

## A Capillary Zone Electrophoresis Study of the Effect of Precursors and Ultrasonic Treatment on the Morphology of Hydroxyapatite Particles

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**Abstract**—A set of analytical techniques is proposed for studying samples of calcium hydroxyapatite (HA) synthesized on the basis of calcium nitrate or calcium hydroxide as precursors. A comparative study of particle size distribution in HA samples is performed using capillary zone electrophoresis (CZE), scanning electron microscopy (SEM), dynamic light scattering (DS), and static light scattering (SS). The effect of heat treatment on the morphology of the synthesized products is evaluated. It is found that the effect of ultrasound (US) on the dispersity of HA particles in aqueous suspensions depends on the nature of the precursor and heat treatment. It is shown that the HA particles synthesized from calcium hydroxide (HA–OH) aggregate more rapidly than the particles synthesized from calcium nitrate (HA–NO<sub>3</sub>). After the ultrasonic treatment of suspensions, the relative concentration of large HA–NO<sub>3</sub> submicroparticles increased, while particle size distribution in the suspension of HA–OH remained virtually the same. The heat treatment of HA powders at 900°C reduced the ability of particles to aggregate. It is found by CZE that, with increasing time of ultrasonication of suspensions, the relative concentration of nanoscaled and similar in size HA–NO<sub>3</sub> submicroparticles increased markedly, while it changed only slightly in the HA–OH suspension. The reasons for the differences are discussed. A procedure for determining the concentration of HA submicroparticles in suspension by CZE is proposed. A linear dependence of normalized peak areas on HA concentration in suspension is obtained.

**Keywords:** calcium hydroxyapatite, ultrasound, capillary zone electrophoresis, scanning electron microscopy, dynamic light scattering, static light scattering

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It is known that calcium orthophosphate nanocrystals possess two properties essential for bone tissue physiology: they are in dynamic equilibrium with the biological environment in the resorption–mineralization cycle, and their composition and structure are similar to those of human bone tissue [1, 2]. Calcium hydroxyapatite, Ca<sub>10</sub>(PO<sub>4</sub>)<sub>6</sub>(OH)<sub>2</sub> is a crystallochemical analogue of the mineral component of bone tissue. Nanocrystalline HA has high bioactivity: it adsorbs proteins necessary for cellular activity and is selective to cell functions forming bone and fibrous tissue [3]. Composite materials based on HA are created for the substitution and recovery of damaged bone tissue [1]; in particular, HA powders are used to produce dental cements, medicinal pastes, and implant coatings.

The morphology of nanostructures may significantly vary depending on the composition, crystal structure, and method of preparation of the material.

The known methods of synthesis offer the creation of particles of various shapes and sizes. Sedimentation is one of promising methods for synthesizing HA; it is actively used to produce nanocrystalline HA [2, 4–6]. Orthophosphates and various calcium compounds, such as CaCO<sub>3</sub>, Ca(NO<sub>3</sub>)<sub>2</sub>, CaCl<sub>2</sub>, and Ca(OH)<sub>2</sub>, can act as starting materials (precursors) in these processes. This method ensures the synthesis of materials differing in composition, stoichiometry, and crystallinity, depending on the synthesis conditions. Nanocrystalline HA was synthesized from Ca(OH)<sub>2</sub> and H<sub>3</sub>PO<sub>4</sub> at proper concentrations under carefully selected conditions [7]. The effect of synthesis conditions on the formation of ultrafine HA powders is studied [8]. It was shown that the proper selection of precursors and their concentrations along with the optimization of the stirring rate, temperature, and duration of the synthesis yield materials with morpho-

logical features similar to those of biogenic materials [8–10]. The effect of ultrasonic radiation on the synthesis process was explored [11]. There are evidences that temperature and ultrasonic radiation affect simultaneously the morphology of HA particles [12, 13]. The prepared materials have a needle-like [12] or a platelet [13] structure. In these works, urea was used as a mild alkaline agent.

In the manufacture of a variety of orthopedic implants, methods of ion-plasma, magnetron, hydrothermal, and electrochemical deposition of HA on Ti, TiO<sub>2</sub>, and other substrates were applied [6]; therefore, the thermal stability of HA is its important property. There is a detailed review of works on the effect of annealing temperature on the morphology of HA samples obtained by sedimentation [2]. Using this method, samples were synthesized from Ca(NO<sub>3</sub>)<sub>2</sub> · 4H<sub>2</sub>O, (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, NH<sub>3</sub> · H<sub>2</sub>O as precursors; then, the samples were freeze-dried for 72 h and annealed at 800°C for 1 h. Calcium hydroxyapatite particles, obtained after annealing at 900°C for 1 h, are needle-shaped crystals up to 100 nm in length [14]. Data of similar studies were published in [8, 15]. Resulting HA has a crystal structure with a Ca : P ratio of 1.66. It is noted that HA with a Ca : P ratio in the crystal lattice between 1.5 and 1.9 is more resistant to heat and dissolution [2]. The conditions for the synthesis of fine HA powders, which are used both without and with annealing at different temperatures to produce ceramics, were studied [15–17]. Ceramics based on these materials had the best mechanical properties, density, hardness, etc.

The dispersion state of HA suspension is the starting point for manufacturing porous matrices and other materials of high quality [1, 14]. For several years, we have conducting research on the effect of dispersion medium [18, 19] and ultrasound [20] on the size distribution of nanoparticles and submicroparticles in aqueous HA suspensions. We found the conditions for stabilizing suspensions containing HA nano- and/or submicroparticles. The surface properties of HA nano- and submicroparticles and the processes of aggregation and dispersion on the basis of their electrophoretic behavior are studied by CZE. This method has been successfully used for the separation of organic, inorganic, and biological nano- and submicroparticles [21]. The main advantages of this method are high efficiency, rapidness, and the absence of stationary or pseudostationary phases, which eliminates undesirable interactions.

The goal of the present work was to study of the effect of the nature of precursor, heat treatment after the synthesis of fine powders, and time of ultrasound exposure on the size distribution of HA particles in aqueous suspensions. In the synthesis, we used calcium nitrate, calcium hydroxide, and dibasic ammonium phosphate as precursors. To evaluate the particle size distribution in suspensions, scanning electron

microscopy, dynamic light scattering, and static light scattering were applied along with capillary zone electrophoresis.

## EXPERIMENTAL

**Reagents.** For electrophoretic studies,  $1.0 \times 10^{-3}$  M calcium hydroxyapatite suspensions were prepared by adding the appropriate amount of a  $4.0 \times 10^{-3}$  M Na<sub>2</sub>HPO<sub>4</sub> solution to HA powders. All solutions were prepared using deionized water.

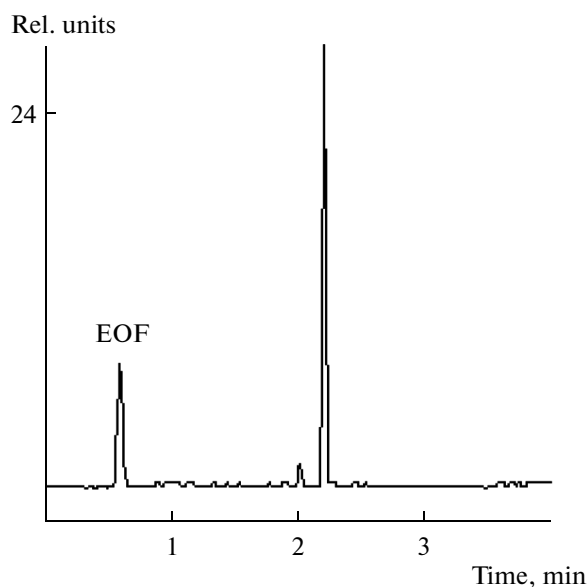
**Synthesis.** Calcium hydroxyapatite powders were synthesized by sedimentation. In the synthesis, we used calcium nitrate or calcium hydroxide and dibasic ammonium phosphate as precursors. All syntheses were carried out at 20°C. After drying, the samples were annealed at 900°C in a SNOL muffle furnace for 1 h.

**Apparatus.** Calcium hydroxyapatite suspension were analyzed using a Kapel'-105 capillary electrophoresis system (Lumex, Russia), equipped with a spectrophotometric detector with variable wavelength (190–380 nm). A quartz capillary tube, 75 μm in inner diameter, with a total length of 39.0 cm and a length to detector of 29.5 cm was used. Experiments were performed at 25°C and  $\lambda = 220$  nm with a source of high voltage of positive polarity. Benzyl alcohol served as a marker of electroosmotic flow (EOF). To recover the active surface, the capillary tube was washed between dimensions successively for 2 min each with water, a 0.1 M NaOH solution, and a buffer solution ( $4.0 \times 10^{-3}$  M Na<sub>2</sub>HPO<sub>4</sub>, pH 8.4); the degree of purification of the capillary tube was monitored by the baseline of electropherogram.

The results were processed using Multikhrom software for Windows-95/98/NT. The normalized values of peak areas were calculated from the peak areas ( $S$ ) as  $S_{\text{norm}} = S/t$ , where  $t$  was the peak migration time. The electrophoretic mobility of particles was determined by the difference between the observed and electroosmotic mobilities. From the obtained electropherograms, the weighted averages of electrophoretic mobilities ( $\mu_{\text{wa}}$ ) of HA particles were calculated as  $\mu_{\text{wa}} = \mu_1 x_{11} + \mu_2 x_2 + \mu_3 x_3 + \dots + \mu_n x_n$ , where  $\mu_n$  was the electrophoretic mobility corresponding to the position of a single peak and  $x_n$  was the area fraction of this peak.

As a power supply for the electroacoustic oscillating system, we applied a UZG13-0.1/22 ultrasonic generator ( $22 \pm 1.65$  kHz,  $100 \pm 10$  W) produced by All-Russian Research Institute of High-Frequency Currents (Russia) with a PP1-0.1/22 rod piezoceramic transducer for the sonic treatment of liquid media (22 kHz, 100 W, 3.54 W/cm<sup>2</sup>). The specific acoustic power of ultrasound was 0.85 W/cm<sup>2</sup>.

Samples of HA suspensions were examined using a JEOL JSM-6700F scanning electron microscope (Japan) equipped with a cold field emission cathode.



**Fig. 1.** Electropherogram of a  $1.1 \times 10^{-3}$  M HA-NO<sub>3</sub> suspension: a  $4 \times 10^{-3}$  M Na<sub>2</sub>HPO<sub>4</sub> buffer solution; pH 8.4; marker, benzyl alcohol; sample injection 30/10 mbar/s; voltage, 20 kV; 220 nm.

An initial HA suspension was previously applied to a cleaned polished silicon substrate, which gave a better contrast in observing individual HA particles.

The particle sizes were determined by dynamic light scattering using a Zetasizer Nanoseries Nano-ZS instrument (Malvern, UK) with a helium–neon laser as a light source at  $\lambda = 633$  nm. The measurement was conducted at 25°C in a thermostated cuvette with  $l = 10$  mm. The prepared 0.01–0.02% HA suspension was allowed to stand for 10 min to remove large particles, and a 2-mL aliquot portion was sampled, or the resulting suspension was further passed through a filter with a pore size of 0.45  $\mu$ m.

The particle sizes in the range of 0.01–40  $\mu$ m were determined by static light scattering using an Analysette 22 Nanotec Plus instrument (Fritsch, Germany)

equipped with an ultrasonic dispersing unit (60 W, 36 kHz) and three semiconductor lasers with  $\lambda = 532$  and 850 nm as a light source. The obtained suspension was treated for a given time in an ultrasonic dispersion unit and then fed for the determination by a pump.

The phase composition of the powders obtained was found according to the X-ray analysis. We used a D2 Phaser diffractometer (Bruker) with a CuK $\alpha$  radiation at  $\lambda = 0.1548$  nm. The diffraction patterns were processed by means of universal programs TOPAS and DIFFRAC.EVA.

## RESULTS AND DISCUSSION

**Characteristics of particles in the suspensions of HA synthesized without heat treatment.** With the use of CZE, the electropherograms of suspensions HA-NO<sub>3</sub> and HA-OH were obtained; they are similar and, besides the EOF marker peak, comprise one narrow peak. The electropherogram of HA-NO<sub>3</sub> suspension is presented in Fig. 1 as an example. The electrophoretic mobilities (EPM), corresponding to the position of the peak, are  $-23 \times 10^{-5}$  and  $-28 \times 10^{-5}$  cm<sup>2</sup> V<sup>-1</sup> s<sup>-1</sup> for HA-NO<sub>3</sub> and HA-OH, respectively.

The particle size of HA in suspensions, evaluated by methods of SS, DS, and SEM, are given in the table. According to the SS data, more than 90% of the integrated intensity of peaks in the histograms of particle size distribution corresponds to the size range of 30–850 nm. The histograms of particle size distribution in the suspension above HA sediment, obtained by DS, indicate the ranges of 530–830 and 700–1100 nm for HA-NO<sub>3</sub> and HA-OH, respectively. The specified maximum sizes of particles are probably the limit values determining the sedimentation stability of suspensions. A comparison of these two methods reveals a significant difference in the smallest particle sizes determined, which can be caused by the masking effect of large particles on the determination of nanoparticles and similar submicrosized particles. To reduce this effect, the suspensions were prefiltered

Particle size distribution ( $\mu$ m) in HA suspensions

Suspension	Method	HA-NO <sub>3</sub>	HA-NO <sub>3</sub> -900	HA-OH	HA-OH-900
Initial suspension	SS	0.07–0.85	0.04–0.95 >1	0.06–0.85	0.11–0.62 >1
Initial suspension + ultrasonication	SS	0.17–0.76 >1	0.04–0.60 0.6–1.5	0.02–0.32	0.8–10
Initial suspension	SEM	0.03–2.60	0.04–2.50	0.03–2.60	0.04–2.50
Suspension above sediment	DS	0.53–0.83	0.70–0.95	0.70–1.10	0.70–1.10
Filtrate	DS	0.09–0.62	0.04–0.60	0.14–0.22	0.25–0.46
Filtrate after 20 min	DS	0.25–0.95	0.04–0.62	0.60–1.30	0.25–1.70

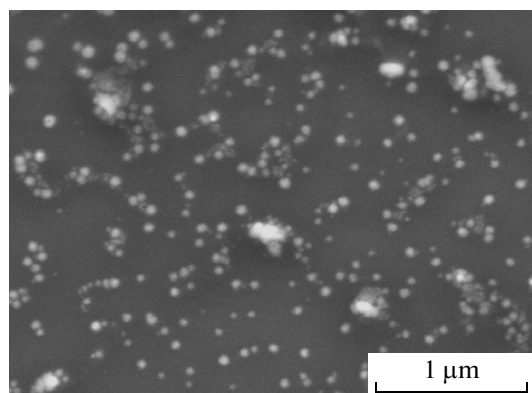


Fig. 2. Micrograph of HA-NO<sub>3</sub> suspension.

through a 0.45- $\mu\text{m}$  filter before the DS measurements. As seen from the table, the filtrate of HA-NO<sub>3</sub> includes particles of 90 nm in size, that is, smaller than those contained in the suspension above the sediment. The minimum size of particles in the filtrate of HA-OH is larger, namely, 140 nm. This difference in behavior of HA particles synthesized from different precursors may be explained by the following features of synthesis: HA-OH was synthesized from a suspension of calcium hydroxide, while HA-NO<sub>3</sub> was obtained by precipitation from a solution of calcium nitrate. Accordingly, in the former case, there were nucleation sites in the system at which the growth of HA particle occurred, and in the latter case, there were not such sites at the beginning. Twenty minutes after the preparation, the minimum size of HA-OH particles in the filtrate increased up to 600 nm, while for

HA-NO<sub>3</sub>, the minimum size was only 250 nm. A greater propensity of HA-OH particles to undergo aggregation is probably due to the properties of their surface. It is found by X-ray fluorescence analysis that in contrast to crystalline HA-NO<sub>3</sub>, HA-OH is an X-ray amorphous compound.

In the micrographs obtained by SEM, HA particles and agglomerates are observed that are 30–2600 nm in size. A micrograph of the initial HA-NO<sub>3</sub> suspension is given in Fig. 2 as an example.

**Effect of ultrasound on the characteristics of particles in the suspensions of HA synthesized without heat treatment.** The effect of ultrasonic treatment on the electrophoretic mobility and the peak area of the HA particles obtained without heat treatment is studied by CZE. It is seen from Fig. 3 that with increasing duration of exposure to ultrasound, the peak area (Fig. 3a) and the absolute value of EPM (Fig. 3b) of HA-NO<sub>3</sub> increase. In the case of HA-OH, only minor changes in these parameters occur. According to the SS data, upon ultrasonication of the HA-NO<sub>3</sub> suspension, along with the disappearance of particles less than 170 nm in size and a decrease in the relative amount of particles with sizes of 170–320 nm, larger submicroparticles and microsized aggregates appear. In the case of HA-OH, on the contrary, the relative amount of smaller particles with sizes below 170 nm increases with decreasing number of larger particles 170–850 nm in size. These data indicate that the electrophoretic behavior of HA particles and the effect of ultrasound on their size distribution in the suspension depend on the nature of precursor used in the synthesis.

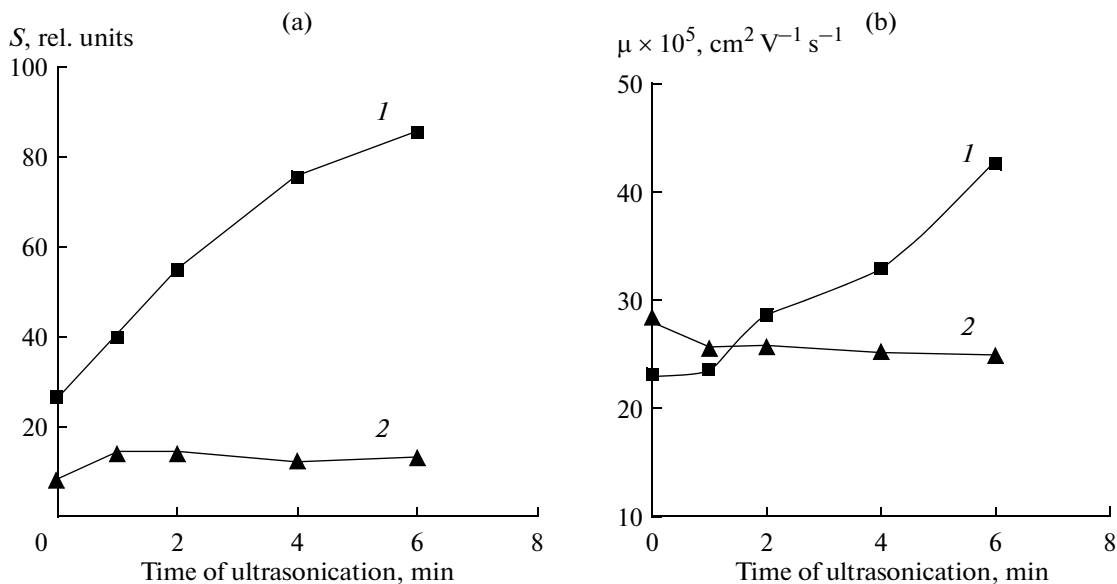


Fig. 3. Effect of the time of ultrasonication on (a) the signal area and (b) and the EPM of suspensions (1) HA-NO<sub>3</sub> and (2) HA-OH.

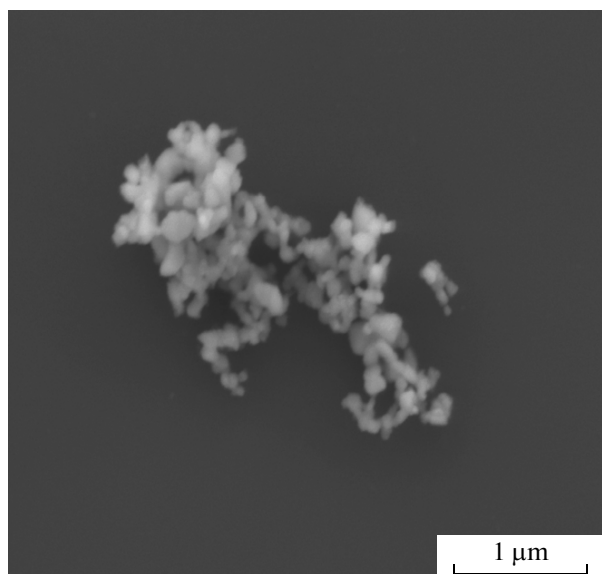


Fig. 4. Micrograph of HA-NO<sub>3</sub>-900 suspension.

The comparison of the results of determination of particle size composition of the suspensions with the data obtained by CZE explains different behavior of particles HA-NO<sub>3</sub> and HA-OH in suspension under ultrasonication. We can assume that in the case of HA-NO<sub>3</sub> suspensions, the enlargement of particles from 30 to 170 nm and a possible increase in the zeta potential lead to a noticeable change in the EPM and the intensity of light scattering; therefore, a growth in the absolute value of EPM is observed and the peak area of HA in the electropherogram increases. The phenomenon of aggregation of particles under the effect of ultrasound is described in publications [22, 23]. Probably, upon the ultrasonic treatment of HA-NO<sub>3</sub> suspension, the thickness of the electrical double layer surrounding a particle decreases, which enhances van der Waals interactions between particles and increases their propensity to undergo aggregation at low values of the zeta potential. The resulting aggregates are more durable than the aggregates present in the initial suspension, and are not destroyed by repeated exposure to ultrasound (the ultrasonic treatment should be stopped shortly every 2 min to prevent overheating of the suspension). As a result, the relative amount of large particles in suspension increases, and hence the absolute value of EPM grows and the peak area in the electropherogram increases. An increase in the absolute value of the zeta potential along with decreasing thickness of the electric double layer can also promote the transition of larger particles from sediment into suspension.

Unlike HA-NO<sub>3</sub>, there are only minor changes in the absolute values of EPM and peak areas in the electropherograms of HA-OH suspension. The electrophoretic mobility and the peak area of HA-OH particles are probably independent on the time of ultraso-

nication because fine particles, formed after dispersion, rapidly aggregate during the interval between two successive treatments, and the suspension almost returns to its initial state. The size distribution obtained by static light scattering also indicates a decrease in the size of particles in suspension under the action of ultrasound. Thus, the precursor of synthesis plays an important role, determining the differences in the behavior of HA-OH and HA-NO<sub>3</sub> particles.

**Characteristics of particles in the suspensions of thermally treated HA.** Suspensions of HA-NO<sub>3</sub> (HA-NO<sub>3</sub>-900) and HA-OH (HA-OH-900), prepared from HA thermally treated at 900°C, are studied by CZE. In the electropherograms of HA-NO<sub>3</sub>-900 and HA-OH-900 suspensions, a narrow peak is observed with the EPM of  $-16 \times 10^{-5}$  and  $-25 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ , respectively.

According to the SS data, the sizes of particles in suspensions HA-NO<sub>3</sub>-900 and HA-OH-900 are in the ranges of 40–950 and 110–620 nm, respectively. Furthermore, particles whose size exceeds 1 μm are found in both suspensions. The results of studies of suspensions HA-NO<sub>3</sub>-900 and HA-OH-900 above the sediment by dynamic light scattering indicate the presence of particles with the sizes of 700–950 and 700–1100 nm, respectively (table); the sizes are almost the same that were determined for suspensions of thermally untreated HA. After filtration of suspensions HA-NO<sub>3</sub>-900 and HA-OH-900 through a 0.45-μm filter, particles 40–600 and 250–460 nm in size, respectively, were found in the filtrates. As in the case of thermally untreated HA, filtration reduces the masking effect of large particles. Twenty minutes after the preparation of filtrate, the particle size distribution of HA-NO<sub>3</sub>-900 remained virtually unchanged (40–620 nm), while the particle size distribution of HA-OH-900 was shifted to larger sizes (up to 1.7 μm). In the SEM micrographs of HA-NO<sub>3</sub>-900 and HA-OH-900, both nanoparticles with sizes more than 30 nm and aggregates with dimensions up to 2.5 μm are observed. The micrograph of HA-NO<sub>3</sub>-900 suspension is presented in Fig. 4 as an example.

**Effect of ultrasound on the characteristics of particles in the suspensions of thermally treated HA.** In the electropherograms of suspensions HA-NO<sub>3</sub>-900 and HA-OH-900, recorded after ultrasonication for 4 min, apart narrow peaks that are more intense than those obtained without ultrasound treatment, a broad peak appears, the position of the maximum of which corresponds to the EPM of  $-(29-32) \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ . As an example, the electropherogram of HA-NO<sub>3</sub>-900 suspension is presented in Fig. 5. The ultrasonic treatment has almost no effect on the EPM of HA particles in suspensions HA-NO<sub>3</sub>-900 and HA-OH-900. With an increase in the duration of ultrasonic exposure, the area of the broad peak in the electropherogram of HA-NO<sub>3</sub>-900

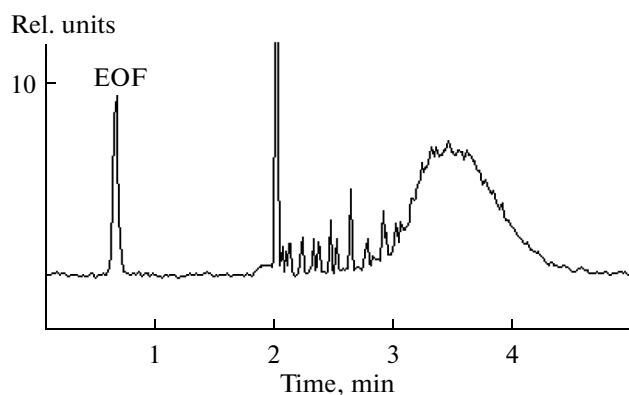


Fig. 5. Electropherogram of a  $1.1 \times 10^{-3}$  M HA-NO<sub>3</sub>-900 suspension; time of ultrasonication, 4 min.

suspension increases significantly relative to the area of narrow peaks (Fig. 6), indicating an increase in the relative concentration of small submicroparticles in suspension. Unlike HA-NO<sub>3</sub>-900, the area of this peak in the electropherograms of HA-OH suspension changes only slightly. The difference in the dependences obtained may be due to a lower relative amount of submicroparticles in HA-OH-900 suspension and their greater tendency to undergo aggregation, as the nature of the precursor may affect the surface properties of the particles and, hence, on the processes of dispersion and aggregation in suspensions.

The effect of HA-NO<sub>3</sub> concentration in the suspension ( $c$ ) to the normalized peak area ( $S$ ) is studied. The obtained dependence is satisfactorily described by linear equation  $S = 38.45c + 0.02$  with a correlation coefficient of 0.99.

The examination of suspensions of thermally treated and untreated HA with different methods can yield a conclusion that the heat treatment alters the surface properties of particles, decreasing their tendency for subsequent aggregation.

In the study of particle size distribution in polydisperse suspensions, the method of static light scattering should be preferred compared to dynamic light scattering and scanning electron microscopy. However, at relatively low concentrations of small submicroparticles in suspensions, the particle sizes obtained by SS may also be erroneous. The study of suspensions above the HA sediment, whose composition corresponds to the composition of sedimentation-stable suspensions, offered an estimation of the maximum particle size (850–1100 nm) that can remain suspended. It is shown that the composition of suspensions above the sediment does not depend on the nature of precursor in the synthesis and the heat treatment and is generally similar for all tested HA samples.

The study showed that capillary zone electrophoresis, along with other methods, enables the evaluation of the effect of precursor nature, heat treatment of samples after synthesis, and the time of ultrasonic

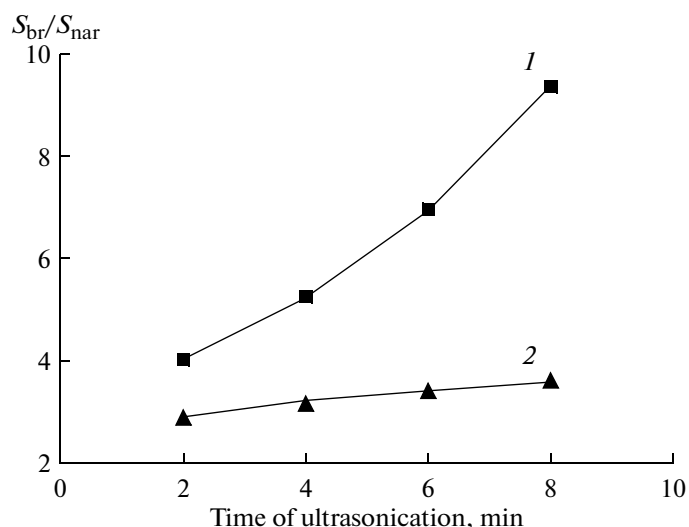


Fig. 6. Effect of the time of ultrasonication on the area ratio of the broad and narrow peaks in the electropherograms of suspensions (1) HA-NO<sub>3</sub> and (2) HA-OH.

treatment on the surface properties and particle size distribution in HA suspensions. The proposed set of analytical techniques allows the control of the quality of HA suspensions, which is a necessary condition for the manufacture of ceramics and composite materials for the medical industry.

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