Mechanical activation effect on structure, physicochemical, and biological properties of potassium/magnesium orotates

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Abstract. In the present paper, the possibility of the formation of the tautomeric structure of potassium orotate and magnesium orotate in the solid state is discussed. Using ball milling, we have received the orotate samples with the predominant content of hydroxy and dihydroxy forms. The crystalline and tautomeric structures have been analyzed by XRD, IR, NEXAFS and XPS spectroscopy. There is a slight difference in the water solubility of the orotate tautomers, but the physicochemical properties (viscosity and volumetric thermal expansion coefficients) of tautomers' water solutions significantly differ. The above differences are related to variation of hydration area of orotate anions due to the composition of the functional groups being changed together with the structure of tautomers. The residual differences in the tautomeric structure of solid orotates formed during the milling process are observed in the orotate water solutions for 1.5-2.5 hours. The biological activity of the water solution of a hydroxy form relative to erythrocytes and epithelial cells is higher than that of oxo- and dihydroxy-form water solutions.

1. Introduction

Due to the well-known notion that the basis of pharmacy is the synthesis of new biologically active substances, leading pharmaceutical companies actively improve the existing pharmaceutical forms. The advantages of known and tested drugs are that their side effects are already identified, while their therapeutic effect is proved by long-term practice. The improvement of drug properties is mainly attained by the formation of crystalline, amorphous, or isomeric structures with a high biological activity without changing the drug composition.

In recent years, a considerable part of the pharmaceutical research has benefited from the advance made in the physics of polymorph transformations [1, 2]. There are many examples of radical differences in physicochemical and biological properties of various crystalline polymorphs sharing the same chemical composition. Amorphous solids are characterized by higher solubility and chemical reactivity [3-5]. Multiple biologically and pharmaceutically important chemical substances are known to exist in different isomeric forms [6]. Given this, many scholars focused their attention on understanding the formation of optical isomers in saccharides [7-9].

Tautomerism is a form of isomerism, in which the intramolecular migration of a hydrogen atom or proton takes place accompanied by the transformation of a single bond into a double one or vice versa.
[10]. The influence of tautomerism on a particular structure of drugs and their potential to interact with biological systems is discussed in [11, 12].

The tautomeric forms of the DNA and RNA have been extensively studied [8]. It has been found that the misuse of the purine and the pyrimidine bases can lead to errors in replication and to point mutations [13-15]. A number of papers are dedicated to the keto-enol tautomerism of p-hydroxyphenylpyruvic acid, which is used to diagnose a congenital metabolic defect [16].

Tautomerism takes place mainly in a solution, gas phase, or amorphous state because the proton transfer occurs easily in such states. In the crystalline state, tautomerism is also possible but only under particular conditions, e.g. at high temperatures [17-20]. Moreover, in this case, the tautomerism rate is much smaller than in a supercooled liquid, solution, or gas phase. Unlike other classes of isomers, tautomers exist in the dynamic equilibrium between one another; therefore, the attempts to prepare separate substances usually result in the formation of a mixture that has all chemical and physical properties of the base components [21].

Recently, "green chemistry" has become popular, which methods involve the synthesis or modification of chemical compounds without the use of solvents or gaseous media, which can harm the environment or contaminate the reaction final product. In this connection, the use of solid-phase methods for modifying properties of medicinal substances is crucial. As a solid-phase method, the method of mechanical activation in high-energy dispersing devices, including ball milling, is very practical [22-28].

In this paper, we investigate the possibility of preparing various tautomeric forms of the solid-state orotates of magnesium and potassium by milling using a planetary ball mill, and compare the biological properties of the prepared tautomers. The lifetime of different solid state tautomers is of special interest from the standpoint of practice. Since dissolution is a limiting stage of an effect of drugs on an organism, such factors as the solubility of preparations and the preservation of differences between tautomers in solution are also very important.

2. Experimental

2.1. Materials
To carry out the work, we used potassium orotate and magnesium orotate obtained from Sigma-Aldrich Co. (US) without any additional purification.

2.2. Sample preparation
For milling substances, a planetary ball mill AGO-2 (Russia) was used. The rotation speed of the drums was 600 rpm. The power density was 2 kJ/g. The dose of mechanical energy supplied to a sample after 1 hour of milling was 7 kJ/g, after 3 hours - 21 kJ/g, after 6 hours - 42 kJ/g. The temperature of the vial walls did not exceed 60°C during the milling procedure due to the forced water-cooling.

2.3. Investigation methods
The elemental composition and solubility of the samples were analyzed by inductively coupled plasma atomic emission spectroscopy (ICP-AES) using a Spectroflame Modula spectrometer (Germany). According to the data of the analysis there were no impurities from the materials of the balls and vials.

The morphology of the prepared powders was examined by atomic force microscopy (AFM) with an Integra Prima (NT-MDT) scanning probe microscope in the semi-contact mode in the air. The powder has been preliminarily attached to a polystyrene film produced by evaporating ethyl-acetate in which polystyrene was dissolved. The film was applied on glass-ceramics with the subsequent fixation of the powder by ultraviolet emission.

The X-ray diffraction patterns (XRD) were obtained using a D8 Advance diffractometer (Bruker AXS) in the parallel-beam geometry (MoKα and CuKα). Calculations of the structure characteristics were made using Topas 4.2. Structure refinement was performed by the Rietveld method [29] using...
MgO as a standard sample [30]. The calculation of the elementary unit cell parameters was based on 205 reflexes. For the Rietveld refinement, the structural data (the initial coordinates of atoms) were obtained from elsewhere [31].

The spectra obtained by Near-edge X-ray absorption fine structure (NEXAFS) and X-ray photoelectron spectroscopy (XPS) were measured at the Russian-German (RG-PGM) dipole beamline at BESSY II, Helmholtz-Zentrum, Berlin. The beamline uses the plane-grating monochromator with the 400 and 1200 lines/mm gratings to select the photon energies in the range of 40 - 1500 eV, with the resolving power of up to E/ΔE =100 000. A detailed description of the beamline can be found elsewhere [32]. All spectra were taken under ultrahigh vacuum conditions (5 × 10⁻¹⁰ Torr).

The NEXAFS data were acquired in a total electron yield (TEY) mode by measuring the sample drain current with varying photon energy. The spectra were normalized to the incident photon flux which was controlled by registering a complete electron exit from the clean surface of the gold crystal mounted on the manipulator holder.

The C K-edge absorption spectral features were calibrated with respect to the energy position of the C1s→ π* resonance of the measured absorption spectrum of the highly oriented pyrolitic graphite (HOPG) at 285.35 eV [33].

The XPS spectra were measured at the photon energy varied in the range of 200 - 1000 eV with the monochromator energy resolution of 125–500 meV. The energy scales of the spectra were calibrated to eliminate the charging effect by referencing the C1s peak of hydrocarbons to 285.0 eV [34]. The high-resolution spectra were analyzed in order to determine the presence of the various chemical substances. Each spectrum was deconvoluted using the methods based on Fourier transform with improved convergence procedure [35].

The shapes of both the NEXAFS and the XPS spectra strongly indicated that no damage of samples under the synchrotron radiation exposure occurred, irrelevant of the irradiation time.

The diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy spectra and the transmission spectra were recorded with the use of an FSM-1202 spectrometer (Russia) with the resolution of 1 cm⁻¹ in the 4000-450 cm⁻¹ region. The transmission measurements were performed on pellets made of the orotate samples and pure KBr powder. The DRIFT spectra were obtained using a diffuse reflectance accessory. The samples were analyzed at room temperature.

The biological properties of the source and milled orotates were analyzed by the microelectrophoresis method, where the amplitude of the cell movement in the field of the microscope was measured. In the electrophoresis chamber, the cells exhibited a constrained reciprocating motion during the stress reversal on the electrodes at 10 V with frequency of 0.1 Hz. The frequency of such motion was equal to the latter one, but its amplitude was different, being controlled by the cell surface charge, which was the marker of physiological state of a cell [36]. The study was conducted using a Cytoexpert complex (Russia).

3. Results and discussion

3.1. Potassium orotate

When the milled powder imparts the mechanical energy dose of 7 kJ/g, it represents separate spherical particles mainly of size 60 nm. The maximum size of some large particles does not exceed 250 nm. When the mechanical energy dose is 21 kJ/g, the particles have the form of ellipsoid of size 80–150 nm. Unstable aggregates up to 1.5 μm are formed (figure 1). The latter can be destroyed under scanning by the silicon probe. After the mechanical energy dose of 42 kJ/g has been exerted to the powder, laminated aggregates of particles of the size over 5 μm are formed. Some layers are formed of disc-shaped particles with the average thickness of 100 nm and diameter of 400-900 nm.
In table 1, the crystallographic parameters of the source and milled potassium orotate powder are shown. Indexing the diffraction pattern (LP-search [37]) shows that the unit cell space-group symmetry of the source powder corresponds to orthorhombic Cmma with the unit cell dimensions $a = 6.842(1)$ Å, $b = 12.535(2)$ Å and $c = 7.685(1)$ Å. The type of the crystal structure remains unchanged (figure 2); however, the main parameters of an elementary unit cell change: $a$ and $b$ increase, and $c$ decreases. The broadening of the diffraction lines indicates the decrease of the crystallites size and the growth of the lattice microstrain ($e_0$). When the mechanical energy dose is 7 kJ/g, the development of the preferred orientation is observed: the relative intensity of the (200) and (201) reflections increases. The molecules (packed in the molecular crystal as lamels) of potassium orotate are oriented parallel to the (100) plane. The increased intensity of the (h00) reflections may arise because of the potassium orotate dispersion with the formation of anisotropically shaped crystallites. It can be assumed that milling at the initial stages takes place due to the shift and detachment of lamels, and the following deforming impactions result in the gradual resizing of the unit cell.

| Table 1. Crystallographic parameters of source and milled potassium orotate |
|---|---|---|
| Parameter | Source | Mechanical energy dose, kJ/g |
| | | 7 | 21 | 42 |
| Space group | Cmma | Ortoromic |
| Crystal system | a, Å | 6.842(1) | 6.844(2) | 6.848(3) | 6.856(5) |
| | b, Å | 12.535(1) | 12.538(2) | 12.533(3) | 12.549(5) |
| | c, Å | 7.685(1) | 7.687(2) | 7.679(2) | 7.673(4) |
| Z | 4 |
| Crystal size, nm | 140 | 28 | 24 | 19 |
| Lvol-IB, nm | 47 | 18 | 15 | 12 |
| StrainL, % | 0.25 | 0.52 | 0.89 | 0.99 |
| e0, % | 0.06 | 0.13 | 0.22 | 0.24 |
| R$_{wp}$ | 7.22 | 7.7 | 6.2 | 6.1 |
| GOF | 1.93 | 2.04 | 1.65 | 1.63 |
| Vol, Å$^3$ | 659.1(2) | 659.6(3) | 659.4(3) | 660.0(5) |
Figure 2. X-ray diffraction patterns of potassium orotate: source (1) and after milling with the mechanical energy dose of 7 kJ/g (2), 21 kJ/g (3) and 42 kJ/g (4).

The tautomeric transformations affect the structure of the hydrogen bonds and the interaction of the double bonds of the pyrimidine base. The tautomerization results in the changes in the IR bands associated with the heterocycle vibrations in the region of "fingerprints". Figure 3 demonstrates the IR spectra of source potassium orotate and potassium orotate milled with the supplied mechanical energy dose of 42 kJ/g. The absorption in the regions 750 to 800 cm\(^{-1}\), 850 to 950 cm\(^{-1}\), and 990 to 1020 cm\(^{-1}\) is characteristic of the deformational vibrations of the pyrimidine ring. The IR spectrum of the milled sample shows the variation of the intensity ratio of the bands and the change in the width of the bands in comparison with the source sample.

Figure 3. Transmission IR spectra of potassium orotate source (1) and after milling with the mechanical energy dose of 42 kJ/g (2).

The nature of the skeleton oscillations of the heterocycle changes due to the formation of the hydroxyl groups. However, in the sample, all tautomeric forms of potassium orotate are present simultaneously, which complicates the detailed interpretation of the spectra.
In addition, the band of stretch vibrations of the associated hydroxyl groups in the region 2500-3600 cm\(^{-1}\) appear and correspond to the hydroxyl and C-N group stretch vibrations.

Figure 4 shows the DRIFT spectra of potassium orotate. The band of the vibrations of the NH, CN, and C=O (1650 to 1750 cm\(^{-1}\)) loses its fine structure due to the tautomeric transformations after milling with the mechanical energy dose of 42 kJ/g.

![Figure 4. DRIFT spectra of potassium orotate source (1) and after milling with the mechanical energy dose of 42 kJ/g (2).](image)

In table 2 the comparative data of NEXAFS and XPS are presented. The changes in the NEXAFS spectra structure (figure 5) correlate with those in the XPS spectra structure (figure 6). Milling with the mechanical energy dose of 7 kJ/g results in the decrease of the intensity of the peaks for C=O and –NH- groups, as well as the formation of the lines of C-OH and –N= groups. After milling with the mechanical energy dose of 42 kJ/g, the peaks of C-OH and –N- dominate, this is the evidence of the domination of the dihydroxy- form in the potassium orotate sample. The sharp resonance (doublet) near 300 eV is assigned to the potassium L\(_{2,3}\) -edge absorption. These resonances are particularly high in the milled samples because of the preferential orientation of the potassium orotate molecules in the flat scaly crystalline samples.

### Table 2. Interpretation of NEXAFS and XPS spectra of orotates

| XPS | NEXAFS |
|-----|--------|
| E\(_{\text{ph}}\), ±0.1, eV | E\(_{\text{ph}}\), eV |
| C1s | O1s | N1s | Group | C | N |
| 285.0 | 533.4 | 400.5 | (CH\(_2\))\(_n\) | 284.2 | C1s→\(\pi\) (C=C) |
| 286.2 | C-OH | 285.0 | C1s→\(\pi\) (C=C) |
| 289.0 | O=C-N | 288.5 | C1s→\(\pi\) (C=O) |
| 290.0 | shake-up | 290.0 | C1s→\(\pi\) (C-OH) |
| 398.4 - heterocycle | 398.0 | N1s→\(\pi\) (-N=; C-N) |
| - 399.0 | 399.7- | N1s→\(\pi\) (-NHC=O) |
| - 400.0 | |

**Wavenumber (cm\(^{-1}\))**

| lg(1/R) |
|--------|
| -0.6 |
| -0.4 |
| -0.2 |
| 0.0 |

**Figure 4.** DRIFT spectra of potassium orotate source (1) and after milling with the mechanical energy dose of 42 kJ/g (2).
Figure 5. NEXAFS C1s K-edge and N1s K-edge spectra of potassium orotate source (1); after milling with the mechanical energy dose of 7 kJ/g (2) and 42 kJ/g (3).

Figure 6. XPS spectra of potassium orotate source (1); after milling with the mechanical energy dose of 7 kJ/g (2), and 42 kJ/g (3).

In the XPS C1s-spectra, the component of the (CH)₂ groups of the adsorbed layer on the surface of the particles is dominant \( E_b = 285.0 \pm 0.2 \) eV. The (C-OH) groups are at \( E_b(C1s) = 286.2 \pm 0.2 \) eV. The component at 289.0±0.2 eV refers to the carbon atoms bonded with the oxygen and nitrogen atoms (O=C-N) in the oxo- and hydroxy-forms of the orotate anions. The fraction of the component of the C-OH groups increases from 12% in the source sample to 20% and 36% in the samples after milling with the mechanical energy dose of 7 kJ/g and 42 kJ/g, respectively. In the O1s-spectra, there are components at 531.8±0.2 eV and 533.4±0.2 eV corresponding to the oxygen atoms in the carbonyl (C=O) and hydroxyl (C-OH) groups. The fraction of the components at \( E_b = 533.4 \) eV increases from 18% to 50% (figure 6). In the N1s-spectra, the intensive component at 400.5±0.2 eV is attributed to the NHC=O group. The components at \( E_b = 398.4 \pm 0.2 \) eV and \( E_b = 399.6 \pm 0.2 \) eV refer to the nitrogen atoms in the aromatic and nonaromatic heterocycles. The fraction of the components of the nitrogen atoms in the aromatic heterocycle increases from 8% to 19.5% (figure 6).

Thus, milling with the mechanical energy dose up to 42 kJ/g does not change the potassium-orotate crystalline structure. The decrease in the size of particles and crystallites is observed. However, the
milling invokes a change in the tautomeric structure of the orotate anion, i.e., the subsequent transformation of the oxo-form into hydroxy- and dihydroxy- forms takes place.

The hydroxy- and dihydroxy- forms of potassium orotate are unstable. Both forms turn into the oxo-form after 2-month’s storage in the exsiccator (apparatus for drying substances or preserving them from moisture) at room temperature.

3.2. Magnesium orotate

Figure 7(a) shows the AFM-image of the magnesium orotate powder after milling with the mechanical energy dose of 7 kJ/g. The powder particles are spheroid-shaped of size 100-200 nm. Single particles of size 40 nm are also observed. The crystalline grains in the particles are 40 nm. The particles are integrated in loosely bound aggregates of up to 5 nm.

When the mechanical energy dose is increased to 21 kJ/g, the aggregate dimensions are almost similar to the above (figure 7(b)); however, the density and shape change. The aggregates become laminated with layers of about 70-180 nm (figure 7(c)). The average particle size is 162 nm. The particles do not demonstrate grain structure.

Upon further milling (the mechanical energy dose is more than 42 kJ/g), the aggregation process prevails over dispersion. The particles are of 300-1000 nm. Particles and dense small aggregates of about 1 nm cannot be distinguished. The particles represent laminated aggregates with particles of the size about 50 nm. Being scanned with an AFM cantilever, such aggregates do not decompose. The milling of magnesium orotate with the mechanical energy dose of 7 kJ/g and 21 kJ/g results in the reflection diffusion (with the preservation of the main set of lines), which is associated with an increase of the structure imperfection and the crush of crystallites (figure 8). These changes are accompanied by an increase in the intensity of the amorphous halo at 19 and 27 deg. Strong reflections of magnesium orotate at 43 and 50 deg can be due to a change in the phase composition of the sample. These lines persist after milling with the mechanical energy dose of 42 kJ/g, when the crystalline
structure of the source magnesium orotate is destroyed (a new set of reflexes appears instead of the initial ones). The complete rearrangement of the initial crystal structure and the formation of a multiphase amorphous-crystalline mixture are observed after milling with the mechanical energy dose of 42 kJ/g.

![Figure 8. X-ray diffraction pattern of magnesium orotate source (1) and after milling with the mechanical energy dose of 7 kJ/g (2), 21 kJ/g (3) and 42kJ/g (4)](image)

Figure 9 shows the IR spectra of the source magnesium orotate and the milled magnesium orotate. The increase in the milling time decreases the band intensity at 533 cm\(^{-1}\) due to the vibrations of \(\delta(CH)+\delta(NH)\). This can be a proof of the deprotonation of NH groups. The absorption between 600 and 800 cm\(^{-1}\) is caused by the deformational vibrations of the N-H pyrimidine ring (there are out-of-plane wagging NH vibrations near \(\sim\)700 cm\(^{-1}\)). Therefore, during milling, the appearance of various (hydroxy- and dihydroxy-) tautomeric forms of magnesium orotate influence the absorption in this region of the IR spectrum. The “breathing” vibrations of the pyrimidine ring in the spectrum region between 1000 and 1050 cm\(^{-1}\) are also changed with the milling time.

![Figure 9. Transmission IR spectra of magnesium orotate source (1) and after milling with the mechanical energy dose of 21 kJ/g (2) and 42kJ/g (3)](image)

The band at 1478 cm\(^{-1}\), which is ascribed to the \(\nu(C=C)\) vibrations, expands due to a shoulder at \(\sim\)1500 cm\(^{-1}\). This indicates the lactam-lactimic tautomeric transformations, which lead to the formation
of the aromatic structures. Milling decreases the intensity of the band at 1518 cm\(^{-1}\) associated with the deformational vibration of \(\delta(NH)\) the pyrimidine ring. After milling with the mechanical energy dose of 42 kJ/g, the band is not observed. This indicates the deprotonation of NH groups due to milling.

The gradual transition of the pyrimidine ring to the completely aromatic form changes the shape of the IR-bands between 1500 and 1600 cm\(^{-1}\) which have appeared due to the NH deformational vibrations. The overlapping of the absorption of the carbonyl groups complicates finding point correlations. For the source sample, in the region 1600-1700 cm\(^{-1}\), the high-resolution bands the amides I (\(\nu(C=O)\)) and the amides II (\(\delta(NH) + \nu(CN)\)) are observed. With an increase in the milling time (and the mechanical energy dose), the bands merge. This indicates the tautomeric transitions which result in protonization of carbonyls. And, since the region 1600-1700 cm\(^{-1}\) is characterized by skeleton vibrations of a completely aromatic pyrimidine ring, it can also evidence that an aromatic structure appears.

The NEXAFS spectra of the K-edge of the C1s of pyrimidine bases have almost the same structure [15]. There are four well-resolved \(\pi^*\)-resonances, two \(\sigma^*\)-resonances near 296 and 304 eV. The peaks of \(\pi^*\)-resonances correspond to the electron transitions from unequal core levels C1s of different carbon electrons in the molecules to the delocalized \(\pi^*\)-nonbonding orbitals. In the uracil spectrum, there are peaks at 284.6 eV, 286.0 eV, 288.0 eV, and 289.5 eV. In the thymine spectrum, the low-energy peak shifts to the higher energy side by 0.4 eV (285 eV). It takes place due to the appearance of the methyl substitute. Still, it should be noted that the figure demonstrating the uracil chemical composition shows the structures corresponding to the oxo-forms and provides no data on the isomeric state of uracil and thymine. The heterocycle containing nitrogen has bonds similar to aromatic heterocycles, but the explicit aromatic nature of the bonds is common only for the dihydroxy-forms.

Figure 10 presents the NEXAFS spectra of the C1s-edge of absorption of magnesium orotate. In all the spectra, there is an intensive peak at 285.0 eV corresponding to the electron transition C1s\(\rightarrow\pi^*\) (C=C) and indicating the presence of an aromatic cycle. The electron transitions C1s\(\rightarrow\pi^*\) in C=O and C-OH contribute to the peaks at 288.5 eV and 290.5 eV. This strongly suggests that magnesium orotate has several tautomeric forms.

![Figure 10. NEXAFS spectra of Cs1-edge of absorption of magnesium orotate: source (1) and after milling with the mechanical energy dose of 7kJ/g (2) and 42kJ/g (3).](image-url)

The shape of the N1s spectra of magnesium orotate and the change of these spectra during milling are very similar to the above-described potassium orotate spectra.

The XPS C1s-, N1s-, O1s- spectra (figure 11) of magnesium orotate are similar to those of potassium orotate. In the XPS C1s-spectra, the component of the (CH)\(_2\) groups dominates in the adsorbed layer on the surface of the particles (E\(_b\) = 285.0±0.2 eV). The (C-OH) groups have E\(_b\) (C1s) = 286.2±0.2 eV. The component at 289.0±0.2 eV refers to the carbon atoms bonded with the oxygen and
the nitrogen atoms in the (O=C-N) group in the oxo- and hydroxy-forms of the orotate anions. The fraction of the component of C-OH groups increases from 25% in the source sample to 33% and 36% in the samples after milling with the mechanical energy dose of 7 kJ/g and 42 kJ/g, respectively. In the O1s-spectra, there are components at 531.8±0.2 eV and 533.4±0.2 eV corresponding to the oxygen atoms in the carbonyl (C=O) and the hydroxyl (C-OH) groups. The fraction of the components at E_b = 533.4 eV increases from 26% to 47% (figure 11). In the N1s-spectra, the intensive component at 400.5±0.2 eV is attributed to the nitrogen atoms in the NHC=O group. The components at Е_b=398.4 ±0.2 eV and Е_b=399.6 ±0.2 eV refer to the nitrogen atoms in the aromatic and nonaromatic heterocycles. The fraction of the nitrogen components in the aromatic heterocycle increases from 15% to 23.5% (figure 11).

Figure 11. XPS spectra of magnesium orotate source (1); after milling with the mechanical energy dose of 7 kJ/g (2), and 42 kJ/g (3)

### 3.3. Orotates water solutions

The pH value of the water solutions of magnesium and potassium orotates decreases in the following sequence: oxo-form > hydroxy-form > mixture of hydroxy-form + dihydroxy-form > dihydroxy-form (table 3). The observed effect is caused by the strong acid properties of the orotate dihydroxy-form (which is OH-acid) rather than by the acid properties of the oxo-form (which is NH-acid). The hydroxy-form is in the intermediate position as it has one OH- and one NH-group. During the storage for a few hours at room temperature, the pH value of the water solutions becomes equal and corresponds to the pH of the oxo-form solution.

| Mechanical energy dose, kJ/g | Magnesium orotate | Potassium orotate |
|-----------------------------|-------------------|-------------------|
| t, °C                       | 0                 | 7                 | 42                | 0                 | 7                 | 42                |
| 10                          | 1.3298            | 1.3242            | 1.3165            | 1.2807            | 1.2689            | 1.1732            |
| 20                          | 1.0265            | 1.0100            | 0.9989            | 0.9977            | 0.9482            | 0.8323            |
| 30                          | 0.5302            | 0.8102            | 0.8002            | 0.8221            | 0.7999            | 0.6929            |
| 40                          | 0.6777            | 0.6897            | 0.6777            | 0.6102            | 0.6809            | 0.5821            |

The capillary viscosity and the expansion coefficients (at 15°-25°C) of the solutions of the tautomers prepared by milling are presented in tables 3 and 4. The differences in the capillary
viscosity and expansion coefficients are due to the change in the area of the hydration of orotate anion because of the changes in the composition of the functional groups (=O and –OH) caused by the change of the tautomeric structures of the orotate anions.

Table 4. Solubility of orotates, volumetric thermal expansion coefficient (Pa·s) of water solutions of orotates

| Mechanical energy doze, kJ/g | Solubility (25°C), g/100gH2O | Volumetric thermal expansion coefficient (15-25°C, 0.1wt.%), K⁻¹ |
|-----------------------------|-------------------------------|---------------------------------------------------------------|
| 0                           | Magnesium orotate 0.12        | Magnesium orotate 0.165                                       |
| 7                           | Potassium orotate 0.25        | Potassium orotate 0.155                                       |
| 42                          | Magnesium orotate 0.15        | Magnesium orotate 0.154                                       |
|                             | Potassium orotate 0.26        | Potassium orotate 0.140                                       |
|                             |                               |                                                               |

The differences in the solutions properties are maintained for 1.5–2.5 hours. Then, the properties become similar to those of the solutions prepared from the orotate oxo-form. It can be concluded that in 1.5-2.5 hours, the hydroxy- and dihydroxy – form solutions transform into the oxo-form solution. The duration of the preservation of the properties depends on the solution concentration and temperature. The systematic studies of the duration of the preservation of the properties of the orotate solutions are still under way.

3.4. Biological properties of potassium orotate and magnesium orotate tautomers

The difference in the microelectrophoretic mobility of live cells in the water solutions reflects different biological activity of the tautomers (table 5).

Table 5. Epiteliocytus microelectrophoresis mobility in water solutions of orotates

| Mechanical energy doze, kJ/g | Magnesium orotate | Potassium orotate |
|-------------------------------|-------------------|-------------------|
|                               | Fraction of active cells, % | Amplitude of cell vibrations, μm | Fraction of active cells, % | Amplitude of cell vibrations, μm |
| 0                             | 52±3.2            | 2.1±0.3           | 36±4.1            | 1.±3.7           |
| 7                             | 84±2.8            | 3.7±0.5           | 100±12.0         | 3.7±0.7           |
| 42                            | 36±3.6            | 0                 | 45±4.7           | 1.5±0.3           |

The studies on laboratory rats have shown the following. After the injection of the hydroxy-form, the amount of magnesium in the blood plasma of rats increased by 64%, but remained unchanged when the oxo- and dihydroxy- forms were injected.

For the laboratory rats with experimental steroid osteoporosis, the content of the overall collagen was lower by 42.8% (p<0.01) than that in the control group of healthy rats. The magnesium orotate injection balances these changes. The usage of oxo-form and dihydroxy-forms of the magnesium orotate makes it possible to reach the level of collagen characteristic of the reference group. The usage of hydroxy-form of magnesium orotate produced the collagen level exceeding that of healthy rats in the control group by 65.3 (p<0.001).

4. Conclusions

In this paper, the effect of milling on the tautomeric structure of magnesium orotate and potassium orotate was investigated. In the source samples, the oxo-forms of orotates were dominant. The hydroxy- and dihydroxy- tautomeric forms were obtained depending on a dose of mechanical energy.

It is shown that the increasing dose of mechanical energy during milling leads to the successive transformations of the chemical structure of orotates: oxo→hydroxy→dihydroxy forms. The solid
tautomers preserve their structure from three to four months. After that, the sample mainly transforms into the oxo-forms of orotates. The tautomers solubility varies slightly. The physicochemical properties (viscosity, volumetric thermal expansion coefficients) of the water solutions of the tautomers differ. The differences are due to the change in the area of the hydration of the orotate anion because of the changes in the composition of the functional groups caused by the change of the tautomeric structures. The isomeric differences formed during milling remain in the orotate water solutions for 1.5 -2.5 hours.

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