

PHYSICO-CHEMICAL AND TOXICOLOGICAL EVALUATIONS OF BETULIN AND BETULINIC ACID INTERACTIONS WITH HYDROPHILIC CYCLODEXTRINS

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Abstract

Betulin and betulinic acid are pentacyclic triterpens with antitumor activity, especially on melanoma and skin cancer. Considering their low water solubility, studies have been conducted in order to improve this aspect. One possible solution is the preparation of inclusion complexes with semisynthetic cyclodextrins using different methods.

The purpose of this study was the evaluation of the stability constants of betulin and betulinic acid complexes with two cyclodextrins: hydroxypropyl-beta-cyclodextrin and randomly methylated-beta-cyclodextrin, using the Higuchi and Connors phase solubility method. Products have been analyzed by scanning electron microscopy and *in vitro* tests. The presence of cyclodextrins increases water solubility and biological activity without toxic activity.

Rezumat

Acidul betulinic și betulina sunt triterpene pentaciclice cu activitate antitumorală mai ales în melanom și cancerul de piele. Solubilitatea lor în apă fiind foarte scăzută, s-au realizat studii pentru remedierea acestui aspect. O posibilă abordare este prepararea unor complecși de incluziune cu ciclodextrine, utilizând diferite metode.

Scopul acestui studiu este evaluarea constantelor de stabilitate ale complexelor acidului betulinic și betulinei cu două ciclodextrine: hidroxipropil-betaciclodextrina și beta-ciclodextrina metilată aleator, utilizând metoda solubilității de fază introdusă de Higuchi și Connors. Produsii au fost analizați prin microscopie de scanare electronică și teste *in vitro*. Prezența ciclodextrinelor conduce la creșterea solubilității în apă și a activității biologice, fără a accentua toxicitatea acestor compuși.

Keywords: betulin, betulinic acid, cyclodextrin.

Introduction

Triterpenes like betulin, lupeol and especially betulinic acid, the main components of birch bark with lupan skeleton (figure 1) display anticancer and other important therapeutic activities [1]. Important pentacyclic triterpenes are found in the outer bark of the birch tree (*Betula*

pendula Roth); of these compounds, betulin is the major one as its content surpasses 20%, correlated with the type of birch tree [7].

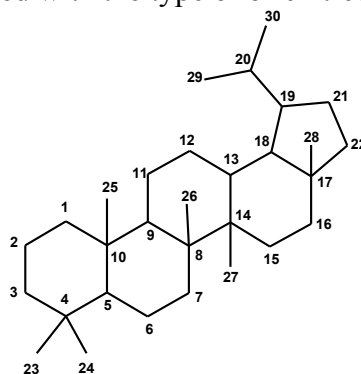


Figure 1.
Lupane skeleton

Betulinic acid (BA) is a selective inducer of apoptosis in tumor cells, inhibits activation of nuclear factor κ B (NF- κ B) and NF- κ B-regulated gene expression induced by carcinogens and inflammatory stimuli, this being the molecular basis for the ability of BA to mediate apoptosis, reduce inflammation and modulate the immune response [8]. BA has a selective action on melanoma cells and does not affect the normal ones as compared to doxorubicine which has a non-selective cytotoxic activity [9]. Betulinic acid selectivity on melanoma cells even compared with the normal cells, makes it unique among the currently used therapeutical substances such as: taxol, camptothecine, elipticine, etoposide, vinblastine and vincristine. Compared to these substances betulinic acid has an implicit low toxicity [4]. Betuline (Bet) presents therapeutic applications, as a glucocorticoid anti-inflammatory agent, but mostly it can be used as a precursor for betulinic acid and other synthetic derivatives, which have anti-viral properties (HIV-1) and anti-tumor properties [4]. Betuline was used in the past, in traditional medicine, in the treatment of skin diseases [5].

Cyclodextrins are torus-shaped oligosaccharides, built up from glucopyranose units, obtained by the fermentation of starch. Cyclodextrins are able to form inclusion complexes with a great number of compounds, which may improve the guest molecule's solubility, bioavailability, physico-chemical stability, both in solid state and in solution [2, 6].

The aim of this study was the formation of inclusion complexes between betulinic acid and betuline and two hydrophilic cyclodextrins: hydroxypropyl- β -cyclodextrin (HPBCD) and randomly methylated- β -cyclodextrin (Rameb); by complexation, the triterpenic compounds are molecularly dispersed in a hydrophilic matrix and become soluble, which

leads to faster dissolution and a better bioavailability. The stability of the complexes between the active substances and cyclodextrins has been evaluated by the Higuchi and Connors method [3]. *In vitro* tests were conducted in order to analyse the pharmaco-toxicity of the complexes.

Materials and methods

Betulinic acid and betulin were purchased from Sigma Aldrich (Germany) and the cyclodextrins were purchased from Cyclolab Res. & Dev. Ltd., (Hungary). All materials were used as received.

1. Calibration curve

Aqueous solutions of different concentration of betulin and betulinic acid were prepared and spectrophotometrically analyzed at 210 nm where both substances present a maximum of absorption.

2. Phase solubility evaluation

The aqueous solubility of betulinic acid and betulin at various concentrations of HPBCD or Rameb was studied by the method reported by Higuchi and Connors. Samples of betulinic acid and betulin in quantities exceeding their aqueous solubility were shaken at room temperature with aqueous solutions of HPBCD and Rameb, respectively, in increasing concentrations (0-300 mmol/L), for a period of five days, until equilibrium was established. The solutions were then filtered and spectrophotometrically analysed at 210nm using the same concentration of cyclodextrin as blank.

The apparent stability constant can be calculated from the solubility data, using the following equation:

$$K = \frac{\text{Slope}}{S_o(1 - \text{slope})}$$

where S_o is the intrinsic solubility of the active substance (BA / Bet) and the slope is the slope of the solubility diagram.

3. Scanning electron microscopy (SEM)

Particle morphology was examined using electronic microscope Hitachi 2400S (Hitachi Scientific Ltd, Japan). A thin-layer covering device (Bio-Rad SC 502, VG Microtech, England) was used to obtain an electric conductivity to the surface of the sample. Air pressure was 1.3-13.0 mPa.

4. MTT-assay.

The test was performed on the MCF-7 breast cancer cell line, 7000 cells/well, media was RPMI + 10% FCS (RPMI – Roswell Park Memorial Institute Medium, FCS – fetal calf serum); L-glutamine=300 mg/L; penicillin and streptomycin=1%. The cells were prepared under typical

standard conditions. All the liquid extracts were sterile filtered (through 0.2 μm filters) before being added to the plate. After the first preparation the cells were suspended in 150 μL of media w/phenol red (RPMI+20%FCS); 15 μL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent were added/well and 150 μL of lysis agent, after 3 h incubation. The reading was performed at 570 nm on an ELISA plate reader with a reference at 650 nm. The tested samples were prepared with a solution of DMSO (dimethyl sulfoxide)/water bidistilled (1:10). The incubation period was 72h. The stock solution was 0.01 mg/mL.

Results and discussion

1. Calibration curves

The calibration curves of BA and Bet are presented in figure 2 a, b. These results permitted to establish the equations of the calibration curves, which were used for further determinations:

$$A = x \times c$$

where

A = the absorbance of the analyzed solution,

c = concentration of the analyzed solution, $\mu\text{g/mL}$.

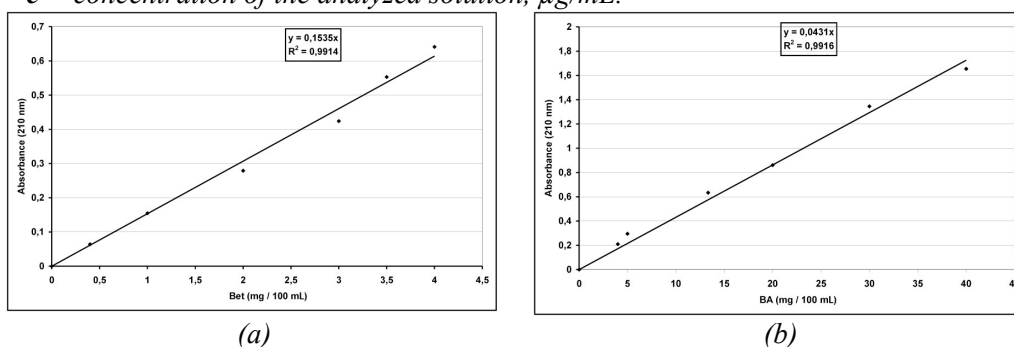
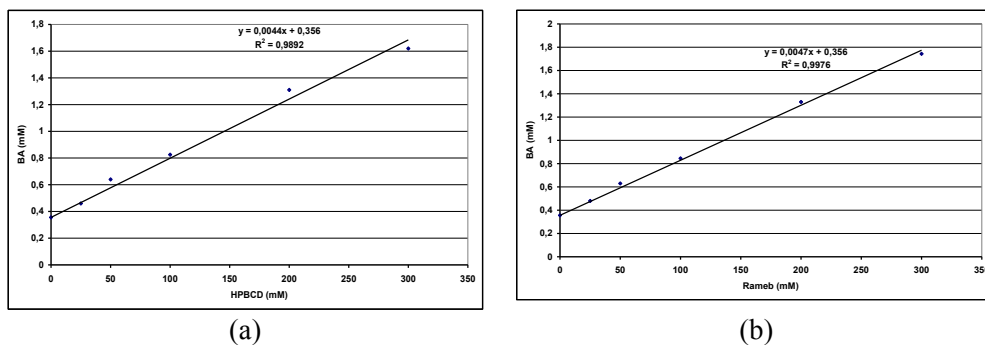


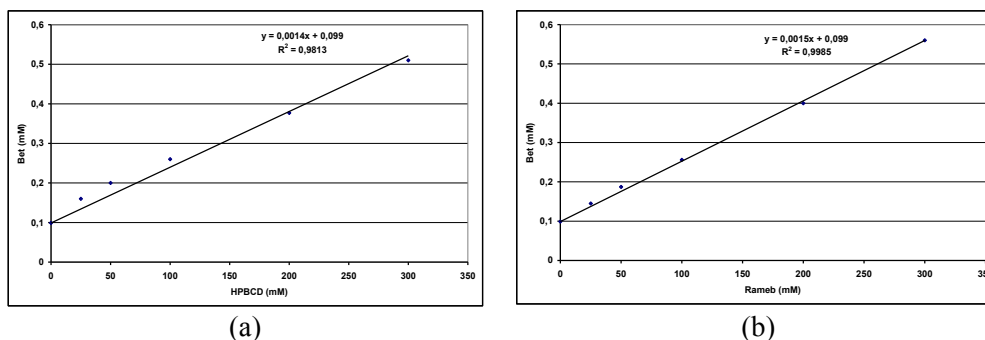
Figure 2.
Calibration curves for Bet (a) and for BA (b)

2. Phase-solubility studies

According to the method introduced by Higuchi and Connors, an A_L type curve was obtained for both substances and the slope of the diagram was less than 1, which leads to the conclusion that an 1:1 inclusion complex was formed. Fig. 3, a and b show the phase solubility diagram of BA with HPBCD and Rameb, respectively, in water. Figure 4 a, b presents the same results for Bet in water in the presence of the two cyclodextrins.

**Figure 3.**

Solubility diagram of betulinic acid with HPBCD (a) and RAMEB (b)

**Figure 4.**

Solubility diagram of betulin with HPBCD (a) and RAMEB (b)

Table I presents the equations obtained by linear regression.

Table I.

Linear regression and stability constants for BA and Bet with cyclodextrins

Active substance	Cyclodextrin	Equation	Correlation coefficient (r^2)	Stability constant (M^{-1})
BA	HPBCD	$y = 0.0044x + 0.356$	0.9892	11.83
BA	Rameb	$y = 0.0047x + 0.356$	0.9976	12.97
Bet	HPBCD	$y = 0.0014x + 0.099$	0.9813	14.14
Bet	Rameb	$y = 0.0015x + 0.099$	0.9985	15.55

where x = cyclodextrin concentration (mmol/L) y = BA/Bet concentration (mmol/L).

Stability constant values are considerably low probably due to a steric inadequacy between the lupanic structure and the cyclodextrin inner hollow. The two active substances present bulky structures which require

cyclodextrins with a larger cavity, possibly γ -derivatives. However the low stability of the complexes influences only the preparation of a solid complex but allows the cyclodextrin to function as a carrier of the active substance into the aqueous phase.

3. SEM pictures

The shape and surface morphology of betulin, betulinic acid and their complexes with the two cyclodextrins are depicted in figures 5 and 6. The microscopic images were recorded with an increase of 200-10000 times of the sample, so one can observe the entire picture as well as the details of the samples.

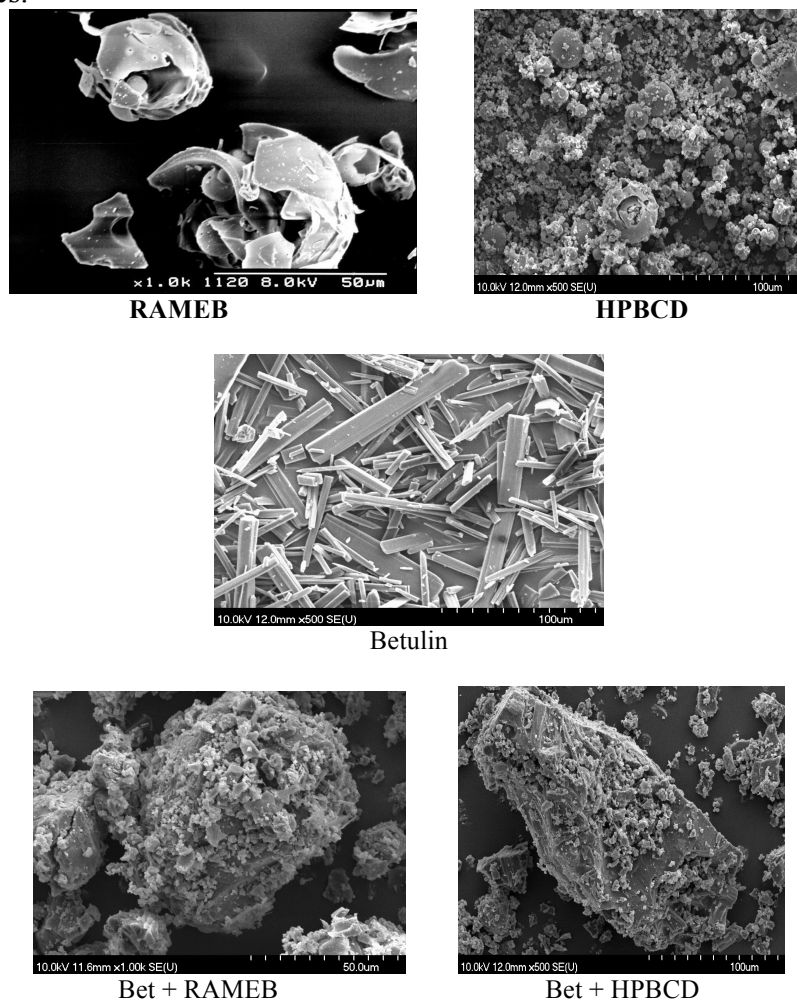
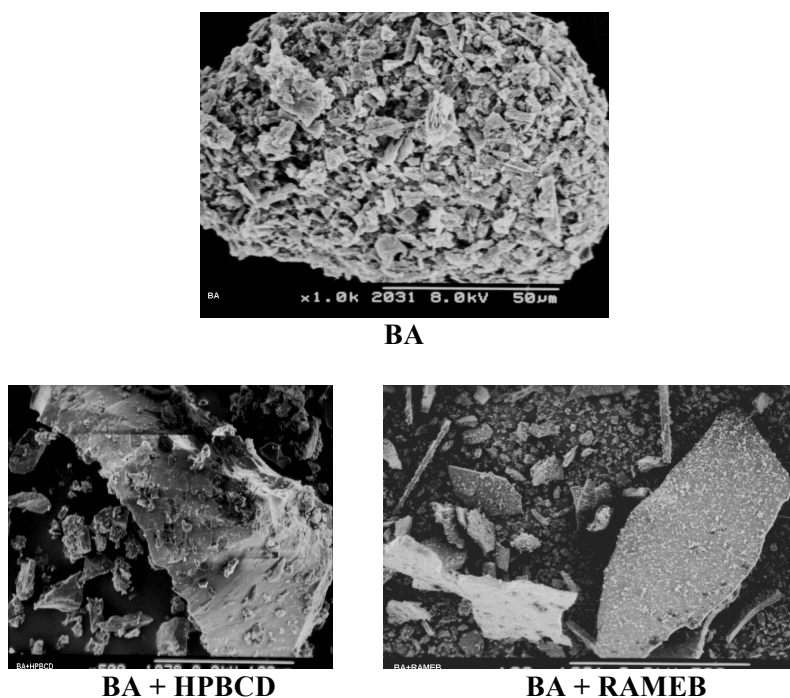


Figure 5.

SEM pictures of betulin and its complexes with RAMEB and HPBCD
Where: RAMEB - randomly methylated- β -cyclodextrin, HPBCD - hydroxypropyl- β -cyclodextrin, Bet – betulin.

**Figure 6.**

SEM pictures of betulinic acid and its complexes with RAMEB and HPBCD
 Where: BA – betulinic acid, HPBCD - hydroxypropyl- β -cyclodextrin, RAMEB - randomly methylated- β -cyclodextrin

Betulin and betulinic acid are formed by needle-shaped crystals of different sizes, with a smooth surface. The hydrophilic cyclodextrins are represented by spherical, smooth, broken particles. For the complexes, a significant change of the substance morphology was noticed, the samples being formed by irregular prismatic crystals of different shapes and sizes.

Consequently, the morphology and size of the complexes particles are fundamentally different from the initial substances leading to the conclusion that an inclusion phenomenon had taken place.

4. MTT-assay

The activity of the tested compounds was reduced to medium regarding the applied cells (figure 7). The differences between the two types of cyclodextrins suggested their intervention in the cell viability process, directly or by increasing the active agent solubility in the medium. The activity of the tested compounds and complexes is not very high but it is significant. Changes in triterpenes solubility can influence their cytotoxic effect.

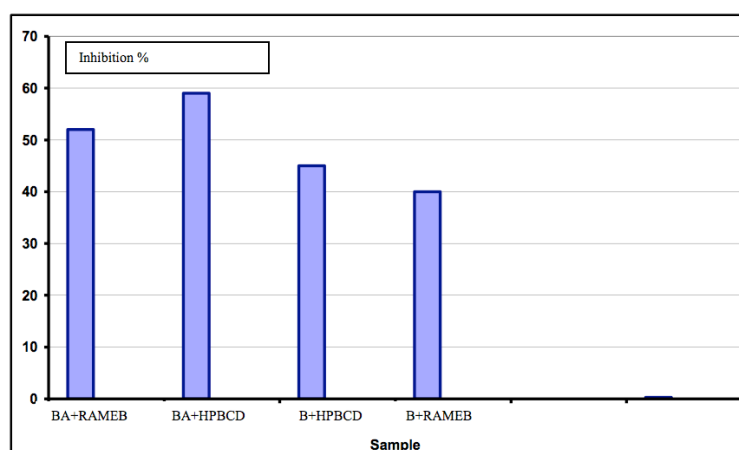


Figure 7.

Citotoxic activity of betulinic acid and betulin mixed with the 2 types cyclodextrins.

Where BA – betulinic acid, RAMEB - randomly methylated- β -cyclodextrin, HPBCD - hydroxypropyl- β -cyclodextrin, B – betulin.

Conclusions

As mentioned in preliminary studies, pentacyclic triterpenes are able to form inclusion complexes with different types of cyclodextrins in order to improve their biopharmaceutical properties.

β -cyclodextrin derivatives do not form very stable complexes with the triterpenic structures aspect underlined by a low stability constant. However, the water solubility of betulin and betulinic acid is significantly increased regardless of the low stability of the inclusion complex.

The *in vitro* activity of the two pentacyclic triterpenes involved in the study is highly influenced by the cyclodextrin presence in a direct manner or by cyclodextrin intervention in increasing the solubility of the active compound.

Acknowledgements

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