

Physico-chemical and Molecular Analysis of Antitumoral Pentacyclic Triterpenes in Complexation with Gamma-cyclodextrin

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*Pentacyclic triterpenes determine anticancer, anti-inflammatory and antiviral activity. The major problem of this type of triterpenes is their low water solubility which can be increased by cyclodextrin complexation. The aim of present study was to analyze the products between pentacyclic triterpenes (betulinic acid, betulin) and γ -cyclodextrin (GCD). GCD is a natural cyclodextrin with 8 units of glucopyranose, obtained by enzymatic degradation of amylose; it is used as host-molecule for the accommodation of large molecules like steroids, spironolactone, triterpenes. In order to obtain the inclusion complexes of pentacyclic triterpenes with GCD, 1:2 molar ratio and two preparation methods (physical mixing, kneading) were used. The inclusion complexes were analyzed by *in vitro* dissolution tests and X-ray diffraction. The molecules of the two components were analyzed by computational chemistry to see if they are sterically compatible.*

Keywords: GCD, betulinic acid, betulin, inclusion complexation, solubility

Saponins are amphiphilic compounds with steroid or terpenic structure [1]. The main components of the birch bark are betulin and betulinic acid, with triterpenic structure, which display anticancer activity [2]. The outer bark of the birch tree contains important pentacyclic triterpenes; of these compounds, betulin is the major one as its content surpasses 20% [3].

Betulinic acid (BA) is an important therapeutic compound, tested on cell lines type: MHH1, MHH3, A172, SK17, SK19, MCF-7 etc.; it is a selective inducer of apoptosis in tumoral cells, inhibits NF- κ B activation, reduces inflammation and modulates immun answer. [4] BA has a selective activity on melanomic cells and does not affect normal cells [5].

Betuline (Bet) is a natural compound obtained from vegetal sources (birch tree, etc.) by sublimation or extraction with chemical solvents (methanol, dichloromethane, chloroform) [6]. Betuline is a glucocorticoid-type anti-inflammatory agent and can be used as precursor in betulinic acid synthesis. [7] It was used in popular medicine for the treatment of skin diseases [8].

Cyclodextrins are torus-shaped oligosaccharides, built up from glucopyranose units, obtained by fermentation of starch. Cyclodextrins are able to form inclusion complexes with a great number of compounds, which may improve the guest solubility, bioavailability, physico-chemical stability, both in solid state and in solution [9, 10].

The aim of this study is the formation of inclusion complexes between triterpenic compounds of birch tree extract (betulinic acid, betuline) and γ -cyclodextrin (GCD); by complexation, the triterpenic compounds are molecularly dispersed in a hydrophilic matrix and become soluble [11], which leads to faster dissolution and a better bioavailability. Products containing 1:2 molar ratio of triterpenic compounds and GCD, prepared by different methods, have been investigated by various techniques.

Experimental part

γ -cyclodextrin was purchased from Cyclolab R&D Ltd. (Budapest, Hungary). It was used as received. Betulinic

acid and betulin (fig. 1) [12] were purchased from Sigma-Aldrich Ltd. Solvents (ethanol, methanol) were of quality standards of Farmacopeea Română Xth ed [13].

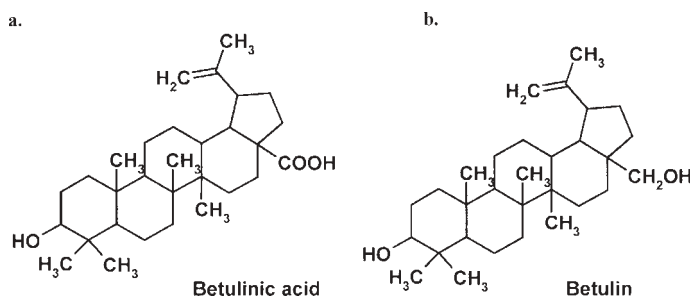


Fig. 1. Betulinic acid (a) and betulin (b) structural formula

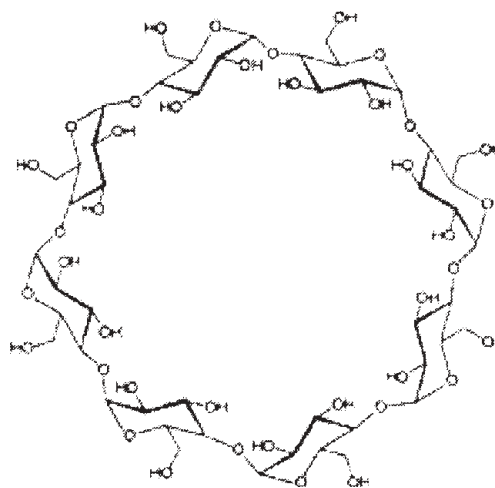


Fig. 2. GCD structural formula

Preliminary studies

In the pre-experiment studies, the absorption curve of BA and Bet, it was recorded the maximum of absorption ($\lambda = 210$ nm) and it was established that the pH of the solution did not influence the profile of the spectrum.

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Preparation of solid products

Different methods were applied in the preparation of the inclusion complexes:

- simple powder mixing, using a mortar and a pestle;
- kneading with a 50% ethanolic solution until the bulk of solvent evaporated; the mixture was then dried at room temperature for 24 h and then was put in the oven, at 105°C for several hours. The final product was pulverized and sieved.

The binary products were prepared using 1:2 molar ratio.

In vitro dissolution studies

The dissolution studies were carried out by using a modified paddle Erweka DT apparatus, in 100 mL buffer solution of pH = 5,5, at 37±1°C, for 120 min. Samples were taken after 5, 10, 15, 30, 60 and 120 min and the dissolved quantity of BA and Bet was determined spectrophotometrically at 276 nm. All studies were done at least in triplicate.

X-ray diffraction analysis

XRD spectra were recorded with a DRONUM-1 X-ray diffractometer (Russia) system with CuK α 1 radiation (λ = 1.54178 Å) over the interval 2-44°/2 θ . The measurement conditions were as follows: target, Cu; filter, Ni; voltage, 35kV; current, 20 mA; time constant, 1S; angular range 2 θ < 2 θ < 44°.

Bonding affinity evaluation

Computational structure-based and virtual screening programs are beginning to be more and more useful in the industrial process of drug development. An important part of these programs is represented by rapid evaluation of bonding energy between low mass organic molecules and macromolecular receptors. The methods used in this purpose are generally called score functions [14].

An ideal score function should estimate the best way possible the free bonding ligand-receptor energy.

Bonding energy evaluation was done using programme pack Open Eye (<http://www.eyesopen.com/> - academic licence), Windows version.

Within this programme cyclodextrins were considered receptor-molecules with an artificially created space region as bonding site for considered ligands. The purpose of this model was to evaluate triterpenes bonding affinity towards β - and γ -cyclodextrin. By definition, the complexes were

1:1 cyclodextrin-ligand, entropic and solvation-desolvation effects were neglected and bonding affinity was evaluated as a sum of electrostatic and van der Waals terms.

Docking program FRED (Fast Rigid Exhaustive Docking) allowed an exhaustive search of all possible positions of every ligand in pre-defined active site; another major advantage of FRED is that it reduces computational time for a medium size ligand when the program is run on a Pentium IV D940 (dual core 3,2 GHz).

Basic structures were obtained from : <http://pubchem.ncbi.nlm.nih.gov/>, using isomeric SMILES formulas:

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CC(=C)[C@@H]1CC[C@]2([C@H]1[C@H]3CC[C@@H]4[C@]5(CC[C@@H](C([C@@H]5CC[C@]4([C@@]3(CC2)C)C)C)O)C)CO betulin  
CC(=C)[C@@H]1CC[C@]2([C@H]1[C@H]3CC[C@@H]4[C@]5(CC[C@@H](C([C@@H]5CC[C@]4([C@@]3(CC2)C)C)C)O)C)C(=O)O betulinic acid
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In order to generate 3D structures OMEGA was used Open Eye conformer generator, (academic licence UMF Timi^ooara)



Fig. 3. 3D structure OMEGA generated for betulin

Atomic electric charges were obtained using QACPAC programmes (OpenEye).

Results and discussions

In vitro dissolution tests

Aqueous solubility of BA and Bet is increased by GCD, which also influences the dissolution rate of the active substances. The physical mixtures prepared with GCD yielded a higher dissolution profile as compared with the triterpene compounds and the kneaded products were better than the physical mixtures. Figures 4-5 show the dissolution profiles for BA, Bet and their kneaded products with GCD in buffer solution.

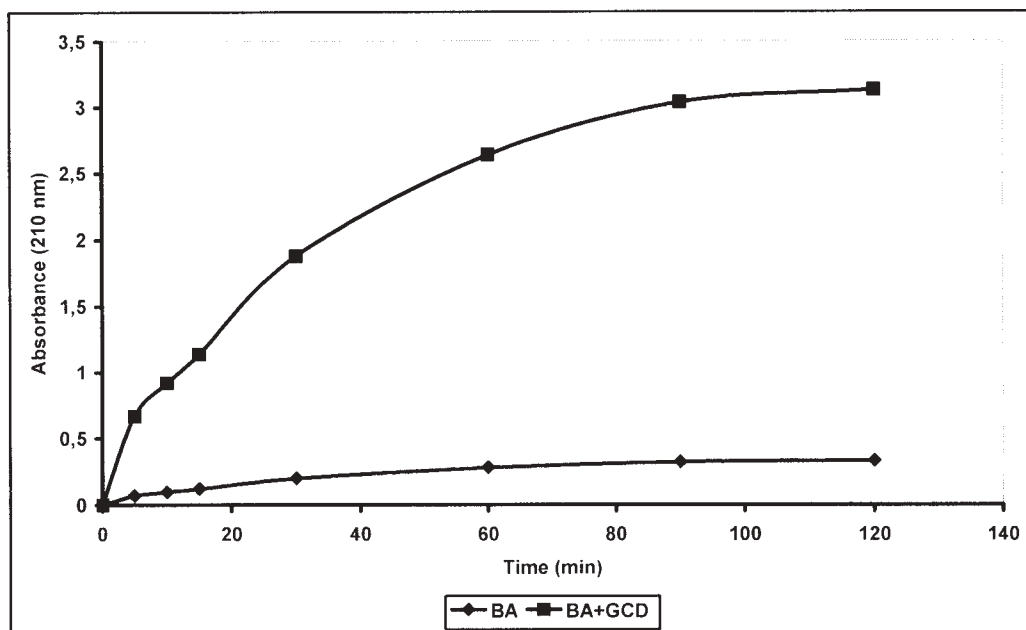


Fig. 4. In vitro dissolution of BA from GCD kneaded products

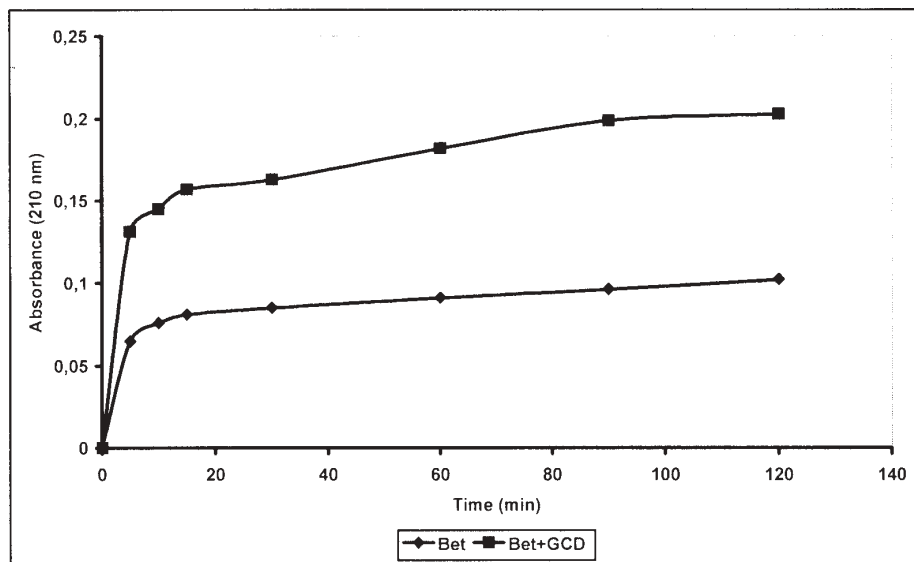


Fig. 5. In vitro dissolution of Bet from GCD kneaded products

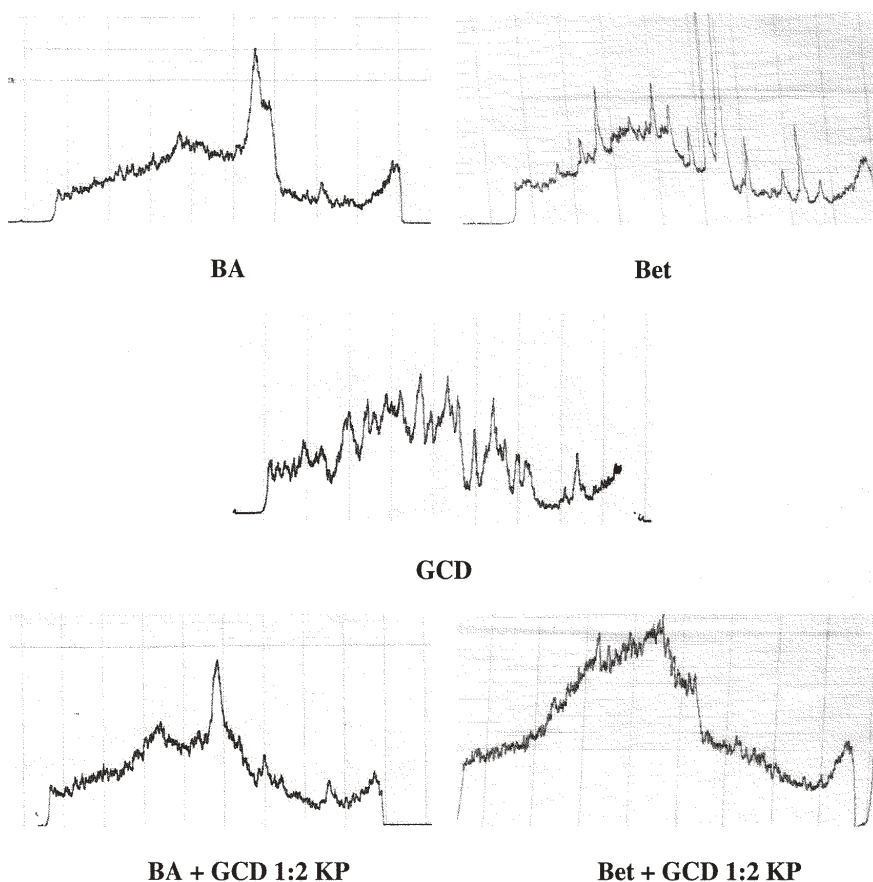


Fig. 6. X-ray diffractograms of BA and Bet and their 1:2 KP products with GCD

X-ray diffraction

X-ray diffraction analysis confirms the thermal analysis results. As can be seen in figure 6 BA and Bet present some peaks, characteristic for crystalline compounds, whereas they are practically absent in the respective KP products. The disappearance of all crystalline peaks leads to the conclusion that an amorphisation phenomenon takes place, which can be interpreted as an inclusion complex formation between triterpenic substances and GCD.

Bonding affinity evaluation

For γ -CD the results were:
 Score function: chemgauss2
 Steric : Steric interactions
 Acc/Metal : ligand acceptor - protein donor and all metal interactions
 Donor : Contribution from ligand donors interaction with protein acceptors
 Aromatic : Aromatic-aromatic interactions

Steric	Acc/Metal	Donor	Aromatic	Total Score	Molecule
-52.14	-0.72	-0.14	0.00	-53.00	betulinic acid
-47.48	-0.28	-0.34	0.00	-48.10	betulin

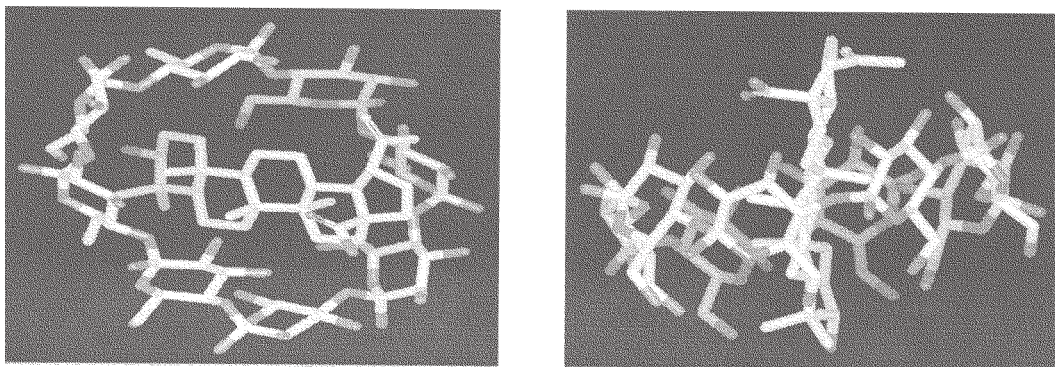


Fig. 7. γ -CD and betulinic acid (hydrogen atoms are hidden in order to simplify the images)

According to score function:chem.gauss 2 the best affinity towards γ -CD is showed by betulinic acid.

Obs. Neither betulin nor betulinic acid were able to be accommodated in β -cyclodextrin cavity because of their sizes.

Conclusions

GCD is able to form inclusion complexes with compounds that fit its cavity dimensions. The solubility of triterpenic compounds was increased in the presence of GCD.

In vitro dissolution tests revealed that the kneading method significantly improved the rate of dissolution especially at a molar ratio of 1:2.

The formation of inclusion complexes has been proved by X-ray diffraction analysis, which confirmed the efficacy of the kneading method in this purpose.

The presence of GCD significantly influences different parameters of the drug such as solubility and dissolution rate; this could prove advantageous in the future, offering the possibility of new pharmaceutical preparations with higher bioavailability and smaller therapeutically dosage.

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