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COMBINED PREPARATION BASED ON CHELATING MAGNESIUM BY PHOSPHORYLATED CASEIN. CHARACTERISTICS OF ITS SYNTESIS

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The article is devoted to the development and synthesis of a new drug for use in animal husbandry and veterinary medicine as an immunostimulatory and adaptogenic agent. The basis of the new drug is a combination of magnesium, phosphorus, and casein of cow's milk.
*Given the important scientific and practical importance for veterinary medicine of innovative drugs that would have pronounced immunostimulatory and adaptogenic properties, **the aim of our research** is to develop a method of obtaining a new drug with an original composition and further studies of its effectiveness and safety.*
*Modern **materials and methods** were used to achieve this goal. In particular, mass spectrometry was used on a Waters H-class UPLC liquid high-pressure liquid chromatography spectrometer with a Waters TQ-S micro three-quadrupole detector; atomic emission spectrometer with inductively coupled plasma Analytik-Jena Plasma Quant PQ 9000 Elite; liquid chromatographer with a three-quadrupole mass detector and with analytical column – Waters ACQUITY UPLC BEH C18 1.7μm 2.1×50mm.*
*The **result of the work** was the development of a method of modification of the casein molecule, which was carried out in several stages: the first stage was the direct phosphorylation of the casein molecule; the second stage of the synthesis was the chelation of magnesium with casein.*
*Based on the results of this work, the following **conclusions** were formed: 1) the synthesis of a new drug is carried out in two stages: the first – modification of casein by direct phosphorylation and the second stage – chelation of magnesium with casein; 2) it was found that the efficiency of phosphorylation directly correlates with the number of treatment cycles of the reaction mixture and is optimal for three treatment cycles*
***Keywords**: casein, magnesium, phosphorus, synthesis, hydrolysis, amino acids, phosphorylation, chelation, complexation*

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1. Introduction

One of the important tasks of today's biological science is the creation of new drugs and improvement of existing ones with biologically active components in an easily accessible form for the animal body. These drugs should provide effective stimulating and corrective action on various metabolic links. To date, the market of drugs for animal husbandry and veterinary medicine are different in composition and mechanism of action. However, there are no casein-based drugs that would also be a source of biologically active forms of magnesium and phosphorus. Therefore, it is reasonable and relevant to develop and study the effectiveness and safety of such a drug and its further implementation in practice.

2. Literature review

The basis for the creation of a new drug is animal protein casein and macronutrients magnesium and phosphorus. Casein has a number of physicochemical and biological features that determine its choice as a carrier of functional groups and ions. First of all, casein is a complete protein, i.e. it has a full range of proteinogenic amino acids in the optimal ratio necessary for the mammalian body. Also, the ability of casein to dehydrolyze at low pH values is an important factor in the choice of casein as a substrate for synthesis [1–3].

Magnesium is the fourth most common cation in the body, so it is indispensable for the body and its deficiency causes serious consequences. The biological effects of magnesium are mainly due to the formation of complexes with intracellular ligands and antagonism with calcium by binding to proteins and membrane structures. The above properties determine the participation of magnesium in the synthesis of macromolecules such as nucleic acids and proteins. About 300 enzymes need magnesium as a cofactor for their activity. Binding of magnesium to the active site of the enzyme leads to a change in the spatial configuration of the peptide chain and, as a consequence, its activation. Magnesium improves the absorption and metabolism of vitamins B, C,

E. The participation of magnesium in the antioxidant defence of the body is due to magnesium-dependent synthesis of glutathione [4].

Assimilation of magnesium in the body is carried out through the digestive system. In order to assimilate and further perform their biological functions, it is necessary that magnesium ions be chelated, i.e. combined with organic molecules. Two carboxyl groups of amino acids are able to combine with one ionic magnesium ionic bonds, amino groups of these amino acids - donor-acceptor bonds. Amino acids act as ligands and such a coordination compound is called a chelate (Fig. 1).

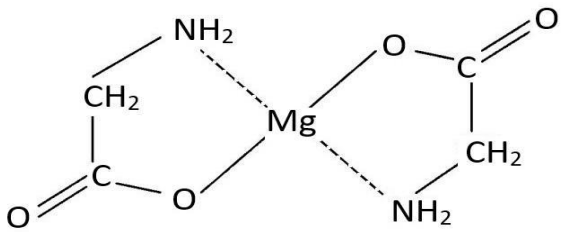

Fig. 1. The structure of the magnesium chelate molecule

Metal ions chelated by amino acids are digested in the small intestine similarly to dipeptides. The intensity of chelation of ions directly correlates with the degree of their assimilation. After assimilation, the chelates are hydrolyzed to release two amino acids, which are then used for the synthesis of peptides, and magnesium ion, which is a cofactor for apoenzymes [2–4].

Preparations containing phosphorus combined with organic radicals are well-known agents. These compounds are used as a source of organic phosphorus to enhance mineral, carbohydrate, fat and protein metabolism. Organic phosphorus preparations are characterized by a high rate of metabolism and low toxicity [5].

It is known that about 30 % of all body proteins are phosphorylated in one or another stoichiometric ratio. Most proteins in the animal body are enzymes. One of the mechanisms of regulation of enzyme activity is their phosphorylation and dephosphorylation, due to which such key vital functions of cells as gene expression, cell cycle and apoptosis are regulated. Phosphorylation of proteins occurs by the formation of an ester bond between the hydroxyl groups of serine, threonine, tyrosine and phosphoric acid residue [5, 6].

The combined composition of magnesium and phosphorus is artificially phosphorylated casein of cow's milk as a ligand that chelates magnesium ions. The drug is a homogeneous powder, which can be easily tableted for oral administration. To enhance and supplement the therapeutic and prophylactic effect, it is possible to add water- or fat-soluble vitamins in crystalline form to the mixture (recommended C and B_{12}).

3. The aim and tasks of the study

The aim of the study is to create a combined drug based on chelation of magnesium with phosphorylated casein for use in animal husbandry and veterinary medicine.

To achieve this goal, the following tasks were set:

1. Formulation of a new drug with high therapeutic and prophylactic, adaptogenic and stimulating effect and could be used as a raw material for further development of more complex aggregates.

2. Development of a protocol for the synthesis of a new drug in the laboratory.

4. Materials and methods

The development and testing of an effective method of phosphorylation was tested on crystalline amino acids. The esterification reaction products of individual amino acids were identified mass spectrometrically on a Waters H-class UPLC liquid high pressure chromatography spectrometer with a Waters TQ-S micro triquadrupole detector. The content of phosphates and magnesium ions in the final product was detected using an atomic emission spectrometer with an inductively coupled plasma Analytik-Jena Plasma Quant PQ 9000 Elite. Sample preparation was performed in a microwave oven for microwave decomposition of samples.

The analytical method using a liquid chromatograph with a three-quadrupole mass detector had the following parameters: mobile phase – acetonitrile/water 5:95 %; analytical column – Waters ACQUITY UPLC BEH C18 1.7μm 2.1×50mm; injection volume of 1 μl; flow rate – 0.4 ml/min; ionization mode – ES+; MRM transition for threonine – 120 → 102 Da, for threonine phosphate – 200 → 102 Da [7, 8].

An atomic emission spectrometer with an inductively coupled plasma operated in the mode of radial monitoring of the power of the source 1800 W. Sample preparation was performed by microwave decomposition in nitric acid. The concentration of magnesium in the finished product was about 10 %, phosphates – 12 %.

To test the ability to hydrolyze was selected digestive enzyme of the pancreas trypsin. The efficiency of hydrolysis of the drug relative to casein was compared, detecting the reaction by electrophoresis in 12 % polyacrylamide gel to confirm and visualize the hydrolysis process. The presence of the process of hydrolytic cleavage of the drug was determined densitometrically by the intensity of the stains of the tracks. The colour intensity of the fractions in the preparation after trypsin treatment is inversely correlated with the number of peptide bonds in the molecules subjected to hydrolytic cleavage.

The course of the analysis. The test samples were dissolved in centrifuge tubes of 1500 μl to a concentration of 50 mg/ml in TBS pH=7.4. Casein was dissolved to a concentration of 25 mg/ml in TBS pH=10.5. Centrifuge tubes with samples for complete dissolution were kept in a water bath for 30 min at 37 °C.

1. After dissolution of the samples, sampling was performed according to the following scheme: from each sample solution was taken 20 μl using a micropipette in a test tube with a capacity of 200 μl. 20 μl of sample buffer was added to each sample and mixed.

2. 50 μl of trypsin solution was added to the test samples, the contents were stirred to prevent the formation of foam by vortex. After adding trypsin solution, the samples were incubated in a water bath for 45 min at 37 °C.

3. After 45 min of incubation from the samples, sampling was performed according to a scheme similar to the previous sampling.

4. Samples after sampling were again placed in a water bath under the same conditions for another 45 minutes.

5. At the end of incubation (a total of 90 min) from the samples again took samples according to the same scheme.

6. Before applying to the polyacryamide gel (12 % SDS-PAAG, [9]) all samples were incubated in a boiling water bath for 3 min. The gel was introduced into 30 µl of each sample in the following order of wells: 1–standard, 2–casein, 3-source raw material for synthesis, 4-source raw material after trypsin treatment for 45 min, 5-drug before trypsin treatment (parallel 1), 6-drug after trypsin treatment 45 min (parallel 1), 7-drug after trypsin treatment 90 min (parallel 1), 8-drug before trypsin treatment (parallel 2), 9-drug after trypsin treatment 45 min (parallel 2), 10-drug after trypsin treatment for 90 min (parallel 2). Proteins (1–10) were separated by PAAG electrophoresis (4 % concentrating PAAG and 12 % separating PAAG) in the presence of 0.1 % LTO according to conventional procedures [9]. Proteins in the gel were stained with 0.2 % Kumasi R-250 and washed with a solution containing 7 % acetic acid and 40 % ethanol.

5. Research results

The first step in the modification of casein was direct phosphorylation. Direct phosphorylation of casein occurs as a nucleophilic substitution reaction, which is widely described in a number of sources. In this system, this reaction mainly occurs by the mechanism of SN_2, because the reaction by the asynchronous mechanism of SN_1 involves a slow first stage of dissociation of the nucleophile. The attacking groups in this reaction are the hydroxyl groups of threonine, serine and tyrosine. The electrophilic center is the phosphorus atom of orthophosphoric acid, from which the hydroxyl group is cleaved [2].

The scheme of this reaction on the example of esterification of threonine with orthophosphoric acid is shown in Fig.2.

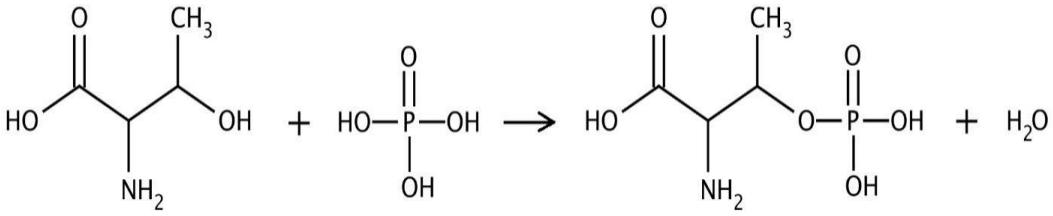

Fig. 2. The reaction of esterification of threonine with orthophosphoric acid

The second stage of the synthesis was the chelation of magnesium with casein. For highly efficient complexation, casein peptide bonds must be tautomerized to the enol form to form highly reactive hydroxyl groups. Due to the strong displacement of the electron cloud from hydrogen to oxygen and the partial attraction of this electron cloud by nitrogen, which is combined with carbon, the proton of the newly formed hydroxyl group becomes labile.

The labile proton is able to be replaced by cations with lower electronegativity than hydrogen, so cations with lower electronegativity than hydrogen are metal ions. Metal ions with free unfilled electron orbitals are also able to interact with nitrogen by a donor-acceptor mechanism. With the simultaneous formation of the above bonds, the peptide chain seems to envelop the metal ion, which is called chelation (from the Greek *Chelè* – claw) [10].

Analysis of the obtained data showed that the efficiency of phosphorylation directly correlates with the number of treatment cycles of the reaction mixture and is optimal for three such treatment cycles. The classical method of direct phosphorylation according to Fisher was chosen as a reference method of phosphorylation. The efficiency of phosphorylation reached 20–30 %, which was manifested by a decrease in the intensity of the threonine signal and an increase in the threonine phosphate signal (Fig. 3).

The separation process lasted 2 h in the mode of a direct current of 20 mA at room temperature. Then the gel was immersed in a solution for staining for 24 h, then in a solution for washing for 48 h.

In Fig. 4 presents an electrophoregram of tracks of native casein and the finished product, which clearly shows the comparability of the molecular weights of the casein fraction, the raw material for synthesis and the synthesized drug "Biophosphomag" (2, 3, 5, 8).

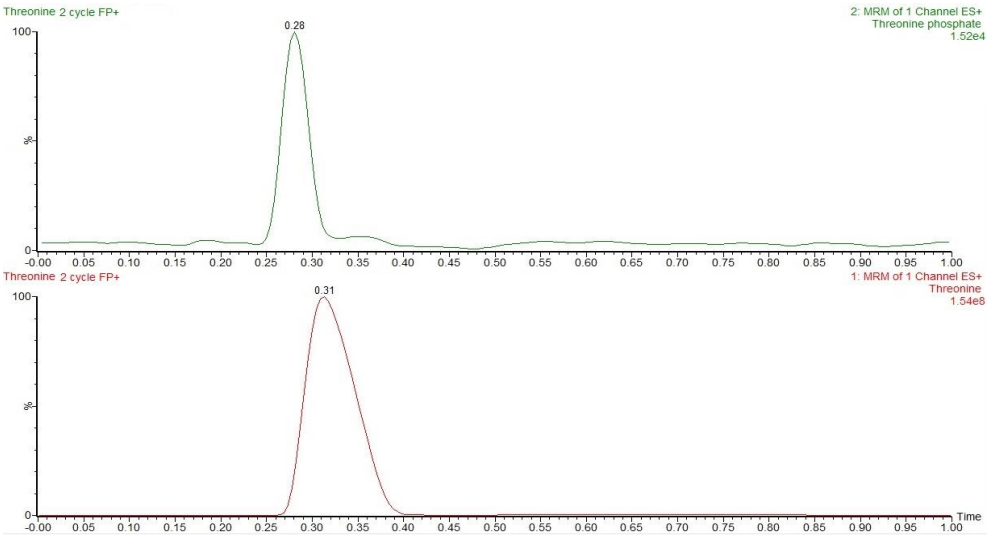

Fig. 3. Chromatograms comparing the efficiency of phosphorylation of raw materials (1-threonine phosphate and 2-threonine)

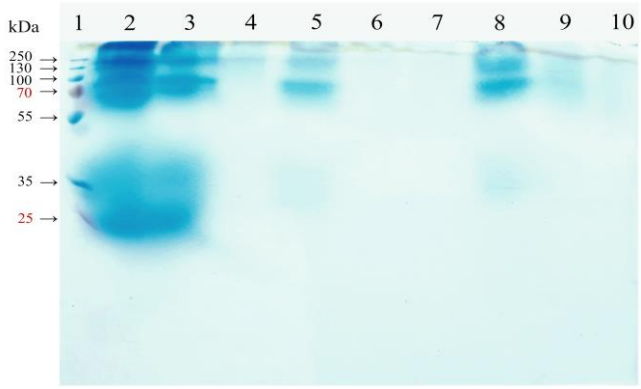

Fig. 4. Electrophoregram of tracks of native casein, raw materials, finished product and their trypsin hydrolysates

The decrease in the colour intensity of tracks 4, 6, 7, 9, 10 clearly indicates that the drug has no worse ability to hydrolyze trypsin than the raw material for synthesis based on casein.

6. Discussion of research results

The results of research on the creation of a new drug have shown its obvious **advantages**, in particular: the synthesis of a new drug can be carried out in the laboratory using known methods and devices; available raw materials of domestic origin are used to create the drug. In turn, the disadvantage of the above research is the use of complex and expensive equipment, access to which has certain **limitations** [7, 8].

Scientific work, both fundamental and applied, to study the properties of substances such as magnesium, phosphorus and casein has been conducted by scientists for a long time and the geography of these studies is quite wide. The obtained scientific achievements are used for practical purposes by creating both drugs and biologically active additives, which are widely represented in the modern market. At the same time, as the analysis of recent studies shows, along with the modernization of the methodological and material base, practical tasks are changing, regarding the wider and more efficient use of the biological properties of these substances [11, 12].

The results of the studies presented in the article show the possibility of achieving a high therapeutic and prophylactic effect due to the synergism of the active components of the drug, which are magnesium, phosphorus and casein. The effectiveness of a new drug is ensured by its composition and method of production, because as a complex effect on metabolism is greater than the sum of the effects of each substance separately. In turn, the developed formulation and method of obtaining potentially provide easy availability for the body of the drug components as it is based on the natural protein casein, which is easily hydrolyzed in the digestive tract [13].

The new drug was created for widespread use in animal husbandry and veterinary medicine, which involves research with both laboratory and productive animals. Instead, research work with animals imposes certain **study limitations**, which are regulated by relevant international and national regulations [14].

The creation of a new drug opens up **prospects for further research**. In particular, the priority is to establish the safety of the drug for living organisms. In this context, a research on cell cultures is relevant. It is important to determine the effectiveness of the drug for various indications, especially for the correction of certain metabolic disorders affected by various pathogens.

7. Conclusions

1. The synthesis of a new drug is carried out in two stages: the first - modification of casein by direct

phosphorylation and the second stage – chelation of magnesium with casein;

2. It was found that the efficiency of phosphorylation directly correlates with the number of treatment cycles of the reaction mixture and is optimal for three treatment cycles.

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Conflict of interests

The authors declare that they have no conflicts of interest.

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