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The Role of β-Antagonists on the Structure of Human Bone – A Spectroscopic Study

J. Anastassopoulou[1], P. Kolovou[1],
P. Papagelopoulos[2] and T. Theophanides[1]
[1]*National Technical University of Athens, Chemical Engineering Department, Radiation Chemistry and Biospectroscopy, Zografou Campus, Zografou, Athens*
[2]*National and Kapodistrian University of Athens, School of Medicine, Department of Orthopaedic Surgery and Traumatology, Athens*
Greece

1. Introduction

Human bones are inherently complex materials consisting of minerals, collagen, water, non-collagenous proteins, lipids, vascular elements and cells. The bone is a physiologically active and reactive tissue (Petra et al., 2005). Through hormonal or mechanical signals the osteoblasts and osteoclasts are forming the bones. It is known that the role of osteoblasts is to create a collagen-rich extracellular matrix, which will become mineralized (bone formation) with calcium. On the other hand, the main role of osteoclasts is to degrade calcified bone tissue (resorption) (Shier et al., 1996). In the bone microenvironment, there is a dynamic balance between resorption and formation that maintains skeletal homeostasis. This process between bone formation and bone resorption is called remodelling. Bone remodelling and bone loss, is in function of age, external mechanical loads originating from physical activity and diseases.

The inorganic component of bone accounts approximately to 65% of the wet weight of bone and it is not pure hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, but a poorly crystalline calcium hydroxide in a deficient biological apatite, containing numerous trace anions, the most abundant of which is carbonate (CO_3^{2-}) and acid phosphate (HPO_4^{2-}), fluoride (F^-) and citrate ($C_6H_5O_7^{3-}$) anions, as well as, magnesium (Mg^{2+}), potassium (K^+) cations. These anions and cations are common substitutes of calcium and hydroxide (OH) in hydroxyapatite (Petra et al., 2005; Maguire and Cowan, 2002). The major organic component of bone is collagen, predominantly type I, which provides the bone with elasticity and flexibility and directs the organisation of matrix. Water accounts for 5-10% and collagen with proteins are about 25-30% of the weight of bone tissue. Hydrogen bonds between water and collagen contribute to the stabilisation of a triple helix, and there have been suggestions that dehydration of the collagen may take place during demineralization of bone (Petra et al., 2005; Buckwalter et al., 2000).

In the last decade, there is an increasing interest in using infrared spectroscopy to evaluate and study biological systems (Petra et al., 2005; Mantsch et al., 1986; Anastassopoulou et al., 2008, 2009, 2011; Conti et al., 2008; Kolovou and Anastassopoulou, 2007;Pissaridi etal., 2011;

Mamarelis et al., 2010, Theophanides, 1979; Theophanides et al., 1993). FT-IR spectroscopy is a powerful non destructive technique and easy to investigate complex systems as bones with this technique. The absorption of infrared radiation excites the vibrations of the chemical bonds to higher energy levels, by changing the dipole moment of the molecule with which it interacts. This change and interaction gives the absorption spectrum with the characteristic absorption bands based on the total vibrational modes of biological molecules within the sample, such as hydroxyapatite. This powerful technique permits the study of homogenous and inhomogeneous systems, such as, bone, providing information from all tissue components, both organic and inorganic.

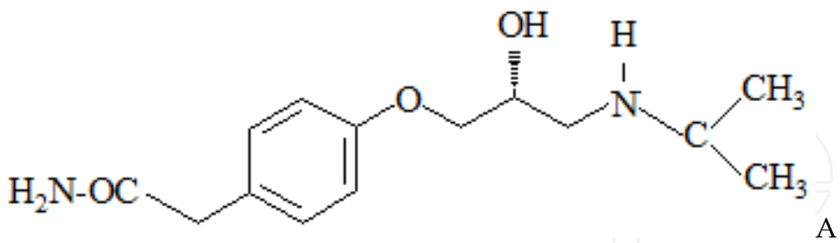
Adrenergic receptors are well known to be present in osteoblastic cells, and it is known that the α- and β-receptors could activate pharmacologically the proliferation of these cells (Suzuki et al., 1998). It is also known that adrenergic agonists efficiently activate β-adrenoreceptors on osteoblasts and can stimulate bone resorption in intact mouse calvaria (Moore et al., 1993). Furthermore, it has been reported that propranolol inhibited cAMP formation induced by β–adrenergic agonists in bone organ cultures (Takeuchi et al., 2000) increased bone strength and the rates of endochondral bone formation in rats (Dietrich et al., 1979; Minkowitz et al., 2005).

The purpose of this *in vitro* work is to study the role of the non-selective beta-adrenergic receptor and beta1-selective adrenoreceptor blocking agents on demineralization of human bones, which was induced by EDTA.

2. Materials and methods

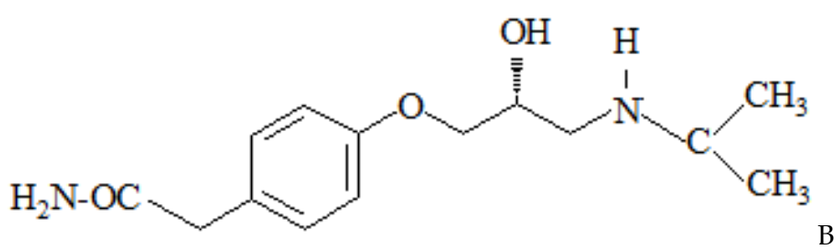
The chemical compounds:

Timolol, (S)-1-[(1,1-dimethylethyl)amino]-3-[(4-4(morpholinyl)-1,2,5-thiadiazol-3-yl] oxy] -2-propanol (Z) –butenedioate, with the empirical formula $C_{13}H_{24}N_4O_3S$ and chemical structure, A:



A

And Atenolol, 4-[2′-hydroxy-3′-[(1-methyl-ethyl) amino] propoxyl, $C_{14}H_{22}N_2O_2$ with chemical structure, B:



B

Ethylene Diamino Tetracetic Acid (EDTA), ($C_{10}H_{14}O_8N_2Na_2$).$2H_2O$ [$CH_2N(CH_2COOH)$ CH_2COO].$2H_2O$ and benzeneacetamide [4-[2′-hydroxy-3′-[(1-methyl-ethyl)amino] propoxyl] were products of Sigma.

The bones were obtained intra-operatively from heads of femur of 65-75 year patients undergoing osteotomy. The samples were prepared in slices in order to preserve better the natural characteristics of the bones. The bone was cut into slices of 2 mm perpendicular to the longitudinal axis of the head (Fig. 1). The histological evaluation of representative sections of the biopsies showed no evidence of any metabolic disease, osteopenia, or bone cancer.

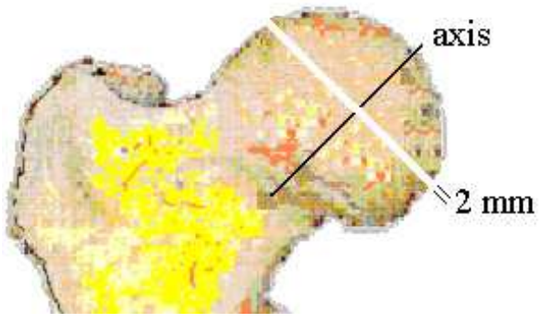


Fig. 1. The anatomic location and the size of the bone sections.

The bone sections were immersed successively in hydrogen peroxide solution (H_2O_2) and in acetone, according to a modification of the method described (Petra et al., 2005). Another bone section was reacted with aqueous EDTA solution and was left for a week in 4 ^oC (Veis and Schlueter, 1964). Twenty mg of bone were mixed with 200 mg of KBr powder in a pestle and was grownded in a mortar and compressed into a pellet. Two more sections were reacted with 2ml aqueous solutions of Na_2EDTA 0,5 M in the presence of timolol 0,5 M and 0,5 M atenolol. The mixture was left for a week at 4^0 C.

2.1 Preparation of bone samples

Fresh cancellous bone was immersed successively in hydrogen peroxide solution (H_2O_2) and in acetone, according to a modification method (Petra et al., 2005). Hydrogen peroxide and acetone processing is known to reduce blood chromophores of fresh bones and the fat tissues of the bone (Petra et al., 2005), but it does not remove the organic components completely. The processed bone sample was cut with microtome in multiple slices of 2 mm thickness each.

One dry slice was demineralized. The demineralization of the bone was carried out by extraction of calcium ions with 0.5 M EDTA at 4^oC, where the pH was adjusted to pH 7.4 with potassium hydroxide (Veis and Schlueter, 1964). Then the bone slice was washed thoroughly with repeated changes of distilled water and acetone.

Two bone slices were left to become demineralized in the same way by extraction at 4^oC with 0.5 M EDTA adjusted to pH 7.4 with potassium hydroxide in the presence of 0.25 M timolol or atenolol, for one week. Twenty milligrams of the cancellous section of the slices were mixed with 200 mg KCl powder in a pestle and mortar and compressed into a pellet to

be studied by FT-IR. A small section of the bone slices was also studied with SEM. It must be noticed that both compounds A and B did not interact with EDTA.

2.2 FT-IR spectroscopy

Fourier Transform Infrared (FT-IR) spectra were recorded in a frequency range of 4000-400 cm-1 using an FTS 3000 MX BioRad, Excalibur Series spectrophotometer and were processed with the Bio-Rad Win-IR Pro 3.0 Software. Twenty mg of fresh bone were mixed with 200 mg of KBr powder in a pestle and mortar and compressed into a pellet. Typically, 32 scans were collected at 4 cm^{-1} resolution over the wavenumber range of 400-4000 cm^{-1}.

2.3 Scanning Electron Microscopy (SEM)

The morphologic and chemical composition of the compounds was obtained by Scanning Electron Microscopy (SEM) with a Quanta 200, (FEI, Hillsboro, Or, Usa) apparatus equipped with an X-ray detector EDS, Saphire CDU, (Edax Int, Mawhaw, NJ, USA). The spectra were obtained with acceleration of 10 kV and beam light 100 μÅ was applied. The samples were covered with graphite with an SCD 004 Sputter-Coater and OCD 30 attachment (Bal-Tec, Vaduz, Liechtenstein). The SEM spectral maps were processed with the Gemin (3.5 version, Edax Int) Software.

3. Results and discussion

3.1 FT-IR spectra

The FT-IR spectra of homogenized bone samples before and after interaction with EDTA solution is shown in Figs. 2a and 2b, respectively.

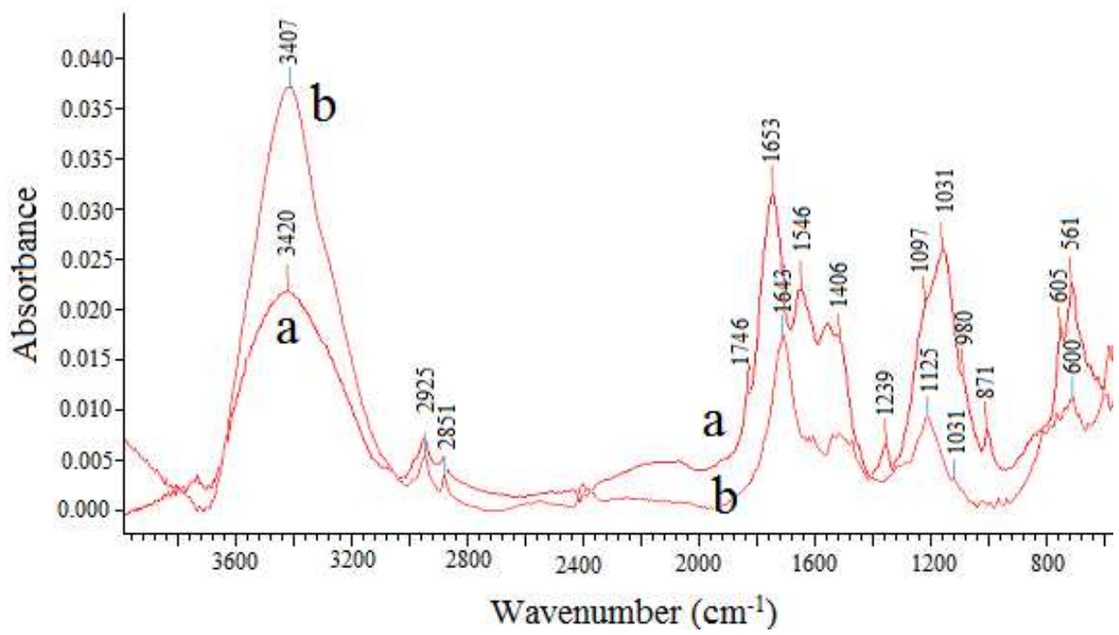


Fig. 2. FT-IR spectra of a) bone and b) bone after the first week of demineralization with EDTA

The infrared absorption bands (cm^{-1}) and their assignments are given in Table 1.

Unprocessed cm^{-1}	**After the 1st week of demineralization with EDTA cm^{-1}**	**Assignment**
3420	3407	υOH, υN-H
3067		υCH=
2925	2924	$\upsilon_{as}CH_2$
2852	2851	$\upsilon_s CH_2$
1746		υC=O non-ionised -COOH
1653	1643	νC=O + δN-H Amide I
1546		δN-H in-plane + νC-N, Amide II
1452		$\delta_{as}CH_3$ + $\nu_3CO_3^{2-}$, AB carbonate
1406		νCOO^- & $\nu_3CO_3^{2-}$, AB carbonate
1337		ρ_w-CH_2 wagging
1239		νCN + δNH in-plane, Amide III
1097 sh		ν_3-PO_4^{3-},non stoich. HA
1031	1031	ν_3-PO_4^{3-}, stoich. HA
960 sh		ν_1-PO_4^{3-}, stoich. HA
871		$\nu_2CO_3^{2-}$, B carbonate-
605	609	ν_4-PO_4^{3-}, HA
561		ν_4-PO_4^{3-}, HA

Table 1. Peak assignments of the FT-IR spectra of homogenized bone before and after a week of demineralization with EDTA.

Significant differences are shown in the spectra of the bone (Fig. 1b) after interaction with EDTA. The broad band which appears at 3420 cm^{-1}in the bone spectrum shifts to 3407 cm^{-1} after decalcification of the bone. This band is dominated by absorptions from stretching vibration of υOH and υNH functional groups of hydroxyapatite and proteins, respectively and is particularly sensitive by decalcification of the bone. This band shows that the OH groups of HA are reduced, while there are other free NH groups, which do not give neither inter- nor intra-molecular hydrogen bonds, leading to the result that the decalcification changes the secondary structure of proteins. The band at 3067 cm^{-1} is attributed to υ=CH stretching vibration of oxidized lipids (Petra et al., 2005; Mamarelis et al., 2010).

The bands in the spectra between 3000 and 2800 are characteristic of the antisymmetric and symmetric stretching vibrations of methyl (CH_3) and methylene (CH_2) groups. The bands near 2925 cm^{-1} and near 2852 cm^{-1} correspond to antisymmetric and symmetric stretching vibrations of υCH_2, respectively. These bands do not shift after demineralization, but increase in intensity. These changes show that the secondary structure of proteins changed and their environment became less lipophilic (Mamarelis et al., 2010; Anastassopoulou and Theophanides, 1990). The characteristic peak at 1746 cm^{-1} due to the stretching vibration of

νC=O of the non-ionized carboxyl group –COOH (Petra et al., 2005; Kolovou and Anastassopoulou, 2007; Anastassopoulou et al., 2008; Mythili et al., 2000) is absent in the spectrum of the demineralized bone. In the spectrum of the unprocessed cancellous bone, an intense band is seen near 1031 cm-1, which is characteristic of stoichiometric biological apatites, with two shoulders, one at 1097 cm^{-1}, which is assigned to non-stoichiometric hydroxyapatite (HA), containing HPO_4^{2-} and/or CO_3^{2-} groups[3] and one at 960 cm^{-1}, which is assigned to the symmetric stretching vibration of the PO_4^{3-} groups (Petra et al., 2005; Graham et al., 2008). The bands near 1452, 1406 and 871 cm^{-1}, are v_3 and v_2 carbonate groups and the bands near 605 and 561 cm^{-1}, are due to the $\nu_4PO_4^{3-}$ vibrational modes. These are clearly seen in the spectrum of the unprocessed cancellous bone and are absent in the spectrum of the decalcified or demineralized bone, since decalcification eliminates the calcium phosphates, $Ca_3(PO_4)_2$.

Significant changes were observed in the region of 1670 - 1540 cm^{-1}, where the Amide I and Amide II absorb. In many biological samples the Amide I band arises from the C=O stretching vibration with contribution of bending δNH of peptide bond of proteins. These Amide bands shifted to lower frequencies and are found at 1653 cm^{-1} and 1546 cm^{-1}, respectively in demineralized bones. This observation leads to the conclusion that the decalcification changes the secondary structure of collagen matrix from α-helix to β-sheet formation (Anastassopoulou et al., 2008, 2011; Kolovou and Anastassopoulou, 2007; Pissaridi et al., 2011; Mamarelis et al., 2010). This observation shows also that the hydrogen bonds between proteins and hydroxyl apatite have been broken and that the proteins have much more freedom in vibrational movements. This is also in agreement with the increase of the bands, which were observed in the region of 3000-2850 cm^{-1}, where as we noticed, the antisymmetric and symmetric stretching vibrations of methyl vCH3 and methlylen vCH$_2$ groups absorb. The above results were expected, since EDTA subtracts the Ca^{2+} cations from the bone to form complexes (Fig. 3) leading to its demineralization.

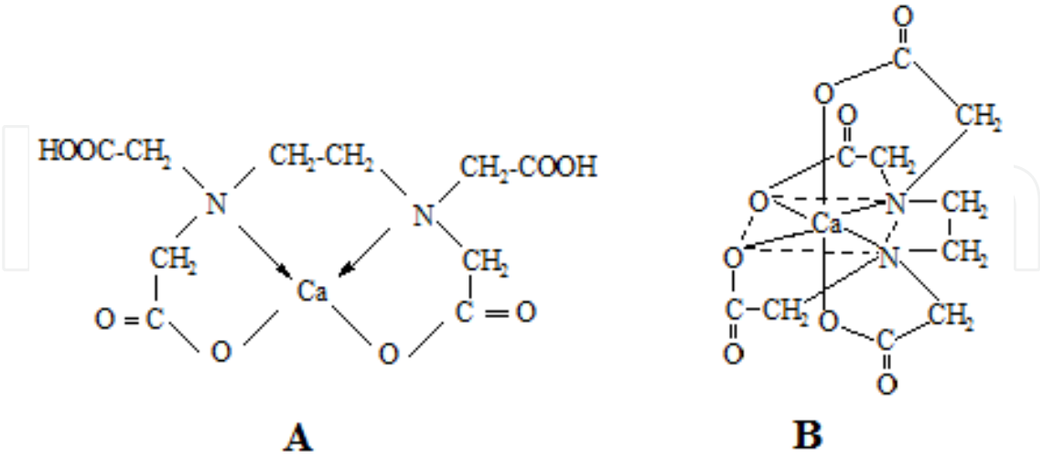


Fig. 3. EDTA-Ca complexes A. Tetrahedral coordination and B. Octahedral coordination

The non-intense band at 1239 cm^{-1} is assigned to Amide III, which arises from the in-phase δN-H in plane bending and vC-N stretching vibrations, which almost disappeared after demineralization. This band is also sensitive to protein structural changes.

In order to study the role of demineralization on the bone structure and to extrapolate the results to possible various bone diseases, we used calcium antagonists during demineralization with EDTA. The FT-IR spectra of bone, which were recorded after demineralization with EDTA in the presence of β- (timolol) and α- (atenolol) blockers after one week of reaction, are shown in Figs. 4b and 4c, respectively, in comparison with the spectra of untreated bone (Fig. 4a). The band assignments are given in Table 2.

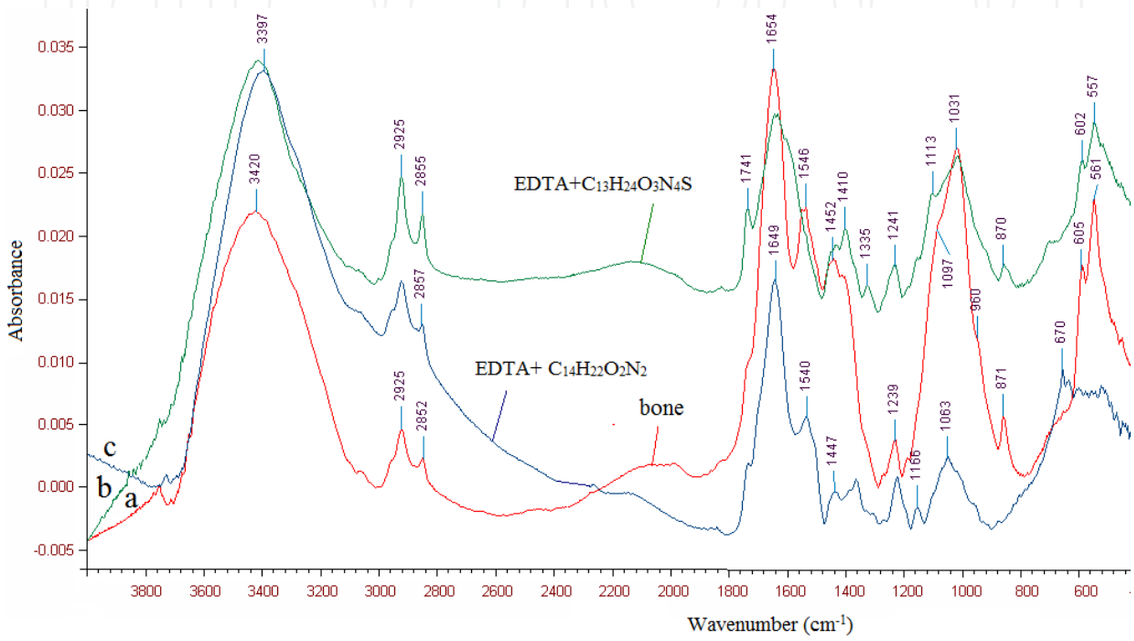


Fig. 4. FT-IR spectra of a) homogenized cancellous bone, b) homogenized and demineralized, cancellous bone with EDTA in the presence of timolol, $C_{13}H_{24}N_4O_3S$, and c) homogenized and demineralized cancellous bone with EDTA in the presence of atenolol, $C_{14}H_{22}O_2N_2$, for 1 week, in the region 4000-400 cm^{-1}.

By comparison of the spectra it was observed that the presence of β-blocker differentiates the reaction between EDTA and the bone. Especially, in the region 1700-1500 cm^{-1}, where the bands of Amide I and Amide II are located, the spectra show that the collagen loses partly the α-helix structure. On the other hand, in demineralized samples and in the presence of α-blocker the secondary structure of collagen changed from α-helix to β-pleated sheet. Interesting are also the results of the spectra in the region of the phosphate groups between 1110 cm^{-1} and 870 cm^{-1} are characteristic. Furthermore, from the spectral data (Fig.3) and Table 2 it results again that there is a competition reaction between EDTA and β-blocker for calcium cations (Ca^{2+}). Taking into account the chemical structure of the two calcium antagonists it was suggested that they should bind to hydroxyapatite of the bones with hydrogen bonds and thus inhibit the demineralization. These results are in accordance with the data of other investigators who reported that β-adrenoblockers prevented bone loss in animals (Mano et al., 2010).

Unprocessed cm^{-1}	Decalcified bone $C_{13}H_{24}N_4O_3S$	Decalcified bone $C_{14}H_{22}O_2N_2$	Assignments
3420	3414	3397	υN-H, υOH
3067	3067	3067	υ=CH
2925	2924	2924	$\upsilon_{as}CH_2$
2852	2855	2857	$\upsilon_s CH_2$
1746	1741	1741	υC=O non-ionised -COOH
1653	1653		νC=O + δN-H Amide I
	1648	1649	νC=O + δN-H Amide I
1546	1550	1540	δN-H in-plane + νC-N, Amide II
1452	1450	1447	$\delta_{as}CH_3$ + $\nu_3CO_3^{2-}$, AB carbonate
1406	1410	1394	νCOO^- & $\nu_3CO_3^{2-}$, AB carbonate
1337	1335	1335	ρ_w-CH_2
1239	1241	1233	νCN + δNH in-plane, Amide III
1097 sh	1113		ν_3-PO_4^{3-},non stoich. HA
1031	1028		ν_3-PO_4^{3-}, stoich. HA
960 sh	960		ν_1-PO_4^{3-}, stoich. HA
871	870		$\nu_2CO_3^{2-}$, B carbonate$^-$
605	602		ν_4-PO_4^{3-}, HA
561	557		ν_4-PO_4^{3-}, HA

Table 2. Band assignments of the FT-IR spectra of homogenized decalcified bones with EDTA in the absence and presence of 0.5 M antagonists $C_{13}H_{24}N_4O_3S$ and $C_{14}H_{22}O_2N_2$ for 1 week.

In the case of the presence of atenolol during demineralization, the pattern of the spectrum in the region 1097-960 cm^{-1} has changed to that of the characteristic amorphous structure of hydroxyapatite (Kolovou and Anastassopoulou, 2007;Anastassopoulou et al., 2008) as shown in Fig. 4.

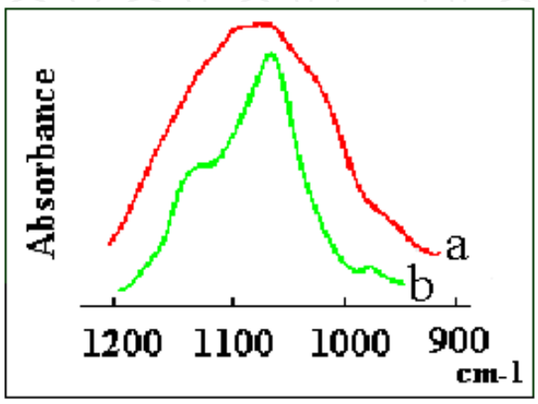


Fig. 5. FT-IR spectra of a) amorphous and b) biological hydroxyapatite.

This pattern of the infrared spectra, which correspond to amorphous structure of hydroxyapatite was also observed upon irradiation of bones (Anastassopoulou et al., 2008; Pissaridi et al., 2011) as well as in cancerous bones (Anastassopoulou et al., 2011), which suggest that under oxidative stress the bone loses its native molecular structure, in the same way as under artificial demineralization.

3.2 SEM spectroscopy

The spectra of the quantitative analysis of all the bone samples, obtained from the scanning electron microscope are shown in Fig. 6 and the results are shown in Table 3.

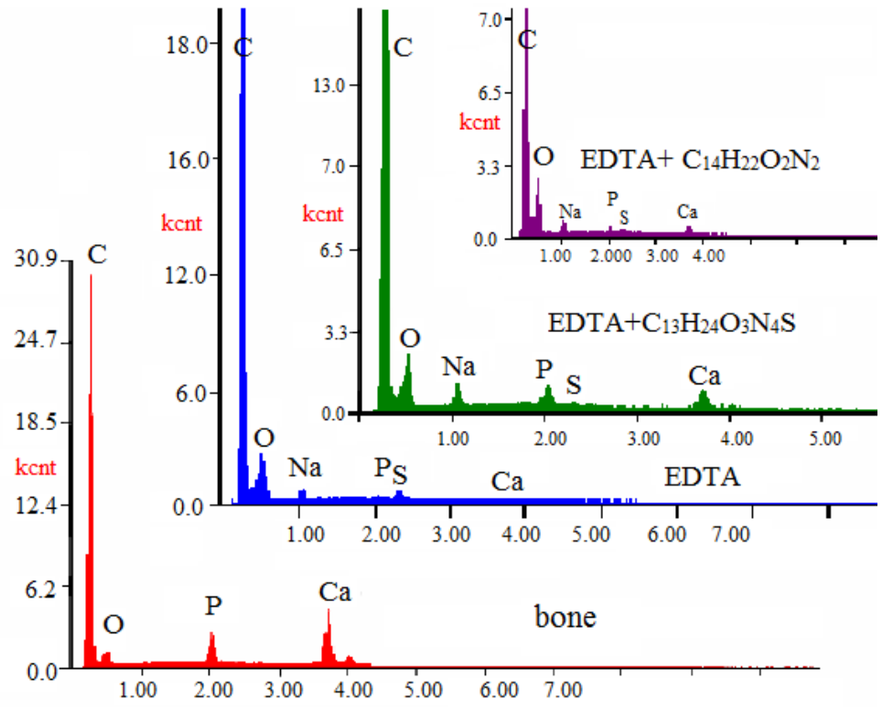


Fig. 6. Spectra of quantitative analysis of the bone samples.

Element	bone	Decalcified bone with EDTA	Decalcified bone EDTA+$C_{13}H_{24}N_4O_3S$	Decalcified bone EDTA+$C_{14}H_{22}O_2N_2$
O	59.01	69.71	64.24	76.90
Na	-	21.33	19.20	15.16
P	11.70	02.54	07.56	01.70
S	-	05.31	01.38	02.31
Ca	29.29	01.11	07.62	03.94

Table 3. Quantitative analysis data of the bone samples (% wt composition)

From the percentage data it is clear that the concentration of calcium has the highest value in the non-demineralized bone. The reaction of the bone tissue with EDTA leads to the disappearance of calcium of the bone. The presence of timolol during the reaction of the bone with EDTA seems to inhibit partially the decalcification of the bone tissues, since the

concentration of calcium increases up to 07.62. A similar result was obtained in the presence of atenolol, $C_{14}H_{22}O_2N_2$, but in both cases the calcium concentration was less than normal concentration.

In Fig. 7 are given the SEM images of cancellous bone sections with enlargement of X80. From the architecture and morphology of the images it is shown that after the reaction of bone with EDTA the sample does not show any bright regions, since the density of the bone is minimized (Fig. 7b) after the elimination of minerals of bone tissue.

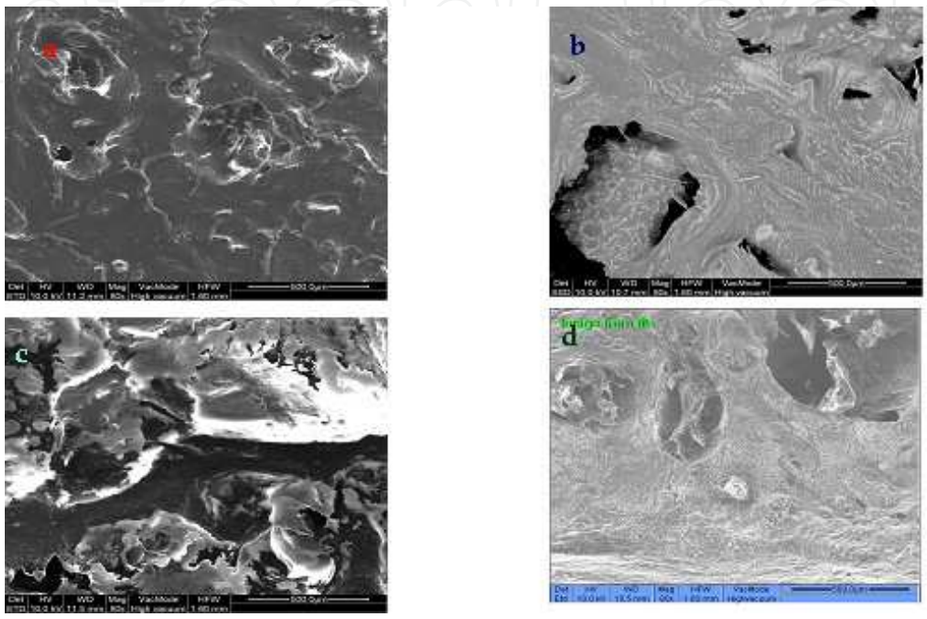


Fig. 7. SEM images of bone a) without any penetration, b) after demineralization with EDTA, c) after demineralization in the presence of $C_{13}H_{24}N_4O_3S$ and d) after demineralization in the presence of $C_{14}H_{22}O_2N_2$.

Significant changes in the brightness of the image are observed, when the demineralization takes place in the presence of $C_{13}H_{24}N_4O_3S$. It is observed an increase in bone density and the deposition of calcium on bone tissue is obvious (Fig. 7c). The image in Figure 6d corresponds to the result, which was obtained after the reaction of bone with EDTA in the presence of $C_{14}H_{22}O_2N_2$. The picture shows that the bone gets a more amorphous structure. These results are in agreement with the FT-IR data, which led to the suggestion that the biological hydroxyapatite changed from low crystallinity to amorphous state.

4. Conclusions

From FT-IR spectra in the region 1700-1500 cm^{-1}, which characterises the secondary structure of proteins, is found that demineralization changes the secondary structure of collagen from α-helix to β-pleated and random coil. Considerable intensity decrease was observed also in the region 1200-900 cm^{-1}, where the absorptions bands of phosphates from hydroxyapatite appear, which are due to bone demineralization and bone damage. The presence of calcium antagonists inhibits partly the demineralization of bones, which is induced from EDTA. These results could lead to the conclusion that the bone diseases

maybe change their secondary structure. These experimental data indicate that β-blockers may prevent bone loss in humans and new drugs to cure osteoporosis could be synthesized on this base.

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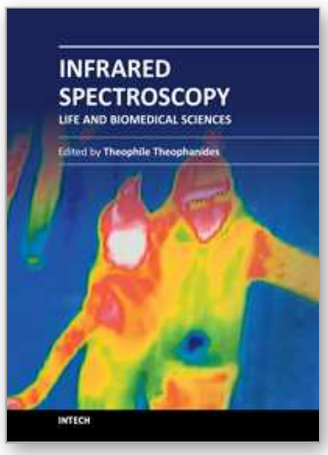

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This informative and state-of-the art book on Infrared Spectroscopy in Life sciences designed for researchers, academics as well as for those working in industry, agriculture and in pharmaceutical companies features 20 chapters of applications of MIRS and NIRS in brain activity and clinical research. It shows excellent FT-IR spectra of breast tissues, atheromatic plaques, human bones and projects assessment of haemodynamic activation in the cerebral cortex, brain oxygenation studies and many interesting insights from a medical perspective.

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InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
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