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DETERMINATION OF MAJOR AND TRACE ELEMENTS IN BONES BY SIMULTANEOUS PIXE/PIGE ANALYSIS

D. L. SAMUDRALWAR, J. D. ROBERTSON*

Department of Chemistry, University of Kentucky, Lexington, KY 40506-0055 (USA)

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Simultaneous PIXE/PIGE was used to determine the concentration of 20 elements including N, O, F, Na, Mg, P, Ca (PIGE) and Cr, Mn, Cu, Zn, Cd, Ba, Pb (PIXE) in a number of animal and human rib samples, marrow, and the IAEA CRM Animal Bone (H-5). Samples and standards were bombarded with 2.5 and 4.0 MeV external proton beams. The minimum detection limits for most of the minor and trace elements ranged from 0.5 to 1.5 ppm. The sample preparation procedure for the nondestructive instrumental analysis of bone is discussed in detail. The analysis method is fast, nondestructive and offers selective analysis of the cortical and cancellous surface of the same bone sample.

Introduction

Bone is a 70% mineralized protein complex, consisting mainly of calcium hydroxyapatite $[Ca_{10}(PO_4)_6(OH)_2]$. It acts as a repository for a number of essential trace elements such as F, Na, Mg, Zn, Sr, and Ba, and as a sink for toxic elements such as Cd and Pb.[1] Because of its very slow remodelling rate, it represents long term dietary habits and environmental exposure of an individual.[2] Moreover, the minor and trace element content of bone plays a vital role in the mineral homeostasis in body fluids through active bone - - blood transfer.[3]

The majority of data on the elemental composition of bone tissue are based upon chemical analysis techniques in which the sample is subjected to one or more chemical separations or matrix reductions.[1,4] Because the analyst must demonstrate that the preparation step(s) can be applied without compromise, most chemical analysis studies have been limited to the determination of a single element. Neutron activation analysis has been applied to the multielemental analysis of bone tissue.[5 - 10] Yet in NAA, the strong bremsstrahlung that results from the activation of the large amount of P in the bone tissue introduces the need for 1. very long counting and decay times and multiple irradiations and/or 2. a pre-irradiation separation of P from the sample.

* Author to whom correspondence should be addressed.

Elsevier Sequoia S. A., Lausanne
Akadémiai Kiadó, Budapest

Another complication in bone analysis is the absence of any standard procedure for removing extraneous tissue from the bone sample. This has resulted in a wide scattering in the reported trace element data.[4] GAWLIK et al.[12] have discussed the problem of contamination from the tools used in the cutting and processing of the bone sample. We propose a simple and efficient method involving a minimal use of solvents for preparing bone samples for instrumental analysis.

The organic matrix is bone tissue, which is mainly collagen protein, is a major contributor to the density of bone. It is an important parameter in clinical studies of bone tissue. The methods used to determine the organic component involve either measuring the amino acids proline and hydroxyproline or estimating the organic content by measuring the N concentration.[3] Both of these methods require sample dissolution. We have utilized PIGE to determine the N content of bone tissue nondestructively.

Experimental procedure

Sample preparation. Rib autopsy samples from twelve subjects were provided by the Kentucky State Medical Examiners Program as a part of coroner authorized protocol and the A.D. Research Center at the University of Kentucky. The age of the 8 male and 4 female patients ranged from 60 to 82 years with an average age of 69 ± 6.3 years. The left 5th rib was removed at autopsy by dissecting away the soft tissues. The ends of the rib were cut with a Strycher autopsy saw. They were then transferred to clean virgin polyethylene bags and stored in a -70 C freezer. Three pork rib samples were provided by the Animal Science Department at the University of Kentucky.

Destilled water, ether, acetone, chloroform, methanol, hydrogen peroxide, glacial acetic acid and their mixtures have been used for defatting and cleaning, while stainless steel, (SS) titanium, quartz and tungsten carbide tools have been used for cutting and processing bone tissue samples.[5-12] We used a Ti blade and SS scissors for removing flesh and cutting bone samples. Extraneous muscle tissue was removed with a high purity Ti scalpel. (All tools were washed with 1% EDTA solution followed by ultrasonic cleaning with demineralized water and methanol (10%, v/v). Oils and lipids were removed by rinsing the sample with an ether : methanol (70:30% v/v) mixture. Although chloroform was also found to be efficient, ether was used for its purely hydrocarbon content. The samples were then freeze dried for approximately 24 h in polyethylene vials to facilitate the removal of remaining muscle tissue from the cortical surface. The dried bone marrow was

removed by scraping with a Ti blade. Most of the previously reported methods involved soaking the samples in distilled water or a solvent for a few days to remove the soft tissue and bone marrow.[7-12] The partially cleaned ribs were then cleaved transversely and the marrow was scraped out using a nylon brush and occasional rinsing with the solvent mixture. Complete removal of blood and marrow was monitored by the Fe, Cr and Rb concentration. The samples were then cut into approximately 1 cm^2 pieces, freeze dried for 12 h, and stored dry at -20 C in virgin polyethylene bottles. The moisture content of 8.5 ± 1.2% is in good agreement with literature values.[4]

In many previous studies, soft animal tissue or plant leaves were used as the reference material (e.g., Refs[6-12]). We prepared a series of single and multielemental synthetic bone standards for elements with Z ranging from 24 to 82 by spiking calcium hydroxyapatite (HAPT, Sigma, USA) with appropriate amounts of NIST SRMs Multielemental *Spectrometric Solutions* 3172 and 3174. NIST SRM Bovine Liver 1577a mixed with HAPT was used as the standard for N determination. In each case, the HAPT suspension was mixed thoroughly with the spike in polypropylene beakers on a vortex mixer and the resulting mixture was dried in an oven at 55 C for 15 h. The cake was powered with a teflon rod (except for the F standard) and pressed into 30-50 mg/cm^2 pellets between two layers of mylar film at 11 MPa. The IAEA CRM Animal Bone (H-5) was used as *secondary standard. Standards and samples were mounted on a kapton film on* polycarbonate holders using a 5% solution of polystyrene in xylene.

IBA measurements. The PIXE/PIGE measurements were performed at the new Ion Beam Analysis (IBA) facility at the University of Kentucky 7.5 MV Van de Graaff accelerator.[13] A Si(Li) detector with FWHM of 165 eV (Mn K_α) was placed 4.3 cm from the target at an angle of 45° relative to the beam direction and a HPGe detector with FWHM of 2.4 keV (1.17 MeV) was placed 3.0 cm from the target at an angle of 90°. The proton beam, normal to the target surface, was rastered over the sample irradiating a spot of 5 mm x 7 mm. Samples and standards were irradiated in a 1 atm helium environment with 2.5 and 4.0 MeV proton beams at an average intensity of 50 to 100 nA for 15 to 30 min. A 925 μm thick mylar filter was used to reduce the Ca X-ray yield in the PIXE spectra. A pulser was used to correct for dead time variation in the γ-ray spectra.

The proton beam energy was optimized by irradiating the standards and animal bone samples in the energy range of 2.2 to 4.0 MeV. As expected, the increasing beam energies produced 2 to 8 times higher X-ray yields. However, the peak/background ratio for these elements with $Z < 40$ dropped by a factor 2 to 20 with increasing bombarding energy. The higher yields for elements with Z

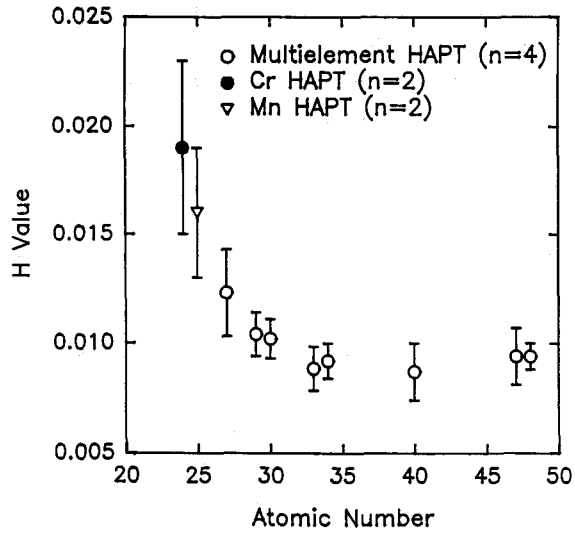

Fig. 1. The H-value curve used in the thick-target PIXE analyses.

> 40 at 4.0 MeV reduced the limit of detection (LOD) for Cd and Ba from 4.5 and 32 ppm to 1.7 and 14 ppm, respectively. Nitrogen was determined by measuring the 2313 keV γ-ray peak produced in the $^{14}N(p,p'\gamma)^{14}N$ reaction at 4.0 MeV.

The GUPIX91 program, which utilizes the H-value formalism, was used for the PIXE analyses.[14] The H value curve shown in Fig. 1 was generated from the HAPT standards. Use of this formalism eliminates the need for a comparator standard for each element to be determined. The X-ray and γ-ray spectra from an IAEA CRM Animal Bone (H-5) sample are shown in Fig. 2. The thick target PIGE calculations were done by the comparator method.

Results and discussion

The results from the IAEA CRM Animal Bone standard,, determined from 8 analyses of 5 different sample pellets, are given in Table 1. The relative standard deviation is, for all elements except Ni, Ba and Pb less than 10%. The concentrations of Ni and Cu are higher and Rb is lower than the suggested values. Our results are, however, well within the accepted range given in the preliminary report.[15] The Mn concentration given in Table 1 is well above the accepted range. We report, for the first time, the concentrations of N and O in this sample. The protein content can be obtained by multiplying the N

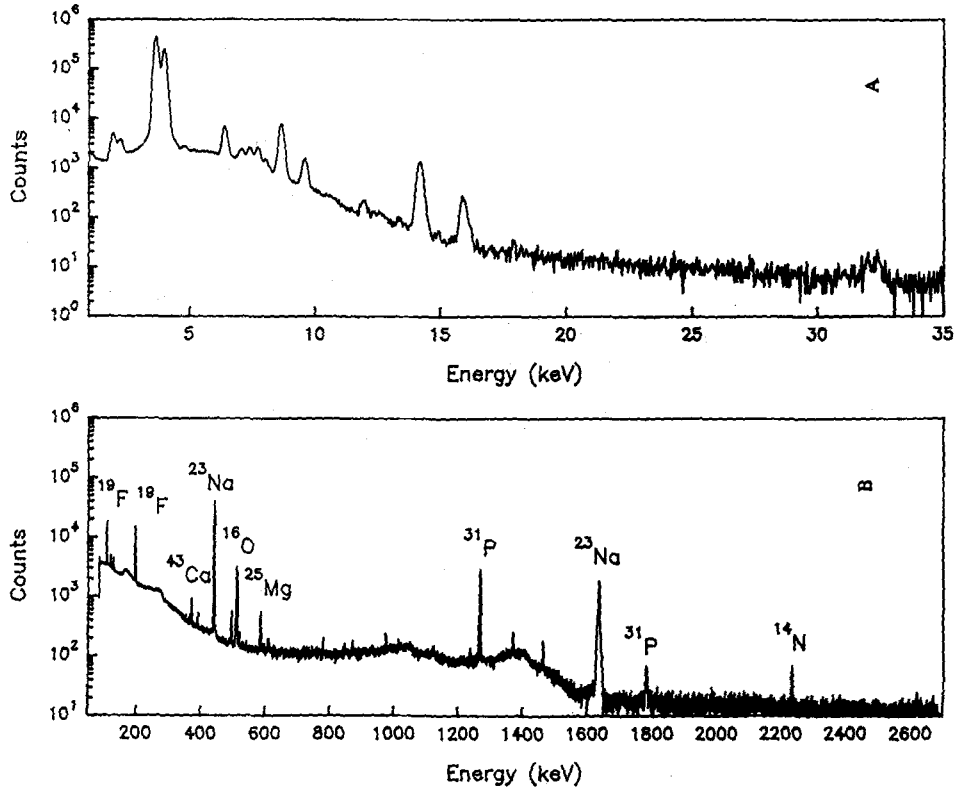

Fig. 2. The PIXE and PIGE spectra of the IAEA CRM Animal Bone (H-5). The two spectra were accumulated at a bombarding energy of 2.5 MeV at an average beam intensity of 70 nA and a total charge of 98 μC (A = PIXE, B = PIGE).

content by a factor of 6.25 if it is assumed that a mixture of pure proteins contains 16% nitrogen.[8,16] The N concentration of 4.2% thus yields a 27.3% collagen content.

In order to determine if the concentration of any element changed during the analysis, three 15 minute irradiations of one IAEA pellet and three 30 minute irradiations of one human bone sample were performed at a beam current of 70 nA. No significant variation was observed in the elemental concentrations in the human bone sample from the repetitive bombardments. Repeat irradiations of the IAEA sample resulted in a 40% decrease in the Pb signal. No change was observed in the concentration of any other elements in this

Table 1

Results of the PIXE/PIGE analysis of the IAEA certified reference material Animal Bone (H-5). All values, unless otherwise noted, are given as μg/g.

Element	PIXE/PIGE Results (n=8) Min	Max	Average	SD	LOD	IAEA Certified Average	SD
PIXE							
Ca (%)	20.2	21.7	21.2	0.50		21.2	0.8
Mn	1.1	3.5	2.36	0.84	1.1	0.45-1.33[a]	
Fe	68	87.5	79.0	5.42	0.8	79	5.9
Ni	1.4	2.3	1.51	0.53	1.4	0.29-2.30[a]	
Cu	<0.9	1.2	1.1	0.1	0.9	0.14-1.23[a]	
Zn	88.7	92.1	90.1	1.25	0.5	89	5.2
Br	3.3	4.4	3.80	0.40	0.5	3.5	0.49
Rb	0.7	0.9	0.8	0.1	0.7	0.28-2.50[a]	
Sr	89.5	104	98.0	4.85	0.6	96	8.2
Ba[b]	79	131	91	21	14	79	12.6
Pb	1.2	4.2	2.81	0.95	1.7	3.1	0.56
PIGE							
N[b] (%)	4.1	4.3	4.2	0.1	0.03		
O (%)	27.1	35.6	32.3	2.99	1.0		
F	417	469	435	18.2	1.0	441-462[a]	
Na	4900	5400	5100	200	5	5000	280
Mg	2900	3800	3500	400	400	3600	89
P (%)	9.4	10.6	9.88	0.36	0.06	10.2	0.86
Ca (%)	19.9	23.3	21.8	0.99	0.8	21.2	0.81

[a] Range of accepted laboratory averages for non-certified values.[15]
[b] Seen only in the 4.0 MeV irradiations.

sample. We attribute the Pb variation to the altered matrix bonding of Pb in powered IAEA standard (defatted) and natural bone.

The IBA measurements can be utilized to determine the elemental composition of the cortical and cancellous surface of the same bone sample. The range of a 2.5 and 4.0 MeV proton beam in bone is 65 and 146 μm, respectively. Typical human or animal rib is 2-3 mm thick with an approximately 0.5 mm thick layer of cancellous tissue. Selective surface analysis is a distinct advantage of IBA for structurally nonhomogeneous biological materials like bone.

The results for the cortical and cancellous bone tissue of pork and human rib samples are given in Tables 2 and 3, respectively. In comparison to the IAEA H-5 standard, the concentrations of Fe, Br, Ba and Pb in pork rib are lower by a factor of 2-3 while Cd and Zn are elevated about the same factor. Compared to the human bone, Mg is elevated by a factor of 2 while, Fe, Sr, Br, F and Pb are depleted by a factor of 2 and F by a factor of 3 in pork ribs.

Table 2

Results of the PIXE/PIGE analysis of 3 pork rib samples. All values, unless otherwise noted, are given as μg/g.

Element	Cortical Average	Cortical SD	Cancellous Average	Cancellous SD
PIXE				
Ca (%)	20.3	1.4	21.5	1.5
Mn	1.2	0.8	nd	nd
Fe	29	7	34	15
Ni	2.2	0.8	2.2	1.0
Cu	1.2	0.9	nd	nd
Zn	158	13	180	3
Br	1.4	0.3	1.2	0.1
Rb	1.3	0.6	1.5	0.1
Sr	33	4	40.4	0.1
Ba*	24	10	3.4	1.6
Pb	0.9	0.6	nd	nd
PIGE				
N* (%)	4.4	0.7	3.5	0.6
F	588	178	610	3
Na	5200	1000	5300	500
Mg	4800	1200	4700	500
P (%)	11	1.8	10.6	0.5
Ca (%)	22	1.4	24	1.2

*Seen only in the 4.0 MeV irradiations.

The depletion in trace elemental concentrations per unit matrix weight is more prominent when elevated Ca and P contents are considered.

The concentrations of F, P, Ca, Mg, Na, Sr, Zn and Rb given in Table 3 are within the range of values in the compilation of IYENGAR et al..[4] The large range of concentrations in this compilation may be attributed to the variations in geographic location and ethnic origin and to differences in sample preparation methods. The need for creating a more systematic database for the bone tissue is clearly demonstrated by this wide range of values. Our results for Ca, P, Na, Mg, Sr, Zn, Fe and N are in excellent agreement with the ICP-ES and AAS analysis of 42 Japanese ribs by YOSHINAGA et al..[11] Moreover, the results for O, Na, Mg, P, Ca, Ni, Cu, Zn, Sr and Pb agree with concentrations in the iliac crest determined by GRYNPAS et al.[9] by INAA and in the shaft of femur determined by HYVONEN-DABEK et al. by PIXE and PIGE.[16,17] This observation indicates identical concentration levels for Na,

Table 3

Results from the PIXE/PIGE analysis of 12 rib autopsy samples.
All values, unless otherwise noted, are in μg/g dry weight.

Element	LOD	Cortical (n=12) Median	Average	SD	Cancellous (n=12) Median	Average	SD	Marrow (n=1)
PIXE								
Ca (%)		21	20	4.1	19	19	2.2	9
Cr	1.2	1.8	1.8 (3)[a]	0.7	2.1	1.9 (3)	0.8	18
Mn	0.8	1.2	1.2 (5)	0.2	1.2	1.01 (8)	0.4	nd
Fe	0.8	21	23	11	73	77	46	308
Ni	1.2	1.5	2.6 (10)	2.1	1.4	2.3 (10)	1.1	nd
Cu	0.7	1.2	6.3 (13)	9.2	0.7	1.4 (13)	4.1	1.5
Zn	0.4	179	180	44	139	144	17	102
Br	0.5	3.0	4.1	4.0	1.2	1.4	0.6	2
Rb	0.6	1.0	2.1 (11)	3.0	1.0	2.1 (12)	3.1	1.3
Sr	0.6	56	62	18	55	58	17	17
Cd	1.5	2.5	2.7 (5)	1.0	2.6	2.4 (4)	1.2	nd
Ba[b]	14	35	36 (5)	13	18	19 (4)	7	nd
Pb	1.5	9.1	13.4	11.3	3.8	5.0	3.3	1.8
PIGE								
O (%)	3.0	32	29	8	33	30	7	31
N[b] (%)	0.03	4.6	4.8	0.6	4.2	4.4	0.6	nd
F	1.0	1769	2074	652	1624	1710	632	899
Na	5.0	5200	5400	1000	5200	5400	600	3800
Mg	200	2500	2600	400	2700	2700	400	2200
P (%)	0.08	9.5	8.8	2.2	9.8	9.6	1.3	3.2
Ca (%)	1.6	21	21	4.0	22	21	2.8	11.7

[a] Number of samples in which the concentration is above the LOD value.
[b] Only seen in the 4.0 MeV irradiations.

Mg, Sr and Zn in various types of bones; i.e. iliac crest, femur and rib. A comparison of the rib and femur results does indicate a 2 to 4 fold accumulation of Fe, Br and F in ribs. The N content of 4.4% corresponds to an approximately 30% organic matter concentration in the matrix. This result agrees well with literature values.[1,4] In the same bone sample, F, Zn, Br, Ba, Cu and Pb tend to accumulate in the cortical layer. Na is the element that is most evenly distributed in cortical and cancellous tissue. Two human rib samples had very high amounts of Pb (20-40 ppm) and one of human sample had high amounts of Cu (37.8 ppm). These values most likely indicate environmental exposure to Pb abd Cu or, in the latter case, the consumption of Cu containing drugs. This premise could not be confirmed due to the absence of detailed subject histories.

There are few reports on the analysis of bone marrow. The multielemental determination of the marrow may be useful, not only in understanding its role as hematopoietic tissue and as a site of osteogenesis, but for calculating the

tissue absorption parameters very often used in non-invasive techniques.[10] The data reported in Table 3 are from a single human sample. The Ca/P ratio of 3.65 in marrow is much higher than in bone. The higher marrow : bone ratios for Fe and Cr can be used as indicators for residual blood or marrow contamination of the bone sample. Mg seems to be evenly distributed between the calcified and noncalcified tissue while the affinity of F towards the bone matrix is evidenced by the fact that the F bone : marrow concentration ratio is greater than 2.

Conclusion

PIXE and PIGE are efficient analytical techniques for the determination of major, minor and trace elements in bone tissue. The method is simple, fast, multielemental and nondestructive. Moreover, if offers selective surface analysis of nonhomogeneous biological samples. The N concentration determined by PIGE may be used to extrapolate the major organic content of the biological samples.

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