International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 11, Issue 5, 2019

Original Article

THE IMPROVEMENT OF BETULIN-3, 28-DIPHOSPHATE WATER-SOLUBILITY BY COMPLEXATION WITH AMINES–MEGLUMINE AND XYMEDON

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Received: 20 Feb 2017 Revised and Accepted: 06 Apr 2019

ABSTRACT

Objective: To study betulin-3,28-diphosphate (BDP) water solubility improved by forming salt complexes with hydrophilic amino alcohols: meglumine as acidosis corrector and xymedon as the water-soluble antioxidant.

Methods: We used ^{13}C-, ^{31}P-NMR, UV-spectroscopy and potentiometric titration to study the BDP-amine salt complexes formation and their solubility using HPLC-analysis.

Results: The participation of xymedon in the proton transfer reaction with BDP in aqueous solutions was confirmed by the bathochromic shift of the carbonyl band from 299.1 nm to 304.2 nm, and by a hyperchromic effect (molar extinction ε from 8508 to 10 441 $l \cdot mol^{-1} \cdot cm^{-1}$) in UV-spectra. BDP complexation with meglumine was estimated by UV-spectral molar ratio method at 256 nm. Molar ratio of BDP-amine complexes (1:4) was proved by ^{31}P-NMR. The chemical shift of phosphorus at C-3 atom of BDP (δ =-0.58 ppm) changed to+3.39 ppm, and at C-28 atom (δ =+0.28 ppm)–to+4.60 ppm. BDP solubility increased 100-600 fold according to HPLC-analysis.

Conclusion: BDP interaction with amine in an aqueous solution was shown to proceed via a proton transfer due to relatively weak forces such as London forces, hydrogen bonding, electrostatic and hydrophobic interactions. In general, the formation of BDP salt complexes with amines in solution determines BDP water solubility. Water-soluble BDP enables to develop hydrophilic dosage forms.

Keywords: Poorly water-soluble, Salt complex engineering, Betulin-3,28-diphosphate, Meglumine, Xymedon

DOI: http://dx.doi.org/10.22159/ijpps.2019v11i5.32707

INTRODUCTION

Lupane triterpenoids such as betulin (lup-20(29)-ene-3,28-diol) and its derivatives extracted from renewable natural resources [1-3], e. g. from birch bark, exhibit various types of pharmacological activity. The antioxidant, anti-inflammatory, hepatoprotective, antimutagenic, and other activities of the compounds were demonstrated by in vitro and in vivo experiments [4-9]. Recent studies have shown that betulin can be a useful structural scaffold in drugs to prevent and treat osteoclast-mediated bone diseases [8].

The major problem to develop a dosage form, for most types of triterpenes, including betulin derivatives, is their low water-solubility [10]. The methods to increase the triterpenoids solubility are the formation of multiarm-polyethylene glycol–betulinic acid prodrugs [11], betulin diacetate complexes with polysaccharides [12], esters of betulin and betulinic acid with amino acids [13], salt of betulinic acid derivatives with organic cations (didecyldimethylammonium, choline and benzalkonium), alkali metals, etc. [14, 15]. The suitable techniques to modify and graft additional polar groups are the formation of sulfates, phosphonate and phosphate groups [16-22]. Moreover, the phospholipid derivatives of betulin with improved solubility were synthesized [17]. The betulin sulphates have been demonstrated to be selective potent inhibitors of C1q interaction of with immunoglobulins and the classical pathway of complement activation [16]. The most favorable modification is the grafting of phosphate derivatives by 3- or 28-OH groups of betulin resulting in solubility improvement in comparison with the extremely low solubility of natural betulin (about $1.0 \cdot 10^{-4}$-$1.9 \cdot 10^{-3}$ $g \cdot l^{-1}$ depending on conditions) [17-22].

Fig. 1a shows betulin-3,28-diphosphate (BDP) obtained by betulin phosphorylation with phosphorus oxychloride, BDP solubility in water is 50-1000 times higher than that of betulin, and its sodium salt (Na-BDP) solubility in water is up to 10 $g \cdot l^{-1}$ [18].

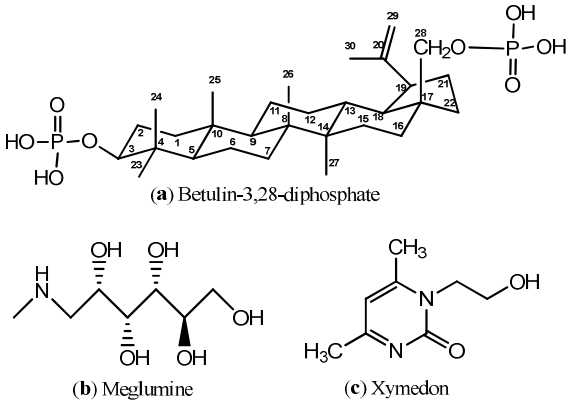


Fig. 1: Formulas of salt complexes components

In addition to improved solubility, betulin phosphates and phosphonates showed high activity as potential drugs [18-23]. *In vitro* and *in vivo* experiments demonstrated antimicrobial [19], antifungal [20], anti-inflammatory, antiviral [21] activities and an antitumor effect in the treatment of ductal carcinoma, glioblastoma, melanoma [22], Ehrlich ascites carcinoma [23] and other diseases.

However, the problem of using both betulin-3, 28-diphosphate and its sodium salts as an active pharmaceutical ingredient is the solubility reduction of these substances in storage. The aging of samples leads to the formation of a different structural modification characterized by more close packing of solid layers. The crystallization water of the hydrate Na-BDP · $8H_2O$ was removed

from molecules with time [18]. In this regard, it is very important to find methods to increase and stabilize BDP solubility. The findings may be useful to design new dosage forms including injectable preparations and hydrophilic gels.

Meglumine ((2R,3R,4R,5S)-6-(methylamino)hexan-1,2,3,4,5-pentol, Megl) traditionally used both for acidosis correction, pH-regulation and making plasma isoosmotic and to improve permeability of poorly soluble lipophilic pharmaceutical substances [24, 25] was chosen as one of these amino alcohols (fig. 1b).

A readily soluble drug having an amino alcohol group, e. g., a pharmaceutical active ingredient–xymedon (fig. 1c), also can be used as a component of a salt complex with BDP. In this case, the BDP molecule exhibiting weak acid properties can act as a component of a drug delivery vector for the group of amines with weak basic properties. Xymedon (1-(β-hydroxyethyl)-4,6-dimethyl-1,2-dihydro-2-hydroxy-pyrimidine, Xym) is a heterocyclic amino alcohol having high antioxidant, reparative, anti-inflammatory and other activities [26]. The abnormal water solubility of xymedon (about 400 $g \cdot l^{-1}$) and high pharmacological properties suggest that a complex of xymedon with betulin-3,28-diphosphate can be a novel antioxidant drug with an optimal BDP dose.

In this paper, we studied the possibility of improving BDP water solubility by forming BDP water-soluble salt complexes with hydrophilic amino alcohols in solution.

MATERIALS AND METHODS

Materials

Betulin was isolated from birch bark (*Betula Pendula*) using the methods [27]. The materials were meglumine (Merck, TD 13015143333), xymedon (Sigma Aldrich, Moscow, Russia), lithium perchlorate (Sigma Aldrich, Moscow, Russia), phytic acid solution 50 % (w/w) in H_2O (Sigma Aldrich, Moscow, Russia), purified water (resistivity ≥18 MΩ·cm, Millipore, Merck, Darmstadt, Germany).

FTIR analysis

FTIR spectra in 400–4000 cm^{-1} range were measured by an IR Prestige-21 FTIR spectrometer (Shimadzu, Kyoto, Japan) equipped with a KBr beam splitter. To perform the measurements, a pellet from a well-dried KBr was prepared according to standard cold pressing. Resolution was 0.5 cm^{-1}. The number of scans was 45.

UV analysis

UV-spectra were recorded by UV-1800 (Shimadzu, Kyoto, Japan).

RP-HPLC analysis

RP-HPLC-analysis was carried out on LC-20Avp (Shimadzu, Kyoto, Japan) with UV-detection, the column is Discovery C18 (25 cm x 4.6 mm, 5 µm, Supelco), retention time τ equals to 5.19 min.

NMR analysis

^{13}C-, ^{1}H-, ^{31}P-NMR spectra were recorded at 101, 400 and 202,46 MHz, respectively, on a JNM-ECX400 NMR-spectrometer (Jeol Ltd., Tokyo, Japan), DMSO-d_6 and D_2O being used as solvents.

Solubility determination

Solubility was measured by shake-flask method [28]. An excess amount of BDP was added to the buffer solution (pH 6.8) by magnetic stirring during 24 h at 20 °C. The added BDP amount was enough to make a saturated solution in equilibrium with solid phase. After phase separation of saturated solution by filtration a liquid phase was analysed by RP-HPLC in isocratic modes (210 nm, 40 °C, mobile phase A30%-B70% v/v, A—acetonitrile, grade 0, B—buffer solution of KH_2PO_4, pH = 6.36; sample volume is equal to 20 µL, flow 1.0 $ml \cdot min^{-1}$. The retention time is 5.19 min.).

Synthesis of betulin-3, 28-diphosphate and its sodium salt

Betulin-3, 28-diphosphate (BDP, 3β, 28-diphosphate-lup-20(29)-ene) and its sodium salt were synthesized according to the procedure [18]. ^{13}C-^{31}P and ^{1}H-NMR spectra and physicochemical constants were in accordance with literature data [18]. BDP and its sodium salt assay were performed by reversed phase HPLC analysis: 210 nm, 40 °C, mobile phase A30%-B70% v/v (A—acetonitrile (grade 0), B—buffer solution of KH_2PO_4, pH = 6.36), flow 1.0 $ml \cdot min^{-1}$. The retention time of BDP and its sodium salt was 5.19 min and 4.51 min, correspondingly.

Biological activity *in vitro*

Biological activity *in vitro* was studied using blood stabilized with sodium citrate (1:9).

Catalase activity (EC 1.11.1.6) was determined by spectrophotometry based on hydrogen peroxide decomposition by the catalase [29]. Glutathione reductase activity (EC 1.8.1.7) was studied spectrophotometrically based on oxidized glutathione reduction [30]. The activity of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was determined in hemolysate of erythrocytes using spectrophotometry based on glucose-6-phosphate oxidation to the phosphoglucolactone with the formation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) [31].

The study as presented was approved by the Local Ethics Committee of Privolzhsky Research Medical University, Russian Federation (Protocol No.2-20.02.16 dated 20 February 2016).

RESULTS

Acid-base interactions of betulin-3, 28-diphosphate with aqueous solutions of amines (xymedon and meglumine)

The pH decreased from the initial values pH $^{0}_{Megl}$ and pH $^{0}_{Xym}$ to pH 3.75 in the potentiometric titration of $5.9 \cdot 10^{-5}$M xymedon (Xym) and meglumine (Megl) aqueous solutions by $2.9 \cdot 10^{-3}$M BDP ethanol solution (fig. 2).

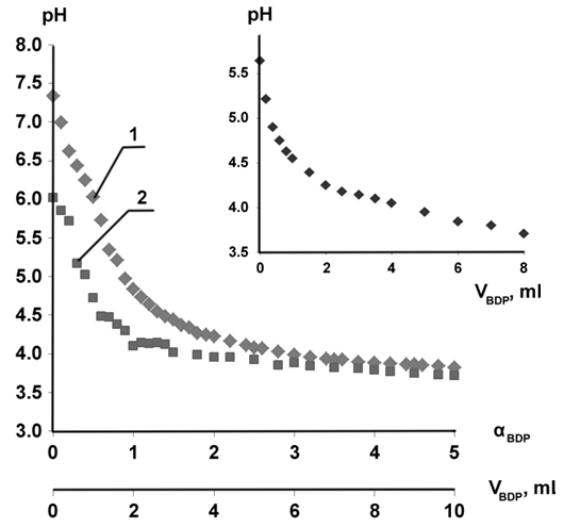


Fig. 2: The pH dependence on molar fraction α, pH = f (α_{BDP}); α = n_{BDP}/n_{amine}. The titration of $5.9 \cdot 10^{-5}$ M amine aqueous solution by $2.9 \cdot 10^{-3}$ M BDP ethanol solution: 1–meglumine; 2–xymedon. The inset shows pH = f(V_{BDP}) after adding $2.9 \cdot 10^{-3}$ M BDP ethanol solution to water in a blank experiment. The initial amine solution volume was 100 ml

The pH=f(α_{BDP}) curves, where molar fraction $\alpha = n_{BDP}/n_{amine}$, leveled off at the molar ratio BDP: amine equal to 4:1 in pH 3.75, which is close to pH of BDP aqueous solution at the same concentration without amine (fig. 2, insert). The results are typical for a weak base titrated by a weak acid, although xymedon is a weak base and meglumine is medium-strength base [24, 25].

According to the dependence of microspecies distributions (%) on pH (fig. 3) calculated using Chemicalize program, the main BDP ionic form at pH 3.75 corresponds to two-charged ion and minor BDP ionic form is a single-charged ion.

The interaction between BDP and amines can be characterized as one-stage proton transfer reactions from each BDP phosphate group.

The study of proton transfer reaction of BDP with xymedon

The acid-base interaction of BDP with amines was studied by UV- and ^{31}P-NMR spectra. BDP phosphate groups have absorption in the range of 240-270 nm [32]. The carbonyl chromophore band (n-π* and π-π* transitions) of xymedon was characterized by intense absorption in the region of 299 nm, while the amino alcohol meglumine does not appear in this UV spectrum region. Therefore, it is possible to study BDP interaction with xymedon using a 299-nm band, while the interaction with meglumine can be controlled by phosphate group absorption only.

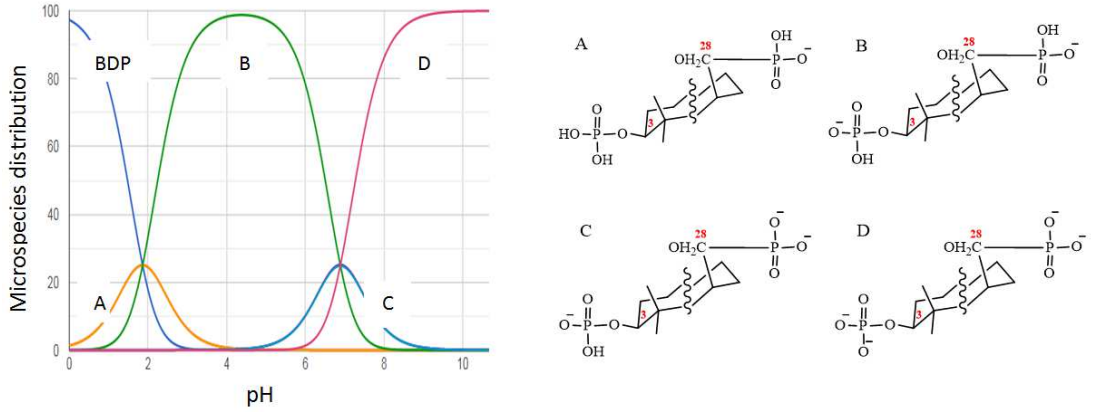


Fig. 3: The dependence of BDP microspecies distributions (%) on pH calculated using Chemicalize program

The fully protonated xymedon form was characterized using xymedon hydrochloride salt as a control sample synthesized from ethylurea and acetylacetone in the presence of hydrochloric acid [20]. UV-spectrum of xymedon hydrochloride shows the absorption band with wavelength λ_{max} = 308.8 nm in contrast to λ_{max} = 299.0 nm for xymedon.

We studied the interaction of BDP with xymedon in aqueous solution in the conditions corresponded to potentiometric titration when xymedon concentration was the same ($5.9 \cdot 10^{-5}$ M), and the ionic strength in the system was provided by $5.9 \cdot 10^{-4}$ M lithium perchlorate.

The participation of xymedon in the proton transfer reaction with BDP in aqueous solutions was confirmed by bathochromic shift of the carbonyl chromophore band from 299.1 nm to 304.2 nm, and a hyperchromic effect manifested in an increased molar absorption coefficient ε from 8508 to 10 441 $l \cdot mol^{-1} \cdot cm^{-1}$ (fig. 4a, table 1).

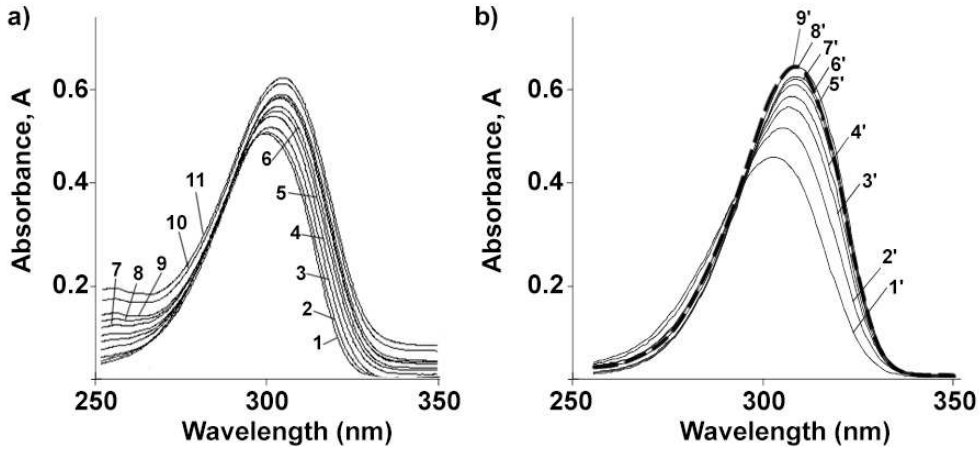


Fig. 4: UV absorption spectra of $5.9 \cdot 10^{-5}$M aqueous solution of xymedon and its protonated form obtained: a) in mixture with BDP; C_{LiClO4} = $5.9 \cdot 10^{-4}$ M; b) in acetate-phosphate-borate buffer solution and in phytic acid (thick dashed line)

Spectra of xymedon and BDP mixtures were compared with those of xymedon salts obtained in aqueous acid solutions. The conditional molar extinction coefficient ε of xymedon hydrochloride (standard sample) was equal to 10 788 $l \cdot mol^{-1} \cdot cm^{-1}$, and the absorption band at λ_{max} equal to 309 nm practically coincided with the spectral characteristics of xymedon at the same concentration in 0.1 M hydrochloric acid solution.

We compared "xymedon-BDP" with "xymedon-phytic acid" aqueous systems to estimate the role of phosphate groups in the interaction. Acid-base properties of organic phosphates–phosphoric acid metabolic derivatives, such as various phosphorylated inositols–$InsP_x$ (x = 1-6) and phytic acid $InsP_6$, glucose phosphates, glycerol phosphates, are similar to BDP. The ionization of phosphate groups of inositol and proton transfer reaction proceeds like the processes in aqueous phosphoric acid solution. The pK_a values of the separation of the first and second protons of these compounds, as well as for phosphoric acid, are 1.5±0.5 and 6.7±0.5, respectively [33]. Moreover, phytic acid forms different kinds of complexes with amines due to not only proton transfer, but also non-specific covalent binding [34].

Table 1: The UV spectral data of 5.9·10^{-5}M xymedon solutions in various aqueous media (betulin-3,28-diphosphate BDP or buffer solution)

№	Conditions		UV spectral data		
	$\alpha = n_{BDP}/n_{Xym}$	pH	λ, nm	A	ε′, L·mol^{-1}·cm^{-1}
1	0	5.76	299.1	0.502	8508
2	0.5	4.75	299.8	0.505	8559
3	1.0	4.10	300.7	0.516	8746
4	1.5	4.02	301.6	0.539	9136
5	2.0	3.96	302.5	0.549	9305
6	2.5	3.92	302.9	0.558	9458
7	3.0	3.88	303.5	0.575	9746
8	3.5	3.82	303.6	0.578	9797
9	4.0	3.79	303.9	0.583	9881
10	4.5	3.75	304.1	0.605	10254
11	5.0	3.72	304.2	0.616	10441
	Acetate-phosphate-borate buffer	12.86	299.7	0.467	7908
		11.86	299.7	0.458	7759
		11.24	299.8	0.458	7754
		10.67	299.8	0.457	7733
		6.00	299.9	0.453	7685
1'		3.99	302.8	0.452	7667
2'		3.72	305.3	0.513	8686
3'		3.40	307.0	0.554	9391
4'		3.22	307.6	0.577	9777
5'		2.92	308.1	0.600	10 173
6'		2.62	308.5	0.611	10 357
7'		2.31	308.7	0.618	10 469
8'		1.33	308.8	0.637	10 788
9'	Phytic acid[1]	–	308.7	0.637	10 575

[1]C_{PA} = 1.0·10^{-5}M, molar ratio of PA: Xym = 1:6. $\alpha = n_{PA}/n_{Xym}$ = 0.17

Previously we showed that BDP like phytic acid forms salt complexes with amines [35] by proton transfer reaction like phosphoric acid.

The spectrum of xymedon in phytic acid solution with a molar ratio of phytic acid: xymedon = 1: 6 was the same as xymedon hydrochloride spectrum (fig. 4b, table 1). It is significant that a conditional molar extinction coefficient ε was close to 10 600±200 l·mol^{-1}·cm^{-1} in all acid solutions under study at various pH values (table 1) and it was different from xymedon in water.

The results can probably be explained by the formation of xymedon and BDP complexes, when xymedon is not fully protonated, in contrast to xymedon salts formed by strong acids.

The significant increase of ε when pH decreases from pH 6.00 to pH 3.75 (corresponding to the plateau on pH = f(α) curve, fig. 2), and practically linear symbatic dependence of ε on BDP molar fraction α in the same pH region (fig. 5 a,b) prove the proton transfer between BDP and Xym.

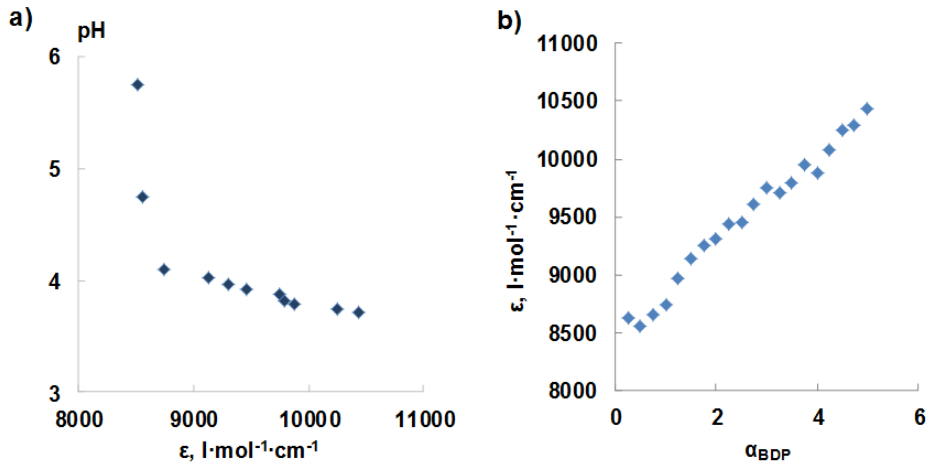


Fig. 5: a) The solution pH as a function of the molar extinction coefficient ε, pH = f (ε); b) Dependence of the extinction coefficient ε on α_{BDP}, ε = f(α_{BDP})

^{13}C-and ^{31}P-NMR spectral data of BDP-Xym mixture in D_2O show the significant changes of spectra compared to the spectra of individual BDP and Xym (table 2, fig. 6). ^{13}C-NMR spectra of xymedon hydrochloride and xymedon phytate were studied as well. Strong changes of chemical shifts of carbonyl carbon signals from 175 ppm to 146-149 ppm were observed in the spectra of all compounds under study (table 2). The signals of carbons designated C-4 and C-6 also changed.

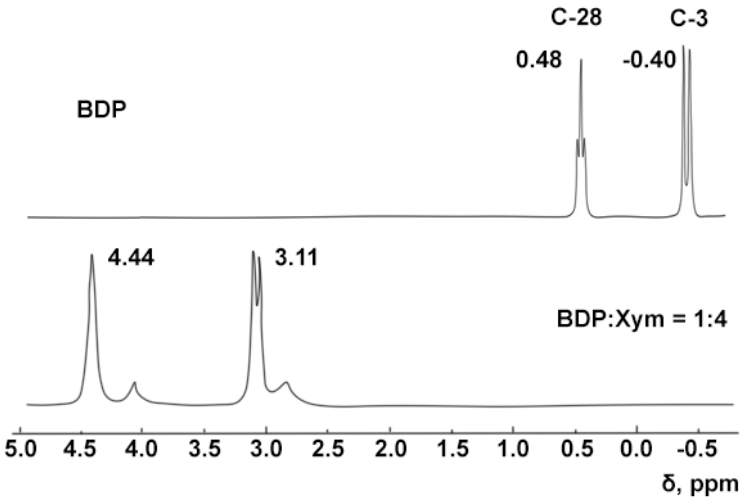
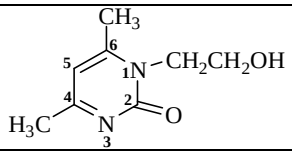


Fig. 6: ^{31}P-NMR spectra of BDP (top) in DMSO-d_6 and BDP-Xym mixture (bottom) in D_2O. PPh_3 is a standard

Table 2: ^{13}C-NMR spectra of Xym, Xym-chloride, Xym-phytate (D_2O) and Xym-BDP (DMSO-d_6)



Group	-CH_3 (at C-6)	-CH_3 (at C-4)	N-CH_2 (1)	-CH_2-OH	HC= (C-5)	C=N (C-4)	C= (C-6)	C=O (C-2)
^{13}C-NMR, δ, ppm								
Xym	20.1	23.1	47.9	58.4	108.3	160.6	157.9	175.5
Xym-chloride	19.4	21.7	49.4	57.8	108.3	172.1	168.5	149.0
Xym-phytate	19.5	23.6	49.4	57.8	108.2	171.9	168.4	148.9
Xym-BDP	18.0	21.3	48.8	57.6	106.5	169.7	166.8	146.4

The chemical shift δ of phosphorus at C-3 atom of the pure BDP is equal to -0.58 ppm (doublet without decoupling from protons) and δ of phosphorus at C-28 is equal to +0.28 ppm. The chemical shifts of phosphorus atoms changed in BDP-Xym mixture spectrum (1:4) to+3.11 ppm at C-3 and to+4.44 ppm at C-28. Two non-intensive and unresolved signals (close to the main signals, δ_1 = 2.7 ppm, δ_2 = 4.0 ppm) may describe the product of xymedon interaction with only one hydroxyl of BDP phosphate group.

Thus, taking into consideration the potentiometric titration data, UV and NMR spectra of reaction mixtures of BDP with xymedon, it is arguable that the interaction of BDP with xymedon proceeds with the formation of salt complexes and via a proton transfer reaction in the solution.

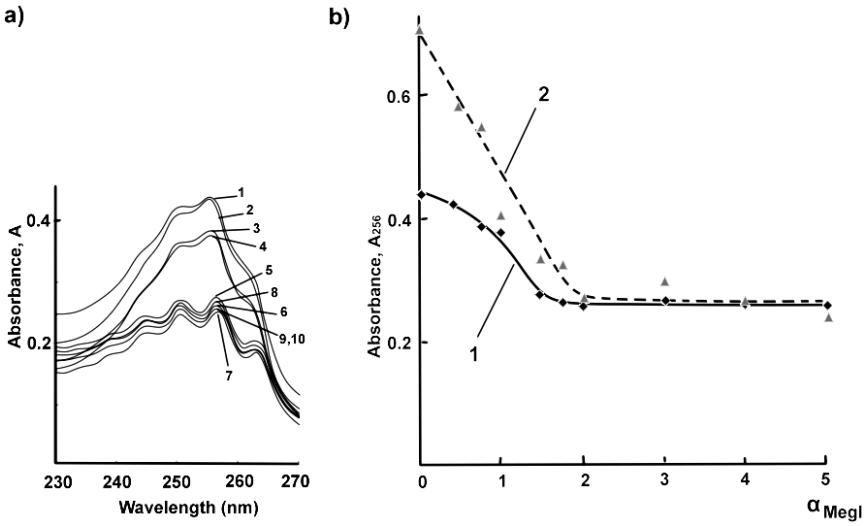


Fig. 7: The UV spectra in 5.1·10^{-4}M solutions of BDP in the presence of meglumine, C_{LiClO4} = 1·10^{-2} M: a) A = f(λ); b) the dependence of A_{256} absorption on the molar ratio of meglumine to BDP, A = f (α_{Megl}) in fresh solutions (1) and after 4 d (2), $\alpha = n_{Megl}/n_{BDP}$

The acid-base interaction of BDP with meglumine

In the aqueous medium BDP UV-spectra were characterized by a band of the phosphate group (n→p*, p→p* transitions) at λ_{max} = 256 nm. BDP phosphate groups under ionization can be considered as an amphoteric ion (B on fig. 3) both as an acid and a base conjugated to the acid. The molar extinction coefficient ε of initial BDP in ethanol solution (curve 1 at fig. 7a) is almost twice as much than ε of BDP in NaOH aqueous solution that characterizes the ionized form, represented by base D presented in fig. 3. Therefore, absorption A values at 265 nm and, correspondingly, conditional molar extinction coefficient ε can characterize BDP ionization and its interaction with Megl, which has no band in this region.

Fig. 7a shows the spectra of $5.0 \cdot 10^{-4}$M BDP and meglumine (Megl) mixtures at molar ratio α (α = n_{Megl}/n_{BDP}) from 0 to 5, when BDP concentration was $5.0 \cdot 10^{-4}$M and an ionic strength was supported by $1 \cdot 10^{-2}$M $LiClO_4$. Fig. 7 and table 3 data show A_{256} decrease while the molar ratio α increases.

It should be noted that the absorption of BDP phosphate groups changed with time during 4 d in aqueous solution without meglumine. Fig. 6b (1 and 2 curves) shows the molar extinction coefficient ε and the absorption increase approximately half as much. It may be explained by the increase with time of the BDP ionic A fraction having higher ε values than ε values of ionic B form in initial BDP solution.

After adding meglumine at essential meglumine excess (α≥2), absorbance (A) and ε values were the same and did not practically depend on α. The "plateau" both on the curve A = f(α) (fig. 7b) and on the curve pH = f(α) (fig. 2) appears in α≥2 region and reflects the same BDP ionization in BDP-Megl mixture under the studied conditions.

It is possible that such BDP ionization has the nature of the salt complex formation similar to BDP salt complexation with trisamine [35]. The composition of the BDP-trisamine complex was proved using Job's method, and the conditional stability constant equal to 1130 $mol \cdot l^{-1}$ was calculated [35].

Table 3: UV-spectra data according to fig. 8 a,b

№ curve	α = n_{Megl}/n_{BDP}	C_{Megl}, $\cdot 10^4$M	A		ε, $l \cdot mol^{-1} \cdot cm^{-1}$	
			A_0	A, τ = 4 d	ε_0	ε, τ = 4 d
1	0.00	0.00	0.437	0.709	874	1418
2	0.50	2.50	0.435	0.580	870	1160
3	0.75	3.75	0.384	0.546	768	1092
4	1.00	5.00	0.376	0.401	752	802
5	1.50	7.50	0.273	0.333	546	666
6	1.75	8.75	0.262	0.327	524	654
7	2.00	10.00	0.251	0.263	502	526
8	3.00	15.00	0.268	0.295	536	590
9	4.00	20.00	0.258	0.263	516	526
10	5.00	25.00	0.258	0.236	516	472

Note: ε of BDP solutions ($l \cdot mol^{-1} \cdot cm^{-1}$): in 0.02M ethanol H_2SO_4 solution is 663; in $4 \cdot 10^{-3}$M aqueous NaOH solution is 418; in 95% ethanol–water (1:1) medium with $LiClO_4$ is 761.

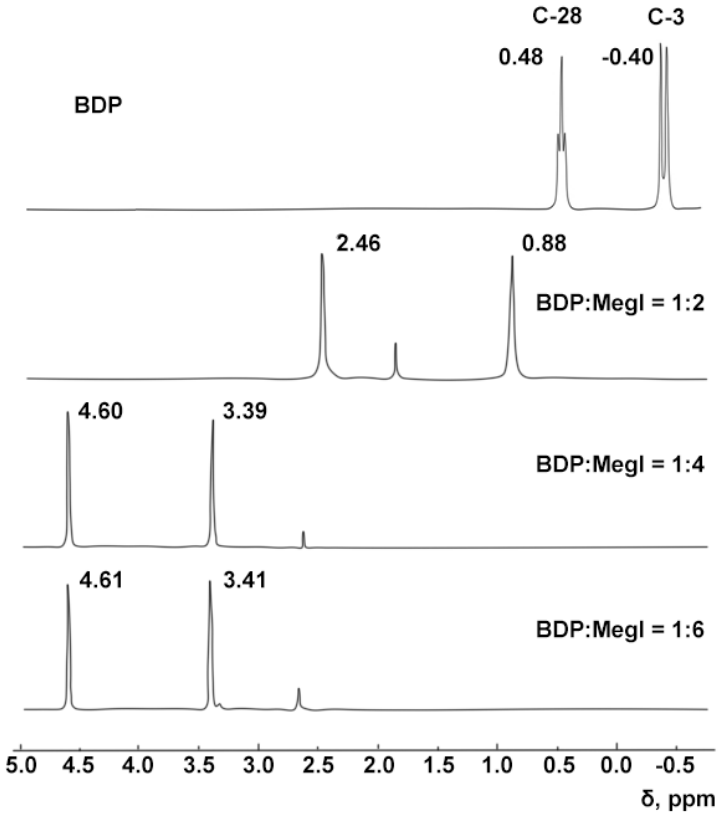


Fig. 8: ^{31}P-NMR spectra of BDP-Megl complexes in D_2O. H_3PO_4 is standard

The interaction of BDP with meglumine in a solution was also studied using ^{31}P-NMR spectra (fig. 8). The chemical shift of phosphorus at C-3 atom of BDP (δ =-0.58 ppm) changed to+0.88 ppm, and at C-28 atom (δ =+0.28 ppm)–to+2.46 ppm in case of BDP-meglumine mixture (1:2). The chemical shifts depended on the BDP: meglumine ratio, and signals appeared in the weaker field with δ=+3.39 ppm (at C-3) and δ=+4.60 (C-28) at molar BDP: meglumine ratio equal to 1:4 (fig. 8), similar to phosphorus signals of BDP-xymedon salt complexes (fig. 6). The molar ratio of BDP: meglumine more than 1:4 did not change chemical shifts.

BDP solubility and antioxidant activity of BDP salt complexes with amines

The argument for strong intermolecular interactions of BDP with Megl, probably resulting in salt complexes formation in solution, is a sharp increase of BDP solubility in aqueous meglumine solutions (table 4). HPLC analysis showed that BDP solubility in water (0.1 g·l^{-1}) significantly increased in the presence of meglumine, and the solubility of complex compounds did not depend on the initial BDP aging in storage (the transformation to BDP structural modification with lower solubility). The maximum BDP solubility in water (59 g·l^{-1} at 20 °C) was achieved in the presence of meglumine at a molar ratio of components 1:4, BDP solubility being nearly 6 times greater than its solubility in the form of sodium salts. We studied the precipitates obtained by freeze-drying of BDP solutions with amines (at molar ratios of 1:1, 1:2, 1:4 and 1:6) as well. The ^{31}P, ^{13}C-NMR and UV spectral data of aqueous solutions (D_2O) of precipitates and initial components of model mixtures at same molar ratios were identical. The data suggest BDP dissolution in the meglumine medium to be largely due to hydrogen bonds, proton transfer interactions, and, ultimately, the interaction between the cationic and anionic parts of the complex components.

Table 4: Water solubility of BDP and its products at 20 °C according to HPLC-analysis

Compound	C_{BDP}, g·l^{-1}
BDP [a]	0.15
BDP [b]	0.09
Na-BDP [a]	10.00
Na-BDP [b]	4.00
BDP: Xym (molar ratio is 1:4)	0.30
BDP [a]: Megl (molar ratio is 1:4)	59.00
BDP [b]: Megl (molar ratio is 1:4)	35.00

[a]fresh prepared, [b]after storage during 3 mo.

The higher water-solubility of amorphous BDP-Megl salt complexes than crystalline Na-BDP can be explained by the stronger decrease of hydrophobic bonding between BDP dimer molecules by Megl due to hydrogen bonds. The effective antioxidant-regenerant xymedon, which has a high ability both to hydrogen bonding and to form stacking structures like pyrimidine, cannot significantly weaken the hydrophobic binding in the BDP dimer and, accordingly, improve its solubility. The poor water-soluble salt complex of xymedon with BDP is of interest as a dosage form of xymedon with an optimal dose, in contrast to its known dosage forms with very high dose (1500-2000 mg per day) due to its high solubility in water.

The antioxidant properties of aqueous solutions formed by BDP and amines: meglumine and xymedon, were studied by *in vitro* experiment on rat blood. The antioxidant activity of glutathione reductase and catalase, activity of glucose-6-phosphate dehydrogenase were studied using data as percentage of control (table 5). The effect of BDP-amine complexes on enzyme activity was compared to the effect of individual components.

Table 5: Biochemical indexes (catalase, glutathione reductase and glucose-6-phosphate dehydrogenase activities) of BDP-Megl and BDP-Xym mixtures in phosphate buffer (pH 6.86). Molar ratio of BDP: amine is 1:4

Compound	Dose of BDP, μg·ml^{-1}	Dose of amine, μg·ml^{-1}	Index, % of control^{-1}		
			Catalase	GR	G6PD
BDP	2.0	-	159±14	215±3	132±3
	5.0	-	172±9	179±3	167±4
	10.0	-	153±7	193±2	137±2
Megl	-	2.6	114±4	112±3	113±2
	-	6.5	112±8	143±3	99±3
	-	13.0	91±3	115±2	110±1
BDP+Megl	2.0	2.6	170±3	239±3	151±3
	5.0	6.5	237±5	204±4	188±5
	10.0	13.0	197±9	225±2	198±3
Xym	-	2.2	134±3	133±2	134±1
	-	5.6	152±5	143±3	99±4
	-	11.2	191±2	185±5	110±2
BDP+Xym	2.0	2.2	195±5	239±4	151±3
	5.0	5.6	187±6	204±8	188±3
	10.0	11.2	157±8	195±3	159±5

[1]Number of replication of experiments was equal to 3. Biochemical indexes values are taken as 100%; catalase—31.35 Ru/mg protein; glutathione reductase — 91.29 nmol nicotinamide adenine dinucleotide phosphate (NADH)/min/mg protein, glucose-6-phosphate dehydrogenase—52.37 nmol NADPH/min/mg protein.

Table 5 data show that the BDP-amine complexes formation promoted the significant increase in catalytic activity of catalase, glutathione reductase and glucose-6-phosphate dehydrogenase compared to the control.

CONCLUSION

Thus, we firstly obtained the aqueous solution of betulin-3,28-diphosphate as a potential antioxidant pharmaceutical active ingredient at BDP concentration up to 59 g·l^{-1} in the presence of meglumine. This fact is very important to prepare injectable and other hydrophilic formulations because a poorly water-soluble BDP loses its initial solubility during aging and storage by transition of structural modifications. On the other hand, the poor water-soluble salt complex of BDP with extremely water-soluble xymedon (up to 300 g·l^{-1}) can be used as an effective antioxidant-regenerant with an optimal dose.

BDP interaction with amine (xymedon and meglumine) in an aqueous solution was shown to proceed via proton transfer due to relatively weak forces such as London forces, hydrogen bonding, electrostatic and hydrophobic interactions. In general, the formation of salt complexes of BDP with amines in solution determines BDP water solubility.

AUTHORS CONTRIBUTIONS

Nina Melnikova has made the contribution to design, analysis and conceptualizes the work. Darina Malygina, Dmitry Panteleev, Olga Vorobyova and Irina Klabukova have made the contribution to physicochemical experiments. Anna Solovyeva and Kseniya Belyaeva have made the contribution to biological activity assay. All authors read manually and approved the final manuscript.

CONFLICTS OF INTERESTS

The authors declare no conflict of interest.

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