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DEVELOPMENT OF THE PHARMACEUTICAL COMPOSITION MICEPHOSPHONE CONTAINING DIMEPHOSPHONE AND ITS STUDY BY MODELING OSTEOPOROSIS

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The effectiveness of the developed combined drug as capsule and suspension dosage forms based on dimephosphone® (dimethyl 1,1-dimethyl-3-oxobutylphosphonate) and calcium carbonate was demonstrated for treatment and prophylaxis of bone diseases. According to IR spectra, dimephosphone® was chemisorbed to calcium ions on the surface of calcium carbonate to form surface calcium phosphonates. Dimephosphone® adsorption (A) as a function of its concentration (C) in aqueous solutions obeyed formally the Langmuir equation. The remodeling action for bone tissue by the suspension dosage form was verified using a glucocorticoid-induced osteoporosis model in rats.

Key words: dimephosphone, calcium carbonate, bone diseases, glucocorticoid-induced osteoporosis model

Bone diseases such as osteopenia and osteoporosis are the fourth leading cause of non-infectious diseases with the frequency of fractures tending to increase worldwide. Bisphosphonates, selective modulators of estrogenic hormones, calcitonins, and strontium salts with organic acids are used to treat these diseases [1]. The drugs of choice belong to the phosphorus-containing class of bisphosphonates, which regulate metabolism in bone tissue on the tissue, cellular, and molecular (to phosphate ions) levels regardless of the mechanism of action [2, 3]. The influence of phosphorus was confirmed by the dependence of the activity and concentration of phosphate-cleaving enzymes, i.e., the total alkalinity and acid phosphatase in addition to the level of disease progression.

Drawbacks of highly effective bisphosphonates in the pharmacotherapy of bone diseases are related to their significant toxicity and numerous side effects [4]. As a result, the search for new drugs for the treatment and prophylaxis of these diseases is important and critical.

Dimephosphone® (DMP) is a domestic pharmaceutical substance of formula dimethyl 1,1-dimethyl-3-oxobutylphosphonate (LD_{50} 2,000-4,000 mg/kg for a 15% solution dosage form) [5]. DMP has a broad spectrum of pharmacological activity (neurotropic, antacid, vaso-active, nootropic, antioxidant) [6] and favorable pharmacokinetic parameters [7].

The goal of the present work was to develop and study P-containing pharmaceutical compositions based on DMP and calcium carbonate ($CaCO_3$) for the prophylaxis and treatment of osteoporosis.

The scope of the investigation included a study of the interaction of DMP and $CaCO_3$ in aqueous solution and the development of a pharmaceutical composition (possible dosage forms) in animal experiments.

Modeling using the PASS computer program [8] showed the DMP had high pharmacological activity for bone diseases (bone-tissue formation stimulator, $P_a = 0.767$; anti-osteoporosis activity, $P_a = 0.672$; Ca regulator, $P_a = 0.660$; acid phosphatase inhibitor, $P_a = 0.604$; osteoclast antagonist, $P_a = 0.474$).

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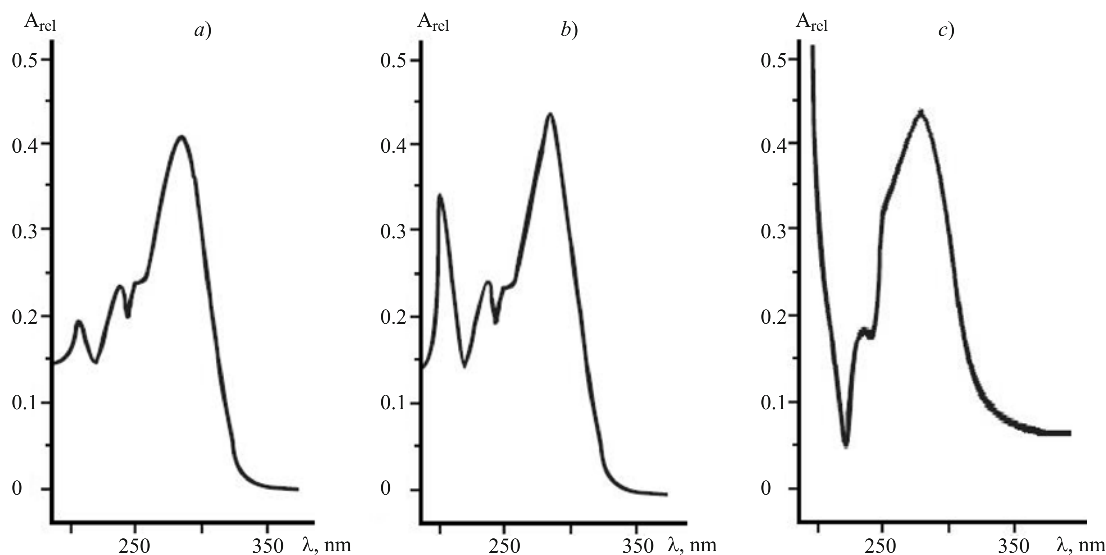


Fig. 1. UV absorption spectra of freshly prepared solutions (0.01 M) of dimephosphone® in a N_2 atmosphere:

Fig. 1	Medium	190 – 210 nm		238 – 240 nm (C=O, P=O)		278 – 286 nm (C=O)	
		λ, nm (± 0.2)	A_{rel} (± 0.001)	λ, nm (± 0.2)	A_{rel} (± 0.001)	λ, nm (± 0.2)	A_{rel} (± 0.001)
a	CH_3CN	208	0.190	238	0.230	285	0.400
b	C_2H_5OH, 95 %	201	0.340	239	0.246	284	0.440
c	H_2O	191	1.910	240	0.180	279	0.430

EXPERIMENTAL PART

Reagents and instruments. Micellate of grade “O” (TU 5743-001-43646913-2006) was supplied by OOO Slavyanskaya Apteka (Russia). Pharmaceutical substance DMP (Dimephosphonum) was produced by OOO Tekhnofos (>99% pure, FS 42-2993-99); $CaCO_3$ (GOST 4530–76); hyaluronic acid sodium salt (powder from bovine vitreous body, 99.8%, 1.63 MDa, Product No. H7630, Sigma); crystalline fructose (TU 911-011-35987677–02); methylparaben [methyl-4-hydroxybenzoate, ≥ 99.0% (GC), Product No. 54750, Sigma-Aldrich]; carbazole [dibenzopyrrole, ≥ 95% (GC), Product No. C5132, Sigma]; and toluidine blue-O (Product No. T3260, Sigma-Aldrich) were used as received. Osteoporosis was modeled using hydrocortisone acetate as a powder for preparing a solution for injection (vials, 500 mg, Hemofarm D. D., Yugoslavia).

Electronic absorption spectra were obtained on a Bio line Specord S-100 instrument (Analytik, Jena, Germany) in the range 190 – 600 nm in a 10-mm quartz cuvette.

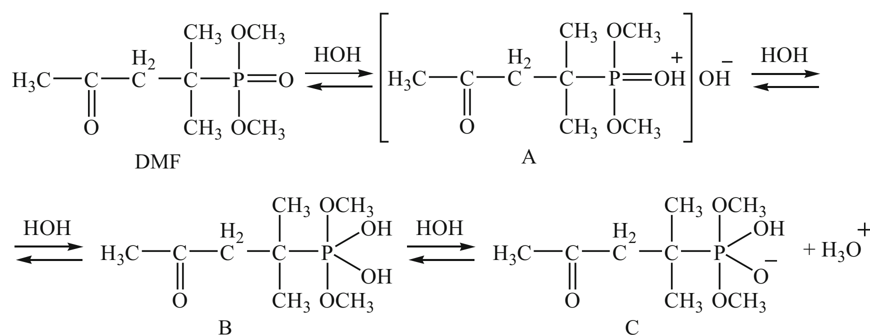
IR spectra were recorded on a Fourier-transform IR Prestige-21 IR spectrophotometer (Shimadzu, Japan) in the range 4,000 – 500 cm^{-1} as mixtures with KBr at sample concentrations 0.1 – 0.3% and as thin layers in mineral oil.

HPLC was carried out using an LC-10Avp HPLC (Shimadzu, Japan) in reversed-phase mode with degassed mobile phase, a thermostatted column, and UV-Vis detection. The column was a Phenomenex® Luna 5u C18 100 A, 250 × 4.6 mm, 5 μm. The sample volume was 20 μL.

Pharmaceutical compositions. The Micellat suspension contained 50% $CaCO_3$ and <1% $MgCO_3$. The Micephosphone composition used in the experiments on rats consisted of (mass%) Micellat (10.0), DMP (10.0), sodium hyaluronate (0.8), fructose (15.0), EtOH (2.0), and methylparaben (0.2). Physicochemical studies were carried out on pharmaceutical compositions of various composition (mass%) with Micellat (1 – 15), DMP (5 – 15), sodium hyaluronate (0.5 – 1.2), fructose (15.0), EtOH (2.0), and methylparaben (0.2) [9]. The capsule form (400 mg in each gelatin shell) included (mg) $CaCO_3$ (50), DMP (100), fructose (15), methylparaben (2), and sodium hyaluronate (the remainder).

Ca determination in Micellat suspension was performed gravimetrically after converting it to the chloride and precipitating it with ammonium oxalate solution. Ca was determined quantitatively in capsules using complex formation (SP XI, No. 1, pp. 186 – 187). Mg content in the suspension was determined in the supernatant liquid (solution A) by EDTA titration at pH 9.5 – 10.0 (ammonium buffer) using chrome dark blue indicator in the presence of NaCl for clearer definition of the indicator color change (SP XI, No. 1, pp. 188 – 189).

Analysis of sodium hyaluronate in the suspension. The suspension (3 g) was treated dropwise with conc. HCl (1 mL) until the precipitate was completely dissolved. The resulting solution was treated with CH_3CN (15 mL). The precipitated hyaluronic acid was centrifuged off. The filtrate (solution B) was used for the analyses of DMP, fructose, and methylparaben. Sodium hyaluronate in capsules was ana-



Scheme 1. Reaction of DMP with H_2O.

lyzed in a similar manner by preliminary placement of the contents of four capsules (1.6 g) into a flask.

The solid was dissolved in H_2O and transferred quantitatively to a 100-mL volumetric flask. Hyaluronic acid was analyzed using a modified Dische method [10, 11]. The solution (2 mL) was treated with sodium tetraborate solution (5 mL, 95%) in H_2SO_4, heated on a boiling-water bath for 15 min, cooled, treated with carbazole in EtOH (0.2 mL, 7.5 mM), and heated on a boiling-water bath for 15 min. UV-Vis analysis was carried out at wavelength 530 ± 2 nm using a solution prepared in a blank experiment as the reference.

Quantitative determination of DMP for total P content after ashing an aliquot of solution B in an oxidizing mixture of HNO_3 and $HClO_4$ (1:1 ratio) was performed photocolorimetrically at wavelength 425 ± 2 nm according to the published procedure [12] that is based on formation of the yellow complex of P with a solution of molybdovanadate heteropoly acid in HNO_3 solution. The initial purity of DMP was monitored by HPLC and UV spectrophotometry methods.

Fructose analysis was carried out during quantitative determination of hyaluronic acid using an aliquot of the aqueous CH_3CN acidic solution B according to the usual method [3]. Photometry of the resorcinol complexes was made at wavelength 480 ± 2 nm.

Methylparaben content in an aliquot of solution B after precipitation of Ca ions by ammonium oxalate was determined using HPLC. The conditions have been described [14]. Spectrophotometric detection was made at wavelengths 205 ± 2 and 254 ± 2 nm.

Biological studies of the effectiveness of Micephosphone suspension using a glucocorticoid-induced osteoporosis model were carried out in winter (November-December 2010) according to the regulations [15 – 17] for animal experiments in the Central Research Laboratory of Nizhnii Novgorod State Medical Academy. The activity of phosphatases in the biological fluids was determined from the Bessey–Lowry–Brock end-point method based on enzymatic hydrolysis of *p*-nitrophenylphosphate by measuring the optical density of the solutions at 405 ± 2 and 420 ± 2 nm [18, 19].

RESULTS AND DISCUSSION

Ionization of DMP is extremely important for bone mineralization because the metabolism of P-containing compounds involves their anionic forms [20]. The solution pH change from 2.7 to 4.0 in a 15% aqueous solution of DMP according to Scheme 1 provides evidence that DMP, which is readily soluble in H_2O, can form ionized species of type A, B, and C owing to hydrolysis.

The reaction of DMP with H_2O was confirmed by UV spectra of pure DMP in anhydrous CH_3CN, EtOH (95%), and H_2O (Fig. 1). Bands characteristic of a charge-transfer transition involving the solvent (190 – 210 nm) were strong for H_2O and very weak for anhydrous CH_3CN. Increasing the solvent polarity produced differences in the $n > \pi^*$ and $\pi > \pi^*$-transitions of P=O and C=O groups. The optical density A_{rel} at 238 – 240 nm decreased; at 278 – 286 nm, increased. A hypsochromic shift and the appearance of a shoulder (λ = 253 nm) in H_2O were noted.

The IR spectrum of DMP (Table 1) in the presence of H_2O (1%) showed in the OH region a single broad and very strong band at 3443 cm^{-1} (intensity increased by six times) instead of two poorly resolved bands. The position of the carbonyl band shifted from 1719 to 1713 cm^{-1} ($\Delta\nu$ = 6 cm^{-1}). The P=O stretching band underwent even greater changes. It changed shape and transformed from a narrow band at 1244 cm^{-1} into a broad double band at 1224 cm^{-1} ($\Delta\nu$ = 20 cm^{-1}) and 1207 cm^{-1}. Two absorption bands (P–OCH_3) remained unchanged at 1033 and 1058 cm^{-1}. The main difference in the spectra was the appearance of a new rather broad band at 1647 cm^{-1} (δOH_2) that was characteristic of inclusion of water in DMP. Because the phosphoryl group (P=O) was the most active functional group in DMP, the formed structure A was most probable. Further transformation of A into B and C (Scheme 1) was possible with an excess of water, e.g., in dilute solutions. These could then react with calcium ions. It was shown earlier [21] that DMP could form complexes with $CaCl_2$ in which the phosphoryl group reacted with H_2O and Ca^{2+} in the hydration sphere of the latter to form H-bonded complexes with the DMP phosphoryl group. IR spectra of a mixture of DMP and

$CaCO_3$ in the presence of 1% H_2O were similar to spectra of mixtures of $CaCl_2$ and DMP and those of starting DMP in the presence of 1% H_2O (Table 1).

The results indicated that DMP reacted with Ca^{2+} on the surface of $CaCO_3$ particles probably with the formation of salt complexes that were capable of transforming partially into type B structures. This was consistent with the similarity of the IR spectra of hydrolyzed DMP (DMP + 1% H_2O) and a mixture of DMP with $CaCO_3$ and H_2O (DMP + 1% H_2O + $CaCO_3$) (Table 1).

Figure 2 shows a typical adsorption isotherm for DMP and its reaction products with H_2O on the surface of $CaCO_3$.

Adsorption (A, mmol/g) of P-containing products was estimated using the equation:

$$A = \frac{(c_0 - c)\cdot V}{m_{ads}}, \quad (1)$$

where c_0 and c are the concentrations of DMP before and after adsorption, respectively (mM); V, the volume of solution from which the adsorption occurred (L); and m_{ads}, the mass of $CaCO_3$ adsorbent (g).

The adsorption isotherms were analyzed in linear coordinates c/A vs. c and showed that the process obeyed formally the Langmuir equation:

$$A = \frac{A_{max} \cdot b_{ef} \cdot c}{(1 + b_{ef} \cdot c)}, \quad (2)$$

where b_{ef} is an empirical coefficient characterizing the adsorption efficiency and A_{max}, the limiting adsorption (mmol/g).

The calculated A_{max}, equal to 29.4 ± 1.8 mmol/g, indicated that the P-containing compounds were strongly bonded to the $CaCO_3$ surface.

Based on the studies of the reaction of DMP and $CaCO_3$, we developed pharmaceutical compositions as suspension and capsule dosage forms.

Micellat suspension was proposed as the source of $CaCO_3$ in the suspension pharmaceutical composition. It also contained Mg ions. Sodium hyaluronate was chosen as a stabilizer because it was highly recommended for treating cartilage diseases and could act as a vector for delivering Ca^{2+}. An EtOH solution of methylparaben was added to the suspension in order to prevent microbial contamination of the polysaccharide. Fructose was added to ameliorate the unpleasant taste of DMP.

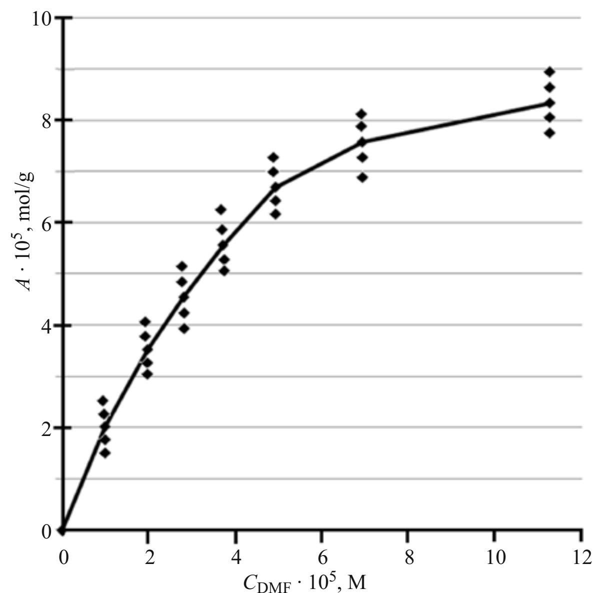


Fig. 2. Sorption isotherm of dimephosphone® (DMP) from aqueous solutions $A = f(C)$ on $CaCO_3$ surface.

Complications were encountered in establishing the identity and quantitative content of the components in the DMP pharmaceutical composition.

The commercial substance, which is a colorless or yellowish transparent liquid with a distinctive aroma, required strict acceptance monitoring because it contained impurities such as water associated with DMP, the monomethyl ester of 1,1-dimethyl-3-oxobutylphosphonic acid, and a certain amount of high-boiling dimers (or oligomers) of DMP. HPLC analysis of distillation fractions (Fig. 3) provided evidence of the existence of several products in the drug substance. Only the third fraction contained pure DMP. This was confirmed by comparing the IR spectra of the sample and a standard (C=O 1718 cm^{-1} and P=O 1244 cm^{-1}).

The Experimental section gives a detailed description of the procedure for determining the other components.

Specific remodeling action of Micephosphone suspension on bone tissue. Observations were carried out for seven weeks on three groups of animals, one of which was untreated (control group I), with 10 Wistar rats in each (males,

TABLE 1. IR Spectra of Dimephosphone® (DMP) and Its Mixtures with Water and Calcium Salts

Sample	Wavenumber, ν, cm^{-1}		
	4000 – 2500 (OH, H_2O)	1750 (C=O) – 1600 (δOH_2)	1250 – 1200 (P=O)
DMP	3522 (s, br), 3470 (s, br)	1719 (vs, n)	1244 (vs, n)
DMP + 1% H_2O	3443 (vs, br)	1713 (vs, n), 1647 (vs, m)	1224 (vs, n), 1207 (vs, n)
DMP + 1% H_2O + $CaCl_2$	3460 (vs, br)	1705 (vs, n), 1635 (vs, n)	1223 (vs, n)
DMP + 1% H_2O + $CaCO_3$	3474 (vs, br)	1719 (vs, n), 1647 (vs, m)	1238 (vs, m), 1205 (vs, m)

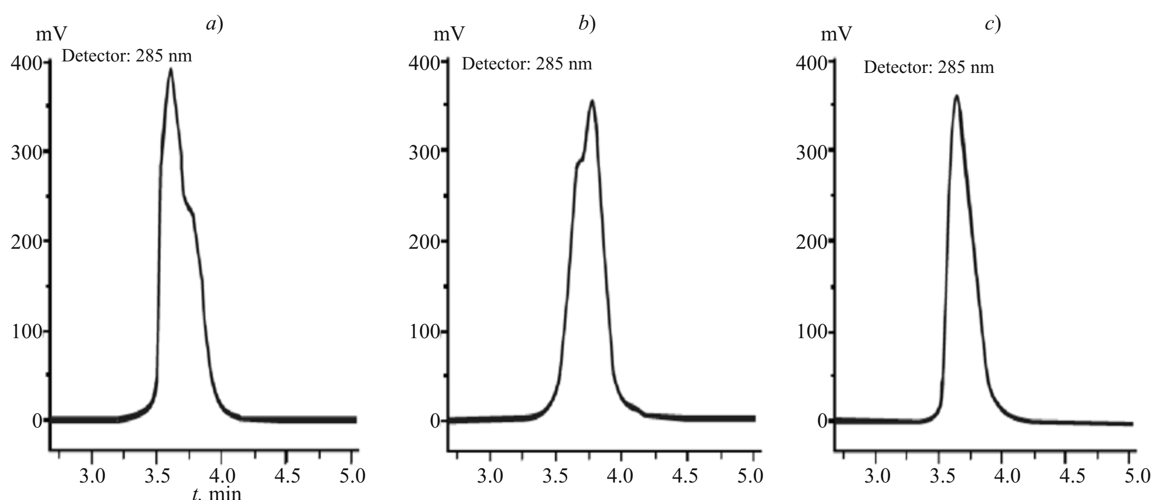


Fig. 3. HPLC plots of dimephosphone® substance before distillation (a) and first (b) and second (c) distillation fractions. Detection at $\lambda = 285$ nm. Eluent, CH_3CN.

3 mo., 160 ± 2.1 g) obtained from the Stolbovaya nursery, BMT, RAMS. Groups II and III were administered i.p. daily beforehand for three weeks hydrocortisone acetate at a dose of 40 mg/kg per day. Rats in control group I received i.p. physiological saline in the same volumes.

Control groups I and II during the subsequent four weeks received starch suspension. Animals in test group III were treated intragastrically with the pharmaceutical composition as Micephosphone suspension in an equivalent therapeutic dose (3.5 mL/kg/d).

The treatment effectiveness was evaluated using the principal disease markers, i.e., the activity of total alkaline phosphatase, acid phosphatase, and tartrate-resistant acid phosphatase, which catalyzes nonspecifically hydrolysis of phosphonate esters to form inorganic phosphates. Furthermore, the Ca and P contents in blood serum and urine were monitored. The results were processed statistically using the Statistica 5.5 program. Differences were considered statistically significant for $p < 0.05$.

Treatment of rat test group III with Micephosphone suspension corrected the changes in their bone structure that were induced by prolonged administration of hydrocortisone acetate, normalized the animals' mass, and improved the general functional condition. The activities of tartrate-resis-

TABLE 2. Dynamics of Rat Blood Serum Biochemical Parameters with Use of Micephosphone Suspension ($M \pm m$)

Parameter	Group, treatment	Initial condition (after osteoporosis modeling)	Condition after treatment
Alkaline phosphatase, U/L	I control (untreated), starch suspension	42.06 ± 3.43	45.39 ± 5.39
	II control, starch suspension	46.40 ± 5.66	56.34 ± 7.60
	III test, Micephosphone suspension	41.82 ± 9.52	50.29 ± 7.75
Acid phosphatase, U/L	I control (untreated), starch suspension	10.91 ± 0.71	10.17 ± 0.21
	II control, starch suspension	14.48 ± 0.95*	15.27 ± 1.15*
	III test, Micephosphone suspension	14.95 ± 0.92*	12.76 ± 0.61**
Tartrate-resistant acid phosphatase, U/L	I control (untreated), starch suspension	4.34 ± 1.15	4.93 ± 1.67
	II control, starch suspension	7.49 ± 2.86*	7.47 ± 0.39*
	III test, Micephosphone suspension	7.33 ± 1.66*	5.45 ± 0.29**
Ca, mM	I control (untreated), starch suspension	1.59 ± 0.14	1.93 ± 0.60
	II control, starch suspension	2.01 ± 0.29	3.34 ± 0.60*
	III test, Micephosphone suspension	1.88 ± 0.10	2.73 ± 0.36*
P, mM	I control (untreated), starch suspension	2.44 ± 0.15	1.93 ± 0.19
	II control, starch suspension	3.88 ± 0.59*	2.59 ± 0.29*
	III test, Micephosphone suspension	3.52 ± 0.21*	2.16 ± 0.06**

* Statistically significant differences with I control (untreated).
** Statistically significant differences with II control, $p < 0.05$.

TABLE 3. Dynamics of Rat Urine Biochemical Parameters with Use of Micephosphone Suspension ($M \pm m$)

Parameter	Series	Condition after treatment
Ca, mM	I control (untreated), starch suspension	2.24 ± 0.67
	II control, starch suspension	5.13 ± 0.65*
	III test, Micephosphone suspension	3.30 ± 0.12**
P, mM	I control (untreated), starch suspension	4.49 ± 0.28
	II control, starch suspension	3.79 ± 0.17*
	III test, Micephosphone suspension	4.22 ± 0.18**

* statistically significant differences with I control (untreated),
** statistically significant differences with II control, $p < 0.05$.

tant acid phosphatase and acid phosphatase decreased and the amounts of P and Ca declined in blood serum compared with the group that received placebo (Table 2).

Use of Micephosphone for therapy normalized the ion content in urine. The P content did not differ statistically significantly from the initial value whereas the Ca content tended to decrease and was statistically significantly less than in the control (Table 3).

The remodeling effect found for DMP was probably similar to the action of bisphosphonates that mimic the structure of endogenous pyrophosphates in bone tissue despite the different mechanism of osteoclast inhibition [22].

Thus, reaction of DMP and $CaCO_3$ in aqueous solutions formed surface complexes of DMP with Ca^{2+}. Based on the studies, compositions for Micephosphone capsules and suspensions were proposed. The use of DMP pharmaceutical substance as the active ingredient in drugs for the treatment and prophylaxis of bone diseases, in particular osteoporosis, was recommended.

REFERENCES

1. P. Hoversten (ed.), *Health Care Guideline: Diagnosis and Treatment of Osteoporosis*, 7th Ed., Institute for Clinical Systems Improvement, Inc. (2011), pp. 24 – 33.
2. R. P. Heaney, *Mayo Clin. Proc.*, **79**(1), 91 – 97 (2004).
3. G. A. Rodan and H. A. Fleisch, *J. Clin. Invest.*, **97**(12), 2692 – 2696 (1996).
4. I. J. Diel, R. Bergner, and K. A. Grotz, *J. Supportive Oncol.*, **5**, 475 – 482 (2007).
5. I. A. Studentsova, R. S. Garaev, I. S. Mokrinskaya, et al., in: *Abstracts of Papers of the Scientific Conference Ecological Problems of Pharmacology and Toxicology* [in Russian], Kazan’ (1990), pp. 101 – 102.
6. V. I. Danilov (ed.), *Dimephosphone, Use in Neurosurgery and Nephrology (Scientific Review of Clinical Trials)* [in Russian], Kazan’ (2008), pp. 4 – 5.
7. A. O. Vizel’, I. A. Studentsova, V. Sh. Ismagilov, and L. I. Shchukina, in: *Abstracts of Papers of the IVth Convention of Pharmacists of the Lithuanian SSR* [in Russian], Kaunas (1987), pp. 132 – 133.
8. Prediction of Activity Spectra for Substances; http: // www.pharmaexpert.ru / PASSOnline /
9. RF Pat. No. 2,442,592.
10. Z. Dische, *J. Biol. Chem.*, **167**(1), 189 – 198 (1946).
11. T. Bitter and H. M. Muir, *Anal. Biochem.*, **4**(4), 330 – 334 (1962).
12. G. M. Pierzynski (ed.), *Methods of Phosphorus Analysis for Soils, Sediments, Residuals, and Waters*, North Carolina State University, Southern Cooperative Series Bulletin No. 396 (2004), pp. 45 – 49.
13. D. N. Olennikov and L. M. Tankhaeva, *Khim. Rastit. Syr’ya*, **2**, 69 – 74 (2008).
14. R. M. Kamble, S. G. Singh, and S. Singh, *E-J. Chem.*, **8**(1), 340 – 346 (2011).
15. R. U. Khabriev (ed.), *Handbook of Experimental (Preclinical) Study of New Drugs* [in Russian], Meditsina, Moscow (2005), pp. 41 – 47, 763 – 774.
16. Order of the RF Ministry of Health and Social Development of Aug. 23, 2010, No. 708n “On Approval of Rules for Laboratory Practice” [in Russian].
17. RF Federal Law of Apr. 12, 2010, No. 61-FZ “On the Handling of Drugs” [in Russian].
18. O. A. Bessey, O. H. Lowry, and M. J. Brock, *J. Biol. Chem.*, **164**, 321 – 329 (1946).
19. C. Wenger, *Alkaline Phosphatase*, A. Kaplan, et al., (eds.), Clin. Chem., The C. V. Mosby Co., St. Louis, Toronto, Princeton (1984), pp. 1094 – 1098.
20. H. Fleisch, *Endocr. Rev.*, **19**(1), 80 – 100 (1998).
21. R. R. Shagidullin, I. I. Vandyukova, A. O. Vizel?, et al., *Khim.-farm. Zh.*, **38**(10), 45 – 47 (2004).
22. R. Graham and G. Russell, *Pediatrics*, **119**, S150-S162 (2007).