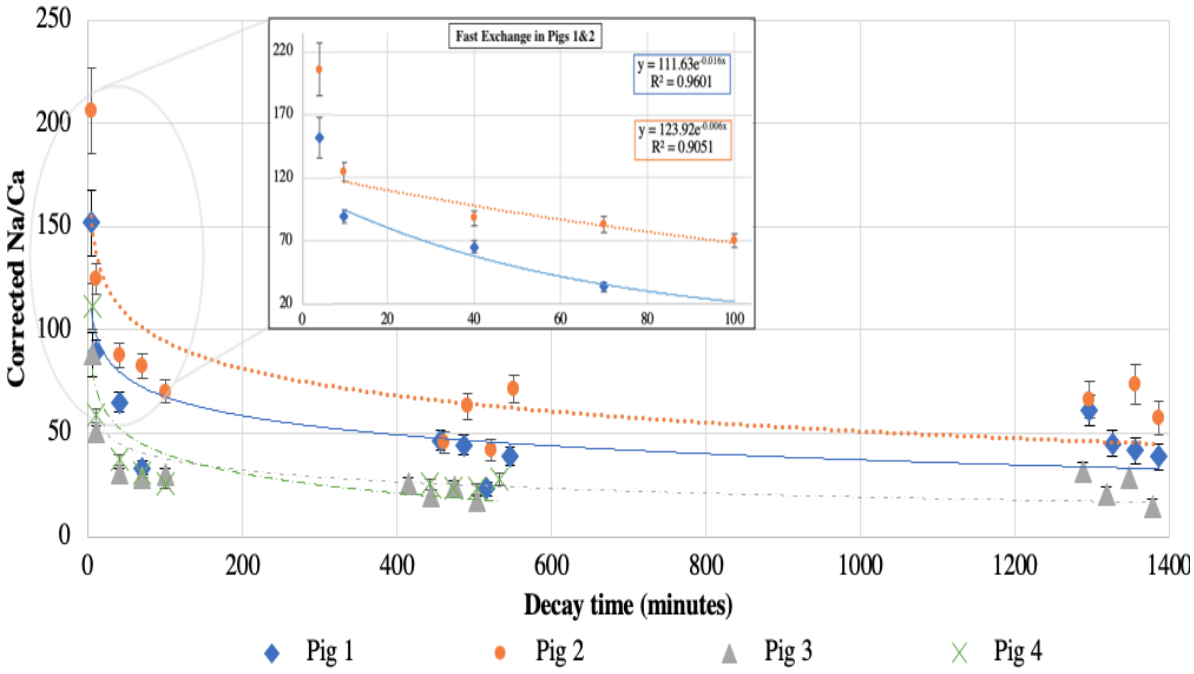


Figure 40 Sodium decay in pigs 1-4

5.2 Future Work

5.2.1 Methodology improvements

Anticipated improvements to the soft tissue estimation methodology include a new experimental soft tissue calibration that uses a phantom more similar to the irradiated subject and shorter counting intervals during the first measurement so that biological exchange can be better determined. The addition of the second detector was anticipated to lower the detection limit, but significant differences in results for the soft tissue estimation between the two detectors raise concerns. This may be due to differences in the n-type detector's settings after the second pig study and before the soft tissue phantoms were measured, which affect the low energy tail of the n-type, so a new calibration will be taken. Other improvements to the IVNAA system in the future include better reflection and lowered external body dose through custom shielding,

5.2.2 ICP Validation

ICP validation is the main priority of future work. The reported ICP results are not directly comparable to IVNAA at this time. The main concern is the weight used to normalize the reported Na g from the ICP sample. The IVNAA bone calibration is based on dry bone, but still has water present. The freeze dried samples of ICP reported as dry bone do not have any water present. Further exploration of how ICP samples are prepared and processed is essential to directly comparing the two methodologies. This will be investigated in future work, so that ICP can be used to validate the IVNAA results.

5.2.3 Human Study

The animal studies performed showed the IVNAA system has great potential for use in nutrition studies. We hope to implement the system in a human nutrition study soon, to determine soft tissue Na, bone Na, and Na biokinetics with high-Na and low-Na diets in mildly hypertensive adults and to assess how Na intake, storage, and biokinetics affect blood pressure in this population. We will recruit mildly hypertensive individuals for a Na dietary intervention study using a crossover design. During both phases, post diet IVNAA, as well as baseline and post diet blood pressure, 24 hour urine, and blood serum will be compared. Data will be analyzed to model Na retention and metabolism in hypertensive adults.

5.2.4 Extended Applications

The IVNAA system is capable of detecting a number of elements other than Na and Ca, including Potassium (K) and Magnesium (Mg). With current research underway to optimize the system for these essential elements, the use of IVNAA in nutrition studies has many more potential applications. Both Na and K are related to muscle movement and blood flow in the body.

Measuring the ratio of the two elements *in vivo* will help researchers better understand the relationship of these elements to bodily functions. Similarly with Mg, direct quantification has not been possible before. A full panel of essential element analysis providing quantification in both bone and soft tissue has great potential to change the current understanding and assumptions about these elements in the body.

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