Original scientific paper
UDC 637.12:546.47]:616.15

NEW COLLOIDAL CHELATED HIGHLY DIGESTIBLE FORM OF ZINC ESSENTIAL TRACE ELEMENT

Andrey Vladimirovich Blinov[1*], Alexander Vladimirovich Serov[1], Alexander Alexandrovich Kravtsov[2,3], Konstantin Vasilevich Kostenko[1], Julia Jurevna Snezhkova[1], Alexey Alexeevich Gvozdenko[1]

[1]Institute of Engineering, North Caucasus Federal University, Kulakova str. 2, 355029 Stavropol, Russia
[2]Scientific-research laboratory of ceramics and techno-chemistry, scientific-laboratory complex of clean rooms, Institute of Engineering, North-Caucasus Federal University, Kulakova str. 2, 355029 Stavropol, Russia
[3]Laboratory of Physics and Technology of Semiconductor Nanoheterostructures for Microwave Electronics and Photonics, Scientific Group of Technology of Heterostructures, Southern Scientific Center of the Russian Academy of Sciences, Chehova str. 41, 344006 Rostov-on-Don, Russia

[*]e-mail: blinov.a@mail.ru

Abstract

Within the framework of the presented study, a synthesis method of the new colloidal chelate form of zinc - zinc lysinate-riboflavinate (LRZn) has been developed. Medical-biological and physicochemical properties of zinc lysinate-riboflavinate have been studied in order to enrich dairy products with zinc-containing compounds.

LRZn was obtained in an aqueous medium as a result of a reaction between L-lysine, B2 vitamin (riboflavin) and a zinc-containing precursor. The phase and dispersion composition of zinc lysinate-riboflavinate was studied by the XRD and photon-correlation spectroscopy methods respectively. The morphology of LRZn was studied by scanning electron microscopy. The elemental composition was investigated using energy dispersive spectroscopy. The paper also presents the results of quantum mechanical modeling of the compound in HyperChem 8.0 and ChemBio3D Ultra 12 software. The acute toxicity of LRZn was investigated in laboratory animals. To determine the acute toxicity of zinc lysinate-riboflavinate, six groups of laboratory white mice of 10 animals in each were formed. The first group served as a control; laboratory animals of other groups were orally administered the developed form of the essential trace element zinc at different doses. The data on the effect of milk, enriched with the essential zinc trace element, on the growth, development and blood biochemical parameters of laboratory animals was obtained. For this purpose, males of white Wistar rats were used as laboratory animals; 3 groups of laboratory animals were formed, all animals were fed on a zinc-deficient diet, once a day animals were fed: the 1st group - milk; the 2nd group - milk enriched with zinc sulfate, and the 3rd group - milk enriched with LRZn. The body weight of laboratory animals was measured daily.

It was found that zinc lysinate-riboflavinate is a monophasic compound with a monoclinic crystal lattice. Investigation of microstructure showed that the samples consist of needle-shaped crystals with a length from hundreds of nanometers to several microns. It was established that zinc lysinate-riboflavinate does not have toxic properties and is a non-toxic compound.

It was determined that the consumption of milk, containing LRZn chelate form has a positive effect on the weight gain of laboratory animals. A decrease in the concentration of urea, total cholesterol and an increase in the concentration of the Alanine aminotransferase and Aspartate transaminase enzymes in the blood of laboratory animals as a result of a higher digestibility of the chelate form of the essential trace element zinc were found.

***Keywords**: Zinc lysinate-riboflavinate, Zinc chelate, Zinc trace element, X-ray diffraction, Scanning electron spectroscopy, Acute toxicity, Blood biochemical parameters.*

1. Introduction

Zinc is one of the most important trace elements in the human body, fulfilling many functions: immunostimulating, regenerative, regulative (regulation of the nervous system, regulation of hormones), antioxidant (included in superoxide dismutase), hypocholesteremic, lipotropic and other [1 - 7].

It is well known that the daily human need for zinc is 12 to 50 mg [1, 2]. However, according to some authors [8 - 13], there are so-called zinc-deficient territories, which include many regions of the world. Therefore, the development of highly digestible, non-toxic forms of the essential trace element of zinc and the enrichment of food products by them is quite an urgent task.

Analysis of zinc preparations used in medicine, food industry, and agriculture, revealed the following facts:

At present, inorganic forms of zinc, such as sulfate, acetate, oxide, and others, are used as active ingredients of the preparations. These compounds have one advantage - a low cost and a considerable disadvantage - low assimilation of zinc, for some forms it is less than 10%. Zinc salts with mineral acids also exhibit high toxicity [5,14 - 19].

- In the treatment of zinc-deficient conditions, organic forms of zinc: asparaginate, glycinate, lactate, ascorbate, and others are used as food additives.

High attention should be paid to triple complexes of metals with vitamins and amino acids. As a source of the essential trace element of zinc in the chelated form, it is suggested to use zinc lysinate-riboflavinate (LRZn).

The aim of this study is to investigate the structure, chemical composition and biological activity of a new colloidal chelate form of the essential zinc trace element - zinc lysinate-riboflavinate.

2. Materials and Methods

The synthesis of LRZn was carried out in an aqueous medium. In the beginning, riboflavin and L-lysine were dissolved in distilled water; then, with heating and stirring, sodium hydroxide was added to the reaction mixture to shift the active acidity of the medium to the alkaline conditions. Further, a zinc-containing precursor was added to the reaction mixture. Heating and stirring were carried out for 5 hours to the end of the reaction. Then, the resulting pulp was filtered for separating the LRZn from other reaction products. The resulting precipitate was washed several times with cold distilled water and dried at 50 ^{0}C. The resulting compound should be stored in a closed, opaque container.

The chemical reactions taking place in the preparation of LRZn are shown in Figure 1.

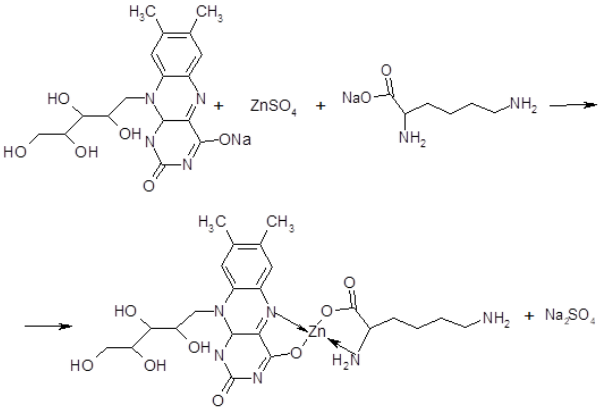

Figure 1. The chemical reaction of obtaining of LRZn

Modeling of the structure of LRZn was carried out in the programs HyperChem 8.0 and ChemBio3D Ultra 12.0 under the principle of minimum energy with geometric optimization, carried out using the conjugate gradient method of Polak-Ribiere in vacuum [20].

The elemental composition and microstructure of the samples were examined using scanning electron microscope "MIRA3-LMH" with the element determination system AZtecEnergy Standart/X-max 20 (standard) ("Tescan", Czech Republic).

The phase composition of LRZn was studied by powder diffractometry on a PANanytical Empyrean X-ray diffractometer (manufactured by PANalytical B.V., the Netherlands).

Disperse composition of aqueous solutions of LRZn with different concentrations was studied by photon-correlation spectroscopy with Spectrometer of dynamic and static light scattering "Photocor Complex" (Photocor, Russia).

To determine the acute toxicity of zinc lysinate-riboflavinate, six groups of laboratory white mice of 10 animals in each were formed. The first group served as a control; Laboratory animals of the second, third, fourth, fifth and sixth groups were orally administered the developed form of the essential trace element zinc at a dose of 5, 10, 20, 50 and 100 mg/kg, respectively.

For the study of the effect of milk, enriched with a zinc trace element, on the growth, development and blood biochemical parameters, studies of the laboratory animals growth and development were conducted. Males of white Wistar rats, whose average weight was about 190 ± 10 grams, were used as laboratory animals; 3 groups of laboratory animals were formed, all animals were fed on a zinc-deficient diet, once a day animals were fed: the 1st group - milk; the 2nd group - milk enriched with LRZn, and the 3rd group - milk enriched with zinc sulfate. The concentration of zinc trace element in both inorganic and organic diets was 10 mg/L. The body weight of laboratory animals was measured daily.

3. Results and Discussion

3.1 Analysis of the morphology and structure of zinc lysinate-riboflavinate complex

Complex compounds of biogenic elements (metals) with organic ligands - vitamins and amino acids are a new class of biologically active substances. Based on the structure, such complex compounds can be classified into molecular (with organic ligands, with organic and inorganic ligands) and chelate (with vitamin and amino acid, with two vitamins). The ternary complex - zinc lysinate-riboflavinate obtained in this work is a chelate mixed-ligand complex of the essential trace element of zinc with the vitamin Riboflavin and the essential amino acid L-lysine.

Figure 2 shows the model of a molecule of LRZn.

The creation of such compounds is caused by the high biological value of vitamin B2 (riboflavin) and the essential amino acid of L-lysine. It is also necessary to note the synergism of this vitamin and essential microelement of zinc, which, probably, should increase its assimilability and bioavailability [1].

As shown in Figure 2, LRZn is a chelate complex in which zinc is bonded to the carboxyl and amino group of the α-amino acid (L-lysine) and to enol oxygen in C_4 and the neighboring nitrogen heteroatom in the riboflavin molecule.

The results of the study of the elemental composition by the method of energy dispersive microanalysis are presented in Figures 3 and 4.

Analysis of the EDX spectrum showed that elements such as O, Zn, N and C are present in LRZn, which is in good agreement with the theoretical notions of the structure of this compound.

Decoding of EDX spectra of LRZn allowed to determine the mass content of each element in the sample of LRZn; The results are presented in the histogram in Figure 4.

The results of riboflavin and zinc lysinate-riboflavinate microstructure research are presented in Figure 5.

It was found that the microstructure of riboflavin and samples of LRZn has a similar structure. Samples consist of needle crystals from hundreds of nanometers to several microns in length. The thickness of the elementary crystals is about 100 nm. The main difference between the microstructure of riboflavin and LRZn is that the riboflavin crystallites are assembled in so-called druses, while in LRZn they are in a separate state.

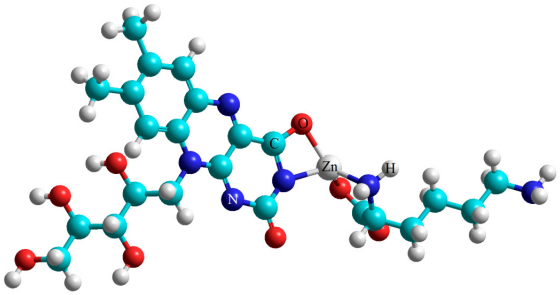

Figure 2. Model of a molecule of LRZn

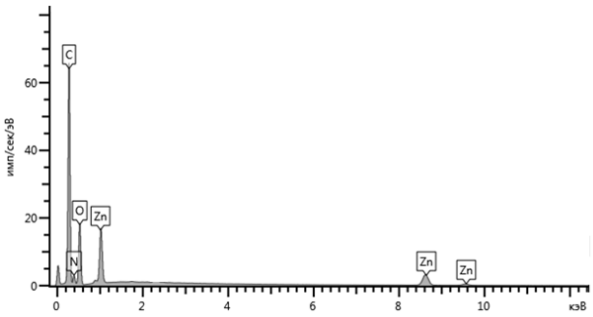

Figure 3. EDX-spectrum of LRZn

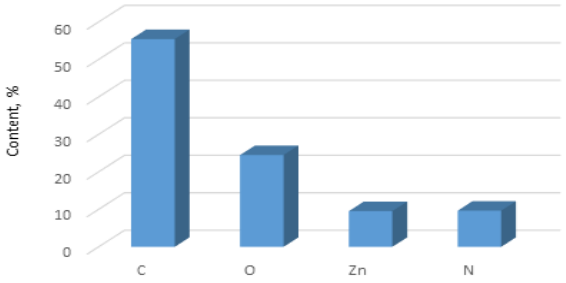

Figure 4. Histogram of the mass content of elements in the sample of LRZn according to the energy-dispersive microanalysis

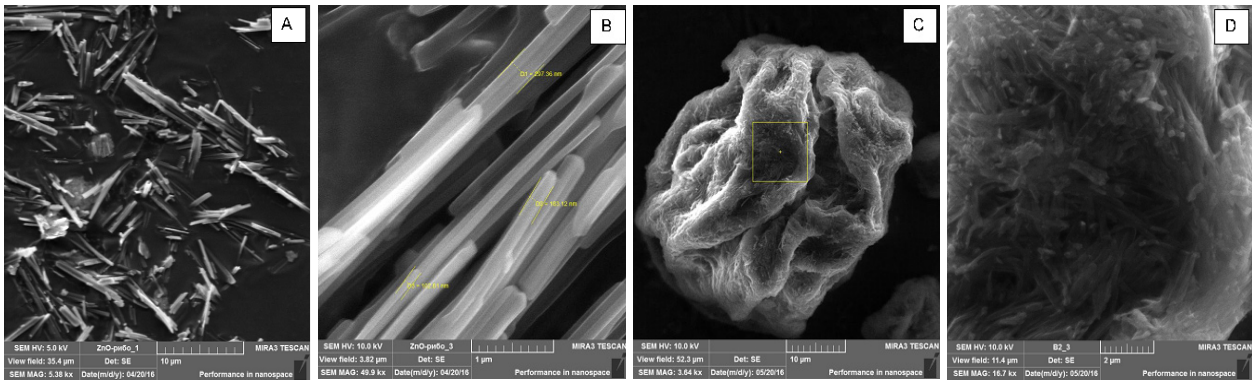

Figure 5. Scanning electron microscope (SEM) images of the samples of LRZn (A, B) and riboflavin (C, D)

At the next stage of the investigation, the phase composition of LRZn was studied, riboflavin and L-lysine hydrochloride served as reference samples. Obtained diffractograms are shown in Figure 6.

As a result of X-ray phase analysis, it was found that LRZn is a single-phase compound with a monoclinic crystal lattice, the space group P 121/c1, unlike riboflavin with an orthorhombic crystal lattice and L-lysine hydrochloride with a monoclinic crystal lattice, the space group P 2_1/c. The crystallographic parameters of the unit cells of the studied compounds are also significantly different, which is shown in Table 1.

Analysis of the obtained data confirms that LRZn is a new compound - the chelate complex of riboflavin and lysine with a zinc trace element, and not a mechanical mixture of riboflavin with lysine and a zinc-containing precursor.

In order to show that zinc lysinate-riboflavinate is a colloidal form of zinc, its aqueous solutions' disperse composition was investigated. The results are shown in Figure 7.

It was found that in aqueous solutions at concentrations about 0.005%, LRZn exists as separate micelles with an average hydrodynamic radius of about 150 nm. At lower concentrations, LRZn forms true solution in an aqueous medium (the average hydrodynamic radius of the particles is about 1 nm), and at higher concentrations, it exists in the form of aggregates of elementary micelles whose mean hydrodynamic radius is about 1 μm.

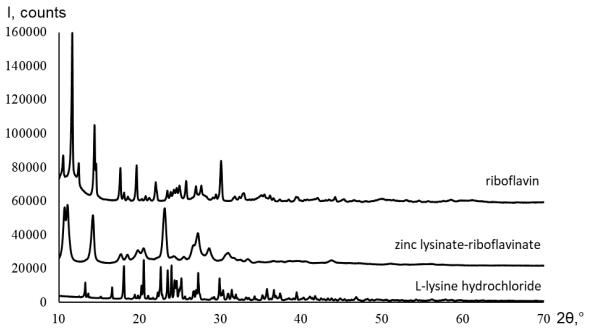

Figure 6. Diffractograms of riboflavin, LRZn, and L-lysine hydrochloride

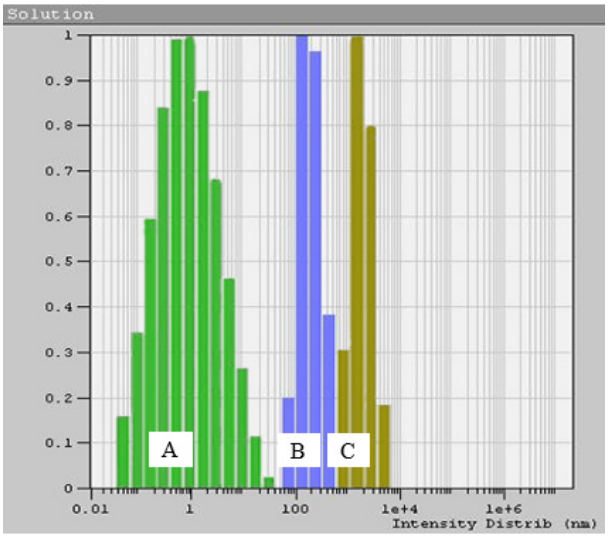

Figure 7. Disperse composition of aqueous solutions of LRZn with different concentrations (A - below 0.005%; B - about 0.005%; C - above 0.005%)

Table 1. Crystallographic parameters of the studied compounds

Name of compound	Space group	Type of crystal lattice	Parameters of the unit cell
LRZn	P 121/c1	Monoclinic	a (Å): 16.69 b (Å): 16.08 c (Å): 11.57 Alpha (°): 90.00 Beta (°): 95.12 Gamma (°): 90.00 V/ 10^6 pm^3: 3092.76
Riboflavin	$P2_12_12_1$	Orthorhombic	a (Å): 15.17 b (Å): 20.15 c (Å): 5.35 Alpha (°): 90.00 Beta (°): 90.00 Gamma (°): 90.00 V/ 10^6 pm^3: 1635.79
L-lysine hydrochloride	$P2_1/c$	Monoclinic	a (Å): 9.20 b (Å): 11.27 c (Å): 8.54 Alpha (°): 90.00 Beta (°):105.60 Gamma (°):90.00 V/ 10^6 pm^3:853.51

3.2 Determination of acute toxicity of zinc lysinate-riboflavinate

Acute toxicity describes the adverse effects of a substance that result either from a single exposure or from multiple exposures in a short time period (usually less than 24 hours). The study of acute toxicity is aimed at determining the quantitative parameters of the toxicity of the test substance, studying of its specific toxic effect, as well as establishing the existing species and sex differences in sensitivity to a toxic agent.

Determination of acute toxicity was carried out in order to establish the absence or presence of toxic effects of the colloidal form of the zinc essential trace element. The safety issue of the developed zinc lysinate-ribobflavinate is quite important and relevant since this additive is planned to be used in the food industry to produce functional foods.

During the experiment, clinically healthy laboratory animals were used. The administration of the study drug to the experimental groups was carried out in compliance with the rules of asepsis and antisepsis. An appropriate volume of distilled water was administered to control animals. The state of health of the animals was observed for 14 days after the administration, the appearance and behavior, the attitude to water and food, the mobility, the condition of the coat and visible mucous membranes were taken into account. Laboratory animals were injected with a certain amount of zinc lysinate-ribobflavinate according to Table 2.

Researches of the acute toxicity of zinc lysinate-riboflavinate after intragastric administration in the maximum allowable solution volumes of 0.5 mL for white mice, corresponding to 100 mg/kg of the active substance showed no signs of toxicological effects. Consequently, zinc lysinate-riboflavinate does not possess toxic properties and belongs to non-toxic compounds.

3.3 The study of the effect of the milk, enriched with zinc essential trace element, on the growth, development and blood biochemical parameters of laboratory animals

In order to show the possibility and prospects for the enrichment of dairy products with a new form of the zinc essential trace element, the influence of milk enriched with the colloidal form of the zinc essential trace element on the growth of laboratory animals, as well as their blood biochemical parameters, was studied.

The concentration of zinc trace element in milk with both inorganic and organic forms of zinc was 10 mg/L. The measurement of the body weight of laboratory animals was carried out daily. Dynamics of change in the average weight of laboratory animals is presented in Figure 8.

Analysis of the data obtained showed that during the first three experimental days there was no difference in the mass of laboratory animals of all experimental groups. Starting from the fourth day and until the end of the experiment, animals of the second group, in whose diet the chelated colloidal form of zinc was present, showed the greatest increase in body weight, then animals of the third group and the least growth was recorded in animals kept completely on a zinc-deficient diet. It is important to consider the mass of laboratory animals on a 21st experimental day (Figure 9).

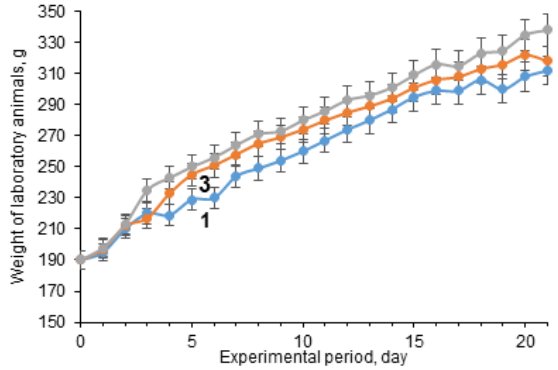

Figure 8. Dynamics of changes in the mass of laboratory animals for the entire experimental period: 1st, 2nd and 3rd experimental groups

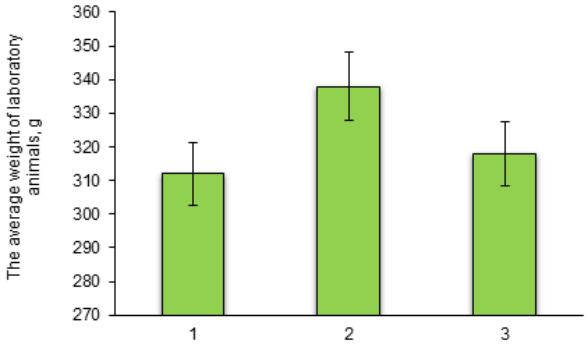

Figure 9. The average weight of laboratory animals on the 21st experimental day: 1st, 2nd and 3rd experimental groups

Table 2. The results of the acute toxicity study of zinc lysinate-riboflavinate

Group	Count of animals in group	Dose, mg/kg	The volume of injected drug, mL	Animal condition
1	10	-	-	Active, appetite saved, signs of intoxication absent
2	10	5	0.1	
3	10	10	0.1	
4	10	20	0.5	
5	10	50	0.5	
6	10	100	0.5	

It was established that the weight of animals on 21st experimental day in the second group (diet with inorganic form of zinc) was 8.33% higher in comparison with the control group and the weight of animals on 21st experimental day in the third group (diet with zinc lysinate-riboflavinate) was 1.92% higher in comparison with the control group. The results of the study show an improvement in the growth parameters of laboratory animals when using zinc lysinate-riboflavinate as a source of essential zinc trace element, which confirms the greater biological activity and digestibility of zinc from zinc lysinate-riboflavinate in compare with inorganic forms.

During the entire experimental period, laboratory animals of all groups were active, with a normal appetite, no behavioral abnormalities were recorded, changes in the state of the skin were not detected, respiration and heart rate were normal.

Upon completion of the experiment, after 3 weeks of keeping the laboratory animals on different rations, blood samples were taken. Biochemical analysis of the blood of laboratory animals was carried out on the base of the FSBI "Stavropol Interregional Veterinary Laboratory" (Stavropol). The results of the analysis are presented in Table 3.

As a result of the data analysis, it was established that the total cholesterol in the blood of laboratory animals of 2nd and 3rd experimental groups, was lower in comparison with the control group by 0.53 and 0.19 mmol/L, respectively. A decrease in the urea content by 2.7 and 1.9 mmol/L and glucose by 0.47 and 1.32 mol/L respectively was also observed. The decrease in the content of these indicators in the blood of laboratory animals indicates an improvement in the digestibility of feed nutrients.

The concentration of enzymes such as alanine aminotransferase, Aspartate transaminase, and bilirubin in the blood of laboratory animals in the 2nd and 3rd experimental groups is higher compared to the control, which, apparently, is due to the improvement of the functioning of the liver cells due to the normalization of the zinc microelement intake in the body of laboratory animals.

The data obtained indicate that the introduction of zinc-containing compounds into the diet increases the synthesis of amino acids, and also improves the speed and quality of protein metabolism. In addition, the concentration of total protein in serum is inextricably linked with the content of aminotransferases, since an increase in the content of total protein causes an increase in the content of transamination enzymes involved in its synthesis.

It was also found that such an indicator as the alkaline phosphatase activity, a zinc-containing enzyme, in laboratory animals of the 2nd experimental group was 733.8 ± 36.7 U/L, while in animals of the 3rd group 1058.0 ± 52.9 U/L. This is due to the fact that zinc, which is part of the chelate complex with lysine and riboflavin, has greater biological activity and, consequently, better digestibility and bioavailability. Thus this form of zinc is able to normalize the activity of zinc-dependent enzymes compared to the inorganic form of zinc, the use of which disrupts the functioning of the liver cells, which leads to the abnormal activity of alkaline phosphatase in the blood of animals.

4. Conclusions

- According to the results of the research, it can be concluded that zinc lysinate-riboflavinate is an individual chemical compound existing in an aqueous medium in the form of colloidal particles, with a monoclinic crystal lattice and a needle-shaped shape with an average hydrodynamic radius of about 150 nm.

- Investigations of the elemental composition confirmed the theoretical understanding of the structure and composition of the molecule of zinc lysinate-riboflavinate.

Acknowledgment

Researches of the acute toxicity of the chelate colloidal form of the zinc essential trace element - zinc lysinate-riboflavinate were carried out under the cooperation agreement №15/2015 between the North Caucasus Federal University and the Stavropol State

Table 3. Results of the biochemical analysis of the blood of laboratory animals

№	Name of the indicator	Sample №1	Sample №2	Sample №3
1	Alanine aminotransferase, units per liter	25.4 ± 1.3	26.3.0 ± 1.3	38.8 ± 1.9
2	Aspartate transaminase, units per liter	202.8 ± 10.1	207.0 ± 10.3	249.9 ± 12.5
3	Total protein, g/L	92.5 ± 2.3	103.2 ± 2.6	100.0 ± 2.5
4	Total bilirubin, μmol/L	17.1 ± 0.8	22.9 ± 1.1	29.5 ± 1.5
5	Glucose, mol/L	2.98 ± 0.07	2.51 ± 0.06	1.66 ± 0.04
6	Creatinine, μmol/L	105.9 ± 5.3	79.8 ± 4.0	100.9 ± 5.0
7	Urea, mmol/L	10.7 ± 0.5	8.0 ± 0.4	8.8 ± 0.4
8	Total cholesterol, mmol/L	2.39 ± 0.06	1.86 ± 0.05	2.20 ± 0.05
9	Alkaline phosphatase, units per liter	718.7 ± 35.9	733.8 ± 36.7	1058.0 ± 52.9

Agrarian University dated 12.03.2015 on the base of Department of Therapy and Pharmacology, Faculty of Veterinary Medicine.

5. References

[1] Regmi B. (2010). *Zinc Deficiency in Agricultural Systems and Its Implication to Human Health*. International Journal of Environmental and Rural Development, 1, 1, pp. 98-103.

[2] Hambidge K. (2007). *Zinc Deficiency: A Special Challenge*. Journal of Nutrition, 137, pp. 1101-1105.

[3] Wani A. (2017). P*ossible role of zinc in diminishing lead-related occupational stress a zinc nutrition concern*. Environmental Science and Pollution Research, 24, pp. 8682-8691.

[4] Uciechowski P., Rink L. (2016). *Zinc*. In: Malavolta M. (Ed.), Molecular basis of nutrition and aging, Elsevier, UK.

[5] Dorea J. (2000). *Zinc in human milk*. Nutrition Research, 20, pp. 1645-1687.

[6] Prasad A. S. (2017). *Discovery of Zinc for Human Health and Biomarkers of Zinc Deficiency*. Molecular, Genetic, and Nutritional Aspects of Major and Trace Minerals, pp. 241-260.

[7] Haase H. (2017). *Zinc Signals and Immune Function*. Molecular, Genetic, and Nutritional Aspects of Major and Trace Minerals, pp. 261-271.

[8] White J. (1997). *Soil Zinc Map of the USA using Geostatistics and Geographic Information Systems*. Soil Science Society of America Journal 61 (1), pp. 185-194.

[9] White J. (1999). *Mapping soil micronutrients*. Field Crops Research, 60, pp. 11-26.

[10] Kubota J. (1967). *Copper, Zinc, Cadmium, and Lead in Human Blood From 19 Locations in the United States*. Archives of Environmental Health, 16, pp. 788-793.

[11] Kapil U. (2011). *Zinc deficiency amongst adolescents in Delhi*. Indian Pediatr., 48, pp. 981-982.

[12] Alloway B. (2008). *Micronutrient deficiencies in australian field crops*, in: Micronutrient Deficiencies in Global Crop Production. Springer Netherlands, Dordrecht, pp. 63-92.

[13] Gupta R. (1988). *Studies on the pathology of experimental zinc deficiency in guinea-pig.* Journal of Comparative Pathology 98, pp. 405–413.

[14] Sahin K. (2005). *Supplementation of zinc from organic or inorganic source improves performance and antioxidant status of heat-distressed quail*. Poultry Science, 84, pp. 882-887.

[15] Krężel A. (2016). *The biological inorganic chemistry of zinc ions*. Archives of Biochemistry and Biophysics, 611, pp. 3-19.

[16] Longchamp M. (2016). *Effects on the accumulation of calcium, magnesium, iron, manganese, copper and zinc of adding the two inorganic forms of selenium to solution cultures of Zea mays.* Plant Physiology and Biochemistry, 98, pp. 128-137.

[17] Rafie M. (2017). *Influence of foliar-applied zinc in the form of mineral and complexed with amino acids on yield and nutritional quality of onion under field conditions.* Scientia Horticulturae, 216, pp. 160-168.

[18] Medani M. (2012). *Zinc sulphate attenuates chloride secretion in Human colonic mucosae in vitro.* European Journal of Pharmacology, 696, pp. 166-171.

[19] López-Córdoba A. (2015). *Co-crystallization of zinc sulfate with sucrose: A promissory strategy to render zinc solid dosage forms more palatable*. Journal of Food Engineering, 170, pp. 100-107.

[20] Grippo L. (1997). *A globally convergent version of the Polak-Ribi re conjugate gradient method.* Mathematical Programming, 78, pp. 375-391.