THE PRESENCE OF HYDROXYAPATITE CRYSTAL IN HETEROTOPIC OSSIFICATIONS ANALYZED BY MEANS OF X-RAY POWDER DIFFRACTION METHOD

F.L.P. SABOU, R. NECULA**, I. ŞAMOTĂ**, ANCA SABOU****

*"Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania,
e-mail: drsabou@yahoo.com
** Faculty of Medicine, Transylvania University Braşov, Romania
*** Elias Emergency University Hospital, Bucharest, Romania

Abstract. X-ray diffraction is a relatively easy method to analyze the crystalline composition of solids, useful in bone crystalline composition determination. We demonstrate the presence of hydroxyapatite crystals existing in normal skeletal bone in all 6 heterotopic ossification samples analyzed by means of X-ray powder diffraction technique. The phenomenon of heterotopic ossification mineralization seems to be similar to that of normal skeletal bone mineralization.

Key words: Heterotopic ossification, mineral composition, X-ray powder diffraction.

INTRODUCTION

X-ray diffraction is a non-destructive analytical technique used to identify and determine different crystalline components present in solids, known as phases. Each solid has its unique characteristic and specific X-ray powder pattern which can be used for its identification [8].

Crystal structure is an internally unique, repeated arrangement of atoms or molecules in crystalline solids which can diffract light. The wavelength of X rays is similar to the distance between atoms 0.1 Å – 100 Å. When an X-ray beam strikes an object, the emitted photons are scattered in all directions, the scattering of X-rays from atoms produces a diffraction pattern into which the angle of reflection is equal to the angle of incidence. That pattern contains information about the atomic arrangement within the crystal [4, 8, 13].

Thus, if we know the wavelength λ of the radiation passing through the crystal and if we can measure the θ angle of diffracted X-rays, we can calculate the

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interplanar spacing d between atom planes using Bragg's law $2d\sin\theta = n\lambda$, which allows phase identification [12].

The mineralization of bones is a specific and complex phenomenon that occurs due to deposition of hydroxyapatite crystals in the organic bone matrix consisting of proteins and type I collagen [6].

Hydroxyapatite with the formula $Ca_{10}(PO_4)_6(OH)_2$ is an important biological component and is the main inorganic constituent of human bones and teeth. It is an insoluble salt of calcium and phosphorus and it represents about 65% of adult bone mass [3, 15].

Heterotopic ossification also known as ectopic ossification or ectopic calcification is defined and described as formation of lamellar bones inside soft-tissue structures where bones normally do not exist (muscles, organs, tendons, skin) and is often the result of physical trauma which induces local and systemic changes leading to soft tissue mineralization [2].

Heterotopic ossification can be a complication of many orthopedic surgical interventions especially in the hip region and they can remain asymptomatic for a long period of time. However, in advanced stages, it can lead from serious limitation of joint mobility to total joint ankylosis [11].

The aim of the study was to determine the presence of hydroxyapatite crystals (existing in normal skeletal bones) in heterotopic ossification, by means of X-ray powder diffraction technique.

MATERIALS AND METHODS

For the study we used heterotopic ossification bone fragments collected from periarticular hip joint muscles and from the knee joint. A number of 6 intraoperatively collected heterotopic ossification bone fragments were processed and analyzed: 4 ectopic bone fragments from different patients from the hip joint (intraarticular free bone fragments and periarticular heterotopic bone fragments from abductor muscles collected after hip replacement) and 2 intraarticular heterotopic bone fragments from different patients, from the knee joint.

X-ray diffraction analysis was performed on samples processed from compact bone fragments using a Philips diffractometer model Micro 101, an existing equipment within the Faculty of Mineral Resources and Environment – North University of Baia Mare, with vertical goniometer, focusing Bragg-Brentano, couple scan $\theta - 2\theta$, X-ray tube with Cu anode, using CuKα characteristic radiation wavelength $\lambda = 1.5405$ Å.

Samples preparation for analysis was made according to the standard technique described in literature and according to laboratory standards [9].

Bone fragments collected during surgery were subjected to immediate mechanical removal of adherent soft tissues; they were washed thoroughly with cold water and stored temporarily for 24–48 hours in a 10% buffered formalin solution, in plastic containers.

The next step consisted of drying the bone fragments within natural conditions, away from contact with external agents that could change the crystalline composition (dust, metal powders and other materials) of the fragments, for a period of 45–60 days, at an ambient temperature of 18–26 °C and at ambient humidity, in order to achieve dehydration and consistency required for experimental processing.

Once dried, bone fragments were subjected to mechanical fragmentation in smaller pieces using mortar and pestle, followed by pulverization using special stone mortar, until fine powder was obtained, needed in order to be introduced into the diffractometer sample holder.

The fine powder resulting after mortaring, an amount of about 0.30–0.40 g of powder, was placed in the diffractometer sample holder, well tamped and with excess powder removed, and introduced in the diffractometer goniometer, in order to be analyzed.

The finer the resulting powder is, the more conclusive the results are.

Data obtained as a result of the analysis was gathered by the X-ray diffractometer, it was recorded and printed in a graphical form on graph paper, as diffractograms. Knowing in advance the angular range into which the hydroxyapatite crystal diffracts, the samples were analyzed within the range of an adjusted 2θ angle.

On each recorded diffractogram we have marked the initial and final 2θ angle for which the X-ray diffractometer has been set for work. We considered the maximum diffraction peaks as being relevant, respectively the most intense peaks where the diffraction phenomenon occurred, excluding low intensity peaks. After determining the important diffraction peaks, by using the ruler we measured the 2θ angle corresponding to the peak of interest.

In order to determine the relative crystalline structure of the analyzed bone powder, by using Bragg's Law we carried out the calculation of the distance between the atom planes depending on the 2θ diffraction angle.

Elements of the crystalline phases of the samples to be examined can be identified by comparing the results with a reference database. If a component is not included in the database used, further investigations are needed that can help in identifying unknown phases, sometimes earlier records can be considered as reference values and may be useful in interpreting test results.

The mineral content of each test, the degree of crystallization should be at least 4% in order to be detected by the X-ray diffractometer.

In order to obtain conclusive results (resolution diffractometry) which highlight the sought crystal, at least 3 peaks/3 angle diffraction peaks at 2 theta values have to be obtained, corresponding to the distance *d*, as mentioned within the theoretical data sheet [7], or within the database of the Joint Committee on Powder Diffraction Standards [1].

Subsequent to calculating the distance between atom planes, data was compared to theoretical data existing in the database of the the Joint Committee on Powder Diffraction Standards (JCPDS card. No 9-432).

RESULTS AND DISCUSSION

The complex phenomenon of heterotopic ossification development is not completely elucidated. Some local and general factors like soft tissue edema, vascular stasis, tissue hypoxia and trauma are involved within the formation process. Heterotopic ossification originates from osteoprogenitor stem cells within the traumatized soft tissues whereas the stem cells have the ability to transform into osteoblasts and they initiate osteoid formation and mineralization leading to mature lamellar heterotopic bone [10].

Until recently it was asserted that the pathological (heterotopic) mineralization would be the result of chemical precipitation of calcium and phosphate in the place where heterotopic ossification was about to get shape, however, recent studies showed that mineralization of soft tissue is a phenomenon which can be compared in many ways with mineralization of skeletal bones [5].

By performing this experimental study, by means of using X-ray diffraction, we pinpointed the presence of hydroxyapatite crystals within examined heterotopic bone fragments, proving the fact that mineralization takes place not only within the skeletal bones, but also within heterotopic bones.

Figures 1 and 2 show recordings of the diffraction patterns for the examined heterotopic bone fragments. The identification and analysis of peaks were done as described in Material and Methods section.

Tables 1 and 2 present calculated distances between atom planes based on the 2θ angle at which the diffraction phenomenon occurred separately for each examined sample. Only highest intensity peaks were selected, for which diffraction occurred.

Subsequent to calculating interplanar distances based on the 2θ diffraction angle and subsequent to listing data for each examined sample, data was compared to data existing on the theoretical data sheet of the X-ray diffractometer. Following Table 2 shows the data extracted from theoretical data sheet which was used as a comparison base.

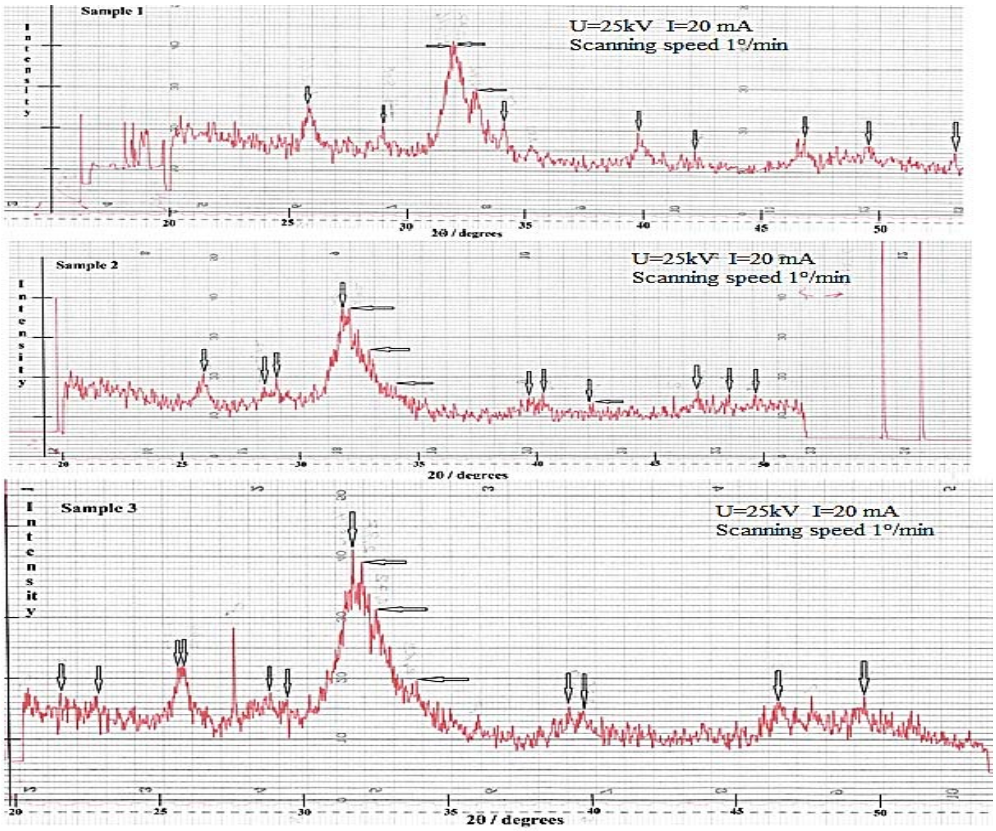

Fig. 1. XRD patterns of analyzed bone fragments. Samples 1 and 2 – Ectopic bone fragments from knee joint. Sample 3 – Periarticular ectopic bone fragments from hip joint. The arrows indicate the peaks taken into account.

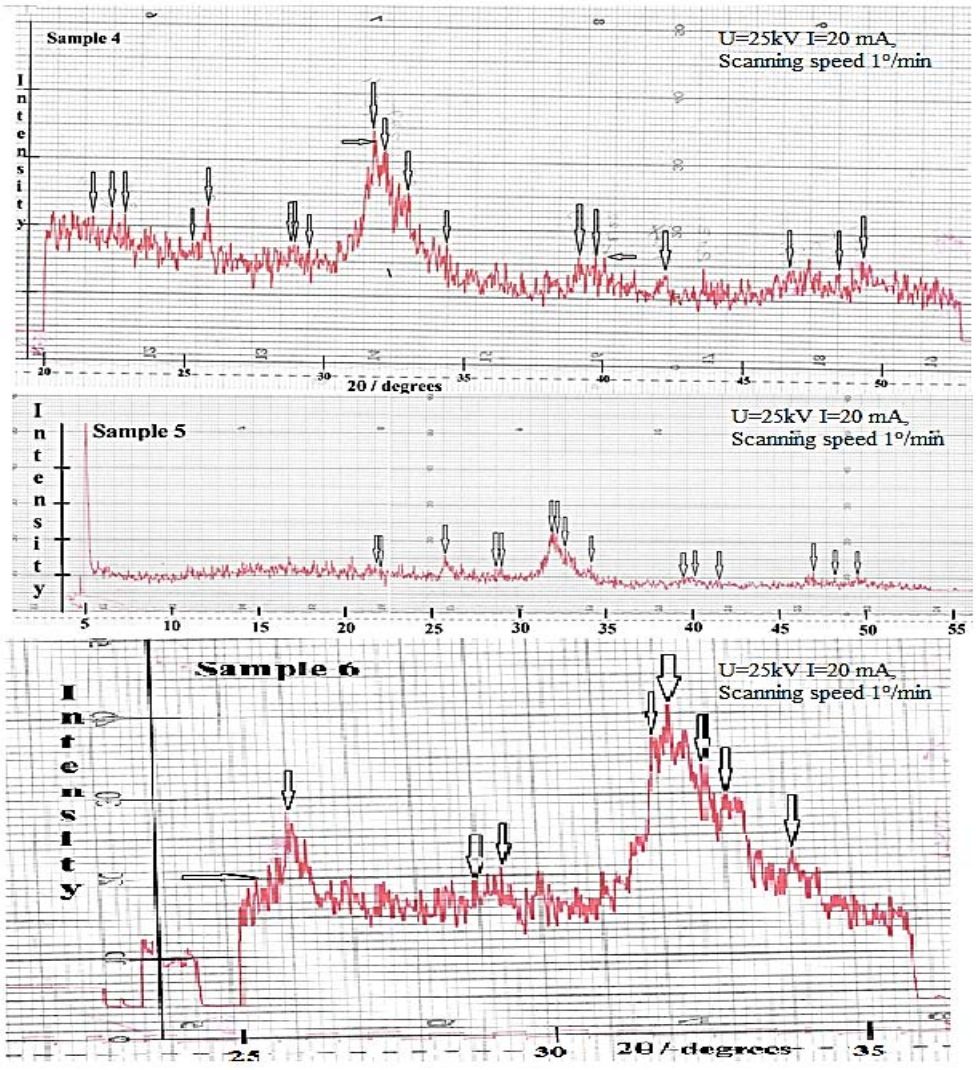

Fig. 2. XRD patterns of analyzed bone fragments. Sample 4 – Intraarticular ectopic bone fragments – hip joint. Samples 5 and 6 – Extraarticular ectopic bone fragments in the abductor hip musculature. The arrows indicate the peaks taken into account.

Table 1

Calculated interplanar distances depending on 2θ angles

Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6	
2θ (°)	*d* (Å)	**2θ (°)**	*d* (Å)	**2θ (°)**	*d* (Å)	**2θ (°)**	*d* (Å)	**2θ (°)**	*d* (Å)	**2θ (°)**	*d* (Å)
25.8	3.44	**25.8**	3.44	**21.8**	4.07	**21.8**	4.07	**21.8**	4.07	**25.3**	3.51
28.1	3.17	**28.1**	3.17	**22.9**	3.88	**22.3**	3.96	**22.3**	3.96	**25.8**	3.44
28.9	3.080	**28.9**	3.08	**25.3**	3.51	**22.9**	3.88	**22.9**	3.88	**28.8**	3.10
31.7	2.814	**31.7**	2.814	**25.8**	3.44	**25.3**	3.51	**25.3**	3.51	**28.9**	3.09
32.1	2.778	**32.1**	2.77	**28.8**	3.10	**25.8**	3.44	**25.8**	3.44	**29.4**	2.29
32.9	2.720	**32.9**	2.72	**29.4**	2.29	**28.8**	3.10	**28.8**	3.10	**31.7**	2.814
34.1	2.63	**34.1**	2.63	**31.7**	2.814	**28.9**	2.09	**28.9**	2.09	**31.8**	2.814
38.8	2.62	**38.8**	2.62	**32.1**	2.77	**29.4**	2.29	**29.4**	2.29	**32.1**	2.778
42.0	2.184	**39.2**	2.29	**32.9**	2.72	**31.7**	2.814	**31.7**	2.814	**32.2**	2.77
46.7	1.94	**40.4**	2.22	**34.04**	2.63	**31.8**	2.814	**31.8**	2.814	**32.6**	2.72
49.4	1.841	**42.0**	2.14	**39.2**	2.29	**32.1**	2.77	**32.1**	2.77	**32.9**	2.72
53.1	1.722	**42.2**	2.14	**39.8**	2.26	**32.8**	2.72	**32.8**	2.72	**34.0**	2.63
		46.7	1.94	**46.7**	1.94	**32.9**	2.72	**32.9**	2.72		
		48.6	1.87	**49.4**	1.84	**34.0**	2.63	**34.0**	2.63		
		49.4	1.84			**39.2**	2.29	**39.2**	2.29		
		49.4	1.841			**39.8**	2.26	**39.8**	2.26		
						40.0	2.22	**40.4**	2.22		
						42.0	2.14	**42.1**	2.14		
						42.3	2.13	**42.3**	2.13		
						46.7	1.94	**47.6**	1.94		
						48.6	1.87	**48.6**	1.87		
						49.4	1.84	**49.4**	1.84		

Table 2

Diffraction data extracted from theoretical data sheet (*d* (Å) = distance between atomic planes; I/I_l = intensity of diffraction; *hkl* = Miller indices); three most intense diffraction peaks are marked in bold

d (Å)	**I/I_l**	***hkl***	**d (Å)**	**I/I_l**	***hkl***
8.17	11	100	2.040	1	400
5.26	5	101	2.000	5	203
4.72	3	110	1.943	30	222
4.07	9	200	1.890	15	312
3.88	9	111	1.871	5	320
3.51	1	201	1.841	40	213
3.44	40	002	1.806	20	321
3.17	11	102	1.780	11	410
3.08	17	210	1.754	15	402.303
2.814	**100**	**211**	1.722	20	004.411
2.778	**60**	**112**	1.684	3	104
2.720	**60**	**300**	1.644	9	322.223
2.631	25	202	1.611	7	313
2.528	5	301	1.587	3	501.204
2.296	7	212	1.542	5	420
2.262	20	310	1.530	5	331
2.228	1	221	1.503	9	214.421
2.148	9	311	1.474	11	502
2.134	3	302	1.465	3	510
2.065	7	113			

As it can be noticed on the theoretical data sheet, the three most intense diffraction peaks occur within the angular range of 31–33°, at 31.7°, *d* distance is of 2.814 Å, at 32.1°, *d* distance is of 2.778 Å, and at 32.9°, *d* distance is of 2.720 Å.

Taking into account the fact that in order to determine the presence of crystal into a substance, a minimum of 3 diffraction peaks is needed at appropriate θ angles and *d* distances between atom planes, the presence of hydroxyapatite within all 6 analyzed heterotopic ossification fragments has been positive. On top of the minimum of 3 diffraction peaks needed in order to pinpoint hydroxyapatite,

diffraction took place at the majority of angles detailed within the standard sheet, and calculated interplanar distances concurred to a large extent.

Comparing the XRD pattern of examined heterotopic bone samples with XRD pattern of normal skeletal cortical and cancellous bone (untreated and treated at different temperatures) reported in the literature [14], a high similarity can be observed (Table 3). Some of the peaks exist in almost all examined heterotopic bone samples, but the peak intensity which reflects the crystallinity grade is lower compared to XRD on synthetic hydroxyapatite or bone treated at 650°C [14].

Table 3

The 2θ values of diffraction peaks found in our heterotopic bone sample in comparison with those reported for cortical and cancellous bone [14]

2θ (°) (this paper)	25.8	28.1			32.9	34.1		46.7	49.4	
2θ (°) according to [14]	25.8	28.1	29	31.8	32.9	34.1	39.6	46.7	49.5	53.2

CONCLUSIONS

The X-ray powder diffraction method is a relatively easy method to analyze crystalline composition of heterotopic ossification.

The angles of the diffracted X-rays recorded for all samples are almost similar with diffraction on hydroxyapatite pure crystal, or normal skeletal bone with few exceptions possibly due to involuntary contamination of samples due to contact with other substances.

The presence of hydroxyapatite crystals within all examined samples was confirmed by the presence of at least three diffraction peaks corresponding to 2Θ angles and the similar interplanar *d* distance calculated using Bragg's law, according to international powder diffraction standards for hydroxyapatite.

There is a difference in the intensity of the diffraction phenomenon on heterotopic ossifications compared with diffraction on pure mineral apatite, probably due to the abundance of organic material content and probably due to the lower degree of crystallization of heterotopic ossification.

The phenomenon of heterotopic ossification mineralization is similar to that of skeletal bone mineralization.

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