DOI 10.1007/s10812-019-00810-8
Journal of Applied Spectroscopy, Vol. 86, No. 2, May, 2019 (Russian Original Vol. 86, No. 2, March–April, 2019)

DETERMINATION OF LABILE AND STRUCTURALLY BOUND TRACE ELEMENTS OF BONE TISSUE BY ATOMIC ABSORPTION SPECTROMETRY

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UDC 548.73:549.753.1

A method of chemical separation of the components of bone tissue, based on their selective solubility, with the subsequent determination of trace elements by atomic absorption spectrometry, is proposed. The total concentrations of Mg, Zn, Fe, Sr, Cu, Mn, and Pb, and the concentrations of these elements in solutions with pH 6.5, 10, and 12 after their interaction with the bone preparation were determined. The obtained concentrations of the "soluble" fractions of trace elements are critically analyzed taking into account the possible reactions of formation and precipitation in alkaline solutions of new insoluble phases. Based on the obtained data, the ability of elements to form mobile fractions in the composition of bone tissue can be represented as follows: Mg > Zn ≥ Fe > Sr > Cu. At the same time, noncrystalline Mg is predominantly localized in water or biological liquids of the bone, and noncrystalline Zn — in the alkali-soluble organic component of the bone. Pb and Mn are practically not detected in solutions, i.e., localized in the crystalline phase.

Keywords: *bone tissue, trace elements, concentration, localization, selective solubility, apatite, atomic absorption spectrometry.*

Introduction. In determining the trace element composition of biomineralized tissues, it is important to know not only the absolute concentrations, but also the fractional distribution of elements between different components of the tissue. In the bone tissue, trace elements can be localized in nanocrystals of biological apatite, replacing the main ions; in the hydration shell on the surface of the crystals; in the organic components of the tissue; in biological fluids and cellular elements. Recently, attempts have been made to determine the preferential localization of trace elements of mineralized tissues by X-ray absorption spectroscopy, although the applicability of this method is significantly limited by the limited availability of high brightness (synchrotron) radiation sources.

The main mineral component of mammalian bone tissue is nanocrystalline defective apatite, which, taking into account the main elements and partial substitutions of the carbonate ion (CO_3^{2-}) with phosphate groups (PO_4^{3-}) and hydroxyl ions (OH^-), can be represented as follows [1, 2]:

$$Ca_{8.3}\square_{1.7}(PO_4)_{4.3}(CO_3)_1(HPO_4)_{0.7}(OH,CO_3)_{0.3}\square_{1.7},$$

where □ are vacancies. In addition to these elements, there are other ions in the bone mineral that can enter the crystal structure of apatite, replacing the main ions. Alkali and alkaline-earth metals, such as magnesium, sodium and potassium, occupy a special place among the metal elements that greatly influence the crystal-chemical characteristics of biogenic apatite. In the bone mineral, these elements are found in relatively large concentrations (Na ~ 1.0 wt.%, Mg ~ 0.2–0.6 wt.%, K ~ 0.07 wt.% [3]), therefore, in the literature they are often defined as "major" impurities or macroelements, in contrast to "minor" or trace elements, whose content in normal bone tissue bioapatite does not exceed a few hundredths or thousandths of a percent [3–6]. Trace elements found in bone tissue are primarily iron, zinc, strontium, lead, and some others [6, 7].

The accumulation of biologically significant ions in the bone mineral is determined by function, since in addition to the supporting function, bones act as a "depot" of trace elements that can be mobilized to maintain the required concentrations in biological fluids of the body. Also, unwanted or even toxic elements that enter the body under adverse environmental

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conditions are "deposited" (and partially inactivated at the same time) in the bone. This is facilitated by the tolerance of the apatite structure to isomorphic substitutions, which ultimately makes it possible to store a completely satisfactory, functionally valuable mineral substance in the skeletal tissues even in conditions of acute fluctuations in the diet and environment.

It is essential that both the main ions and the "major" and "minor" elements of the bone tissue, localized mainly in the crystal lattice of biological apatite, can be found on the surface of the biomineral nanocrystals, in soft tissues and in biological fluids of the bone. In [6, 8], a model of ultrastructural organization of bone mineral was proposed, according to which apatite nanocrystals are surrounded by a relatively labile, but structured, hydration shell containing various cations and anions. Evidently, the non-apatite localization of metal ions yields greater mobility and more active participation in metabolic biochemical processes. For some elements, approximate concentration ratios and fractional distribution between different components of the tissue were experimentally determined or hypothetically proposed [9]. It is assumed that different ions have different capacities for transitioning from the structurally bound state to the labile state [3, 10].

Most often, the elemental composition of mineralized tissues is investigated by energy dispersive X-ray analysis (EDX (WDX)) and proton-induced X-ray emission (PIXE) analysis. These methods can be used to estimate the content of the main elements and "major" impurities at concentrations as low as ≥0.01 wt.%. Their main disadvantages are detection limit and inability to separately determine the fractions of the same element in different chemical states or different localizations. The methods of atomic absorption spectrometry (FAAS, GFAAS) and elemental analysis by mass spectrometry, including those with inductively coupled plasma, having high analytical characteristics, especially a low LOD, are also unable to provide information on the preferential localization of the trace element being determined.

In recent years, a new effective approach has been developed for obtaining information on the local coordination or chemical status of ions. This approach is based on X-ray absorption spectroscopy (XAS), namely, on the analysis of the X-ray absorption near edge structure (XANES) spectra and the extended X-ray absorption fine structure (EXAFS) spectra [9 11, 12]. However, the widespread use of these methods is stifled by the limited availability of the necessary high brightness X-ray source (synchrotron). Nevertheless, XAS data on the percent distribution of Sr^{2+} between the apatite lattice (35–45%), hydrated environment (~30%), and collagen are already available [9]. Using the μ-XANES method, evidence was obtained indicating preferential localization of Pb in the Ca^{2+} positions of the apatite lattice [12]. Information is being accumulated on the chemical status of Zn^{2+} in bones, cartilage and pathological calcifications [13, 14]. In studies of biological mineralized tissue, XAS methods are used in conjunction with micro X-ray fluorescence (μXRF) and micro X-ray diffraction (μXRD) analyses [13]. Based on the accumulated experience, there are prospects for studying the dependence of the primary localization of trace elements on the age, origin and formation conditions of the biominerals.

In this situation, it seems appropriate to supplement the above approaches with more traditional and affordable methods for the quantitative determination of trace elements using simple methods for fractionating biological mineralized tissue samples. Preparation of the material is the key stage of research.

In [15–17], a method was proposed for isolating unchanged bioapatite crystals from bone tissue by combined chemical and ultrasonic treatment. In light of such preparation, the morphological characteristics of bioapatite nanocrystals were investigated by the methods of transmission electron microscopy [15, 16] and atomic force microscopy [17].

In [10, 18], preferential localization of Mg, Na, and K in bone apatite samples was studied using atomic absorption and atomic emission spectrometry. The sample was prepared by stepwise heat treatment (in the range of 560–720^{o}C) followed by ultrasonic dispersion of powdered materials in water. Labile (soluble) fractions of elements were determined in an aqueous medium after ultrasonic treatment. From the data obtained it follows that Mg and Na in bone bioapatite exist in a structurally bound state, replacing calcium in the apatite lattice, as well as in a labile state, localizing outside the crystals. Potassium, on the other hand, cannot enter into the structure of apatite or be chemically bound to it. It was also found that the concentration of labile magnesium in bone samples is about 30–40% of its total content.

In [19], to determine the preferential localization of Na and K in bones, organic material was chemically removed using hydrazine. Ionic composition of the samples prepared in this manner was determined using secondary ion mass spectrometry (SIMS). Most of the sodium and potassium was found precisely in the removed organic bone material.

A method of chemical separation of apatite and non-apatite components of bone tissue is tested in the present work, that excludes multi step slow annealing and ultrasonic dispersion procedures. This approach is based on the fact that calcium apatites of both biological and synthetic origin are practically insoluble in water and alkaline media, although soluble in acids. Other calcium phosphates, presumably present in some biological minerals, dissolve in distilled water (pH ≈ 7) significantly better than hydroxyapatite [3, 20]. In addition, the developed specific surface of the crystalline phase of the bone can accumulate a large number of vital trace elements of the body without binding them in the structure of apatite. Such mobile "labile" forms of storage are justified by the need for a quick retrieval and delivery of the accumulated elements in case of shortages somewhere in the body.

As can be seen, in aqueous media with a pH of 7–8, solutions of weakly bound labile elements present in biological mineralized tissues can be obtained. Increasing the pH to 10–12 leads to the complete dissolution of the organic components of bone tissue (mainly collagen). At the same time, at a pH of 10–12, the precipitation of secondary phases from dissolved ions can be initiated, which is undesirable for solving analytical problems relating to the separation of trace impurities of different localization. Therefore, in the present work, solutions with pH 6.5, 10, and 12 were used, and the obtained concentrations of «labile» fractions of the determined trace elements were critically analyzed taking into account possible reactions of formation and precipitation of new insoluble phases. The obtained values in no way completely describe the localization of trace elements of the bone, however, provide information about their relative ability to migrate in biological tissue.

Experiment. A sample of cortical (dense) thigh bone of an adult cow, obtained from a supplier of meat products immediately after slaughter, was used for the study. Preliminary sample preparation included mechanical cleaning and air drying at 100^{o}C. All procedures were performed with minimal use of reagents and chemical agents to prevent foreign elements from entering the samples. Dried bone samples were carefully ground in a porcelain mortar to a fine powder.

In preparation for determining the total concentrations of elements using atomic absorption spectrometry, a ~0.015 g sample was transferred to a polypropylene tube, 0.5 mL of concentrated (56%) nitric acid was added, and after complete dissolution of the sample, the sample volume was adjusted to 10 mL. Complete dissolution of the sample was confirmed by the absence of the Tyndall effect in the resulting solution.

The samples for measuring concentrations of elements in solutions with pH 6.5, 10 and 12 were prepared with the following steps: 1) preparation of solutions with pH 12: KOH (100 mL, 0.56 g/L), NaOH (100 mL, 0.4 g/L), NH_4OH; 2) dilution of the prepared solutions to pH 10 in separate polypropylene tubes (10 mL); 3) weighing 0.1 g of bone powder and placement in test tubes (7 pcs.); 4) filling the tubes with 5 mL of various solutions; 5) shaking the contents of the tubes for 10–15 min after adding the solution with repeated agitation (5 min) after precipitating the undissolved fraction; 6) settling the contents of the tubes on a rack for 15–16 h before measuring the concentrations.

High purity (≥99% pure) reagents (nitric acid, solutions of alkalis and ammonia) were used for the preparation of samples. All solutions were prepared using double distilled water with an electrical conductivity of ≥1 μS. Polypropylene chemical glassware was used to prevent the interaction of reagents with glass.

Calibration solutions for determining the content of Zn, Cu, Fe, Mn, Pb, Mg, Sr were prepared from state standard solutions.

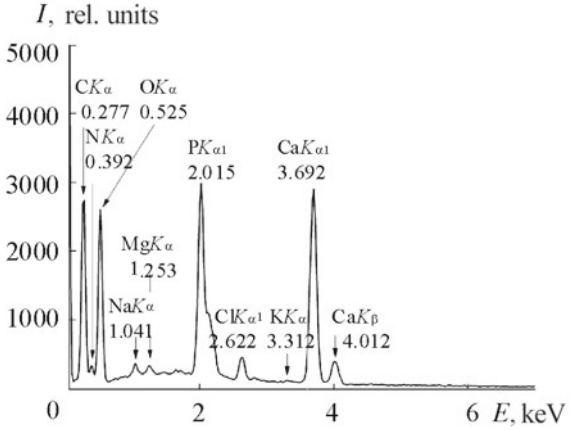
Concentrations were determined on a CAS 120.1 atomic absorption complex (SELMI, Ukraine) with deuterium background correction. The radiation source was an LT-6 hollow cathode lamp. An A-5 electrothermal atomizer 28 mm long with an inner diameter of 6 mm with a standard graphite-coated furnace was used.

The sample was injected into the furnace by means of an MD-20 dispenser with a volume of 20 ± 0.4 μL. Common spectral measurement parameters (wavelength, slit width, lamp current) for Zn, Cu, Fe, Mn, Pb, Mg, Sr were used [21]. The atomic absorption signal was scanned in 0.016 s increments and processed by a computer. The temperature program of the furnace was selected for each element separately to achieve the optimal analysis conditions. To prevent chemical contamination, no additional spectral buffers and modifiers were used.

A preliminary semi-quantitative assessment of the elemental composition of the presented bone sample in the initial state was performed using a Nova NanoSEM 450 scanning electron microscope (FEI, USA) equipped with an energy dispersive X-ray (EDX) silicon drift detector (SDD) X-MaxN (Oxford Instruments, UK) and an ultrathin window, SDD active area 20 mm^2. The accelerating voltage of the electron beam is 15 kV. The spectra were obtained by integrating the signal from an arbitrary scanned area of a smooth cut of the bone. Conductive coatings were not applied to the materials before analysis.

The apatite nature of the biomineral sample and its invariance after treatment in alkaline solutions with different pH levels were confirmed by X-ray diffraction methods in parallel structural studies.

Results and Discussion. As confirmed by EDX-spectra (Fig. 1), elemental analysis of dense bone tissue by electron microprobe X-ray analysis is possible only at the level of basic elements and "major" impurities and even semi-quantitative determination of potassium in most cases causes serious difficulties. The concentrations of Na (≈1.16 wt.%) and Mg (≈0.45 wt.%) estimated from the EDX spectra match the literature data [3, 6]. At the same time, as can be seen from Fig. 2, atomic absorption spectrometry makes it possible to reliably measure the concentrations of both macroelements (Mg) and trace elements, including even Cu and Mn found in the bone tissue at concentrations of only a few ppm. Measured concentrations of bone trace elements are consistent with literature data [3, 6]. The concentrations of Sr and especially Pb slightly exceeding the values given in [6] can be explained by the very high susceptibility of bone tissue to adverse environmental factors. It should be noted that Mg concentrations determined by atomic absorption spectrometry and EDX are in good agreement.



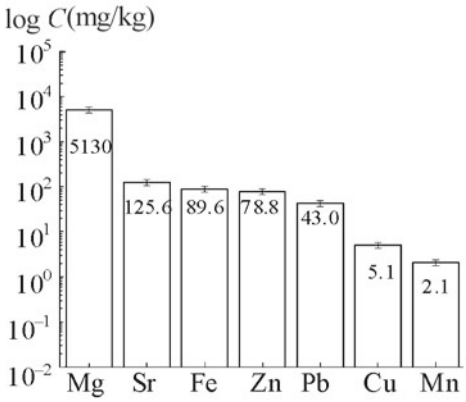


Fig. 1. EDX-spectrum of the studied cortical bone sample.

Fig. 2. Total (absolute) concentrations of elements in the cortical bone sample, determined by atomic absorption spectrometry.

The measured content of selected elements in solutions with different pH values shows that Pb and Mn are not typically found in the dissolved state. This suggests that these elements are predominantly localized in bone apatite. The content of Zn in solutions of different electrolytes is most atypical and is described below. For the remaining elements (Mg, Sr, Fe, and Cu), whose concentrations in decanted solutions can be measured by atomic absorption spectrometry, i.e., exceed the threshold of reliable detection, diagrams have been constructed (Fig. 3). Analysis of these diagrams can be used to draw some conclusions about the ratios of stable (structurally-bound) and labile fractions of elements.

According to Fig. 3a, ~80–85% of total magnesium exists in a bound state (i.e., insoluble in water). From the data of [18] (annealing + ultrasound + atomic absorption), it follows that ~60–70% of the magnesium present in the bone is embedded in the bioapatite structure, replacing calcium. Presumably, the rest of the magnesium localized outside of the bone mineral crystals is removed by ultrasound treatment. This difference in the percentage of bound Mg can be explained primarily by the fact that the exclusion of ultrasonic treatment reduces the efficiency of the release of Mg ions, localized on the surface of the crystals, into solution. In addition, there may be specific features of this bone sample. In general, the data from this study and earlier works are in satisfactory agreement and indicate the presence in bone tissue of a significant fraction (³ 15% of the total) of water-soluble magnesium. The amount of soluble magnesium is significantly lower in ammonia solution and in an alkaline environment (at pH 10 and even more so at pH 12). This decrease in the solubility of magnesium is due to an increase in the concentration of hydroxyl ions with increasing pH.

In contrast to magnesium, only a small fraction of strontium (≈1%) is found in water (Fig. 3b), i.e., it is not associated with the biological apatite structure. The content of strontium in distilled water (1.5 mg/kg) and solutions of alkalis and ammonia (≤ 8.5 mg/kg) is significantly lower than the 1600 mg/kg calculated for the $Sr(OH)_2 \rightarrow SrOH^+ + OH^-$ reaction, solubility product (K_{sp}) = $3.2 \cdot 10^{-4}$ [22]. This means that the formation and precipitation of new insoluble phases cannot be the cause of the detected low concentrations of strontium in solutions. A slight increase in the concentration of soluble strontium in an alkaline medium (at pH 10 and 12) compared to neutral pH can be explained by the presence of a small fraction of strontium in the organic component of the bone, which dissolves completely in alkalis. Thus, the obtained data indicate that most of bone tissue strontium is localized in apatite crystals, replacing calcium, and only a small fraction is found in chemical states unbound to the mineral structure.

Bone tissue iron (Fig. 3c) to a much greater extent than strontium (but less than magnesium), can be in a mobile (soluble) state (≈2% of the total). The content of iron in solutions of alkalis, ammonia and distilled water is an order of magnitude lower than the calculated value (9.6 mg/kg) required for the reaction $Fe(OH)_2 \rightarrow FeOH^+ + OH^-$, $K_{sp} = 3 \cdot 10^{-10}$ [22], meaning low iron content values may not be due to the formation and precipitation of new insoluble phases. The slight increase in the concentration of mobile iron in an alkaline medium compared to neutral can be explained by the presence of a small amount of iron in the organic components of the bone.

The copper content for almost all variants of solutions (Fig. 3d) exceeds the detection limit only slightly, except in solutions of KOH and NH_4OH at pH 12, in which a noticeable increase in the amount of copper was found due to the formation of highly soluble copper complexes [23]. The concentration of copper in water is equal to 0.030 mg/kg,

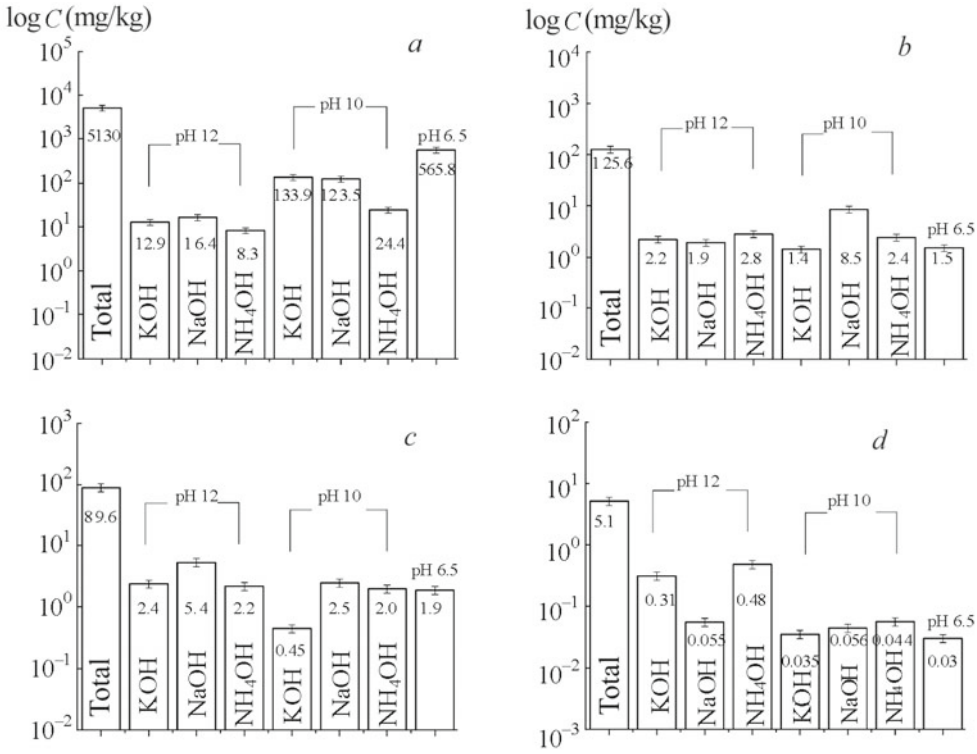


Fig. 3. Content of magnesium (a), strontium (b), iron (c) and copper (d) in the studied cortical bone sample.

the calculated value for the reaction $Cu(OH)_2 \rightarrow CuOH^+ + OH^-$, $K_{sp}= 2.2\cdot10^{-13}$ [22]. In general, the obtained data indicate that almost all bone tissue copper exists in the bioapatite structure.

Data on the concentrations of Zn are different for solutions of different electrolytes. No zinc was detected in distilled water and alkali solutions of NaOH and KOH. In ammonia solutions, 0.93 and 10.0 mg/kg Zn were found at pH 12 and 10, respectively. It is likely that zinc hydroxide formed in alkaline medium $Zn(OH)_2$ produces an insoluble precipitate upon interaction with NaOH and KOH and, on the contrary, in solutions of ammonia, a soluble complex is formed according to the reaction $Zn(OH)_2 + 4NH_3 \rightarrow 3[Zn(NH_3)_4](OH)_2$. Based on this, zinc should be considered as an element capable of both being bound in the crystal lattice of bioapatite and included in the composition of the organic components of bone tissue, and the non-apatite fraction of zinc appears to be quite significant.

Comparing the data obtained for zinc and magnesium, it is noteworthy that the ratio of the "insoluble" and "soluble" fractions for them is approximately equal with the only difference being that in the case of zinc the best solvent is ammonia, and in the case of magnesium — water. This indicates that non-crystalline zinc is predominantly localized in alkali-soluble organic component, and magnesium is found in unbound water or biological liquids of the bone.

Conclusions. A method is proposed for determining the ratio of labile (soluble) and structurally-bound trace elements of bone tissue by chemical separation of bone components with subsequent measurement of concentrations by atomic absorption spectrometry. The total concentrations of Mg, Zn, Fe, Sr, Cu, Mn, Pb in the bone sample and the concentrations of these elements in solutions of various electrolytes at pH 6.5, 10 and 12 after their interaction with the crushed bone preparation were determined. The ability of elements to have mobile (non-apatite) fractions in the composition of bone tissue can be represented as a sequence: Mg > Zn ≥ Fe > Sr > Cu; and noncrystalline Mg is predominantly localized in water or biofluids of the bone, while noncrystalline Zn can be found in the alkali-soluble organic component; no Pb and Mn was found in the tested solutions, meaning that these elements are localized in the crystalline phase.

Apparently, preferential localization and relative lability of biologically significant elements are determined by the functional purpose of the bone tissue, which means that any deviation from the normal ratio of "insoluble" and "soluble" fractions can be associated with a particular pathology. We hope that this work and the subsequent studies of bone tissue of various types (compact and spongy) in healthy bone and in case of pathologies (for example, osteoporosis) will supplement and refine the existing model of the ultrastructural organization of the bone mineral [6] and help to identify correlations

between the distribution of trace elements and functional impairment. This in turn should contribute to selecting the correct strategy for the treatment and prevention of diseases and pathologies of the skeleton.

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