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ORIGINAL ARTICLE

Inclusion complexes with cyclodextrin and usnic acid

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Abstract Molecular inclusion complexes of usnic acid (UA) with β-cyclodextrin (β-CD) and 2-hydroxypropyl β-cyclodextrin (HP β-CD) were prepared by the co-precipitation method in the solid state in the molar ratio of 1:1. Structural complexes characterization was based on different methods, FTIR, ^{1}H NMR, XRD and DSC. Parallel to the complex by the above methods, corresponding physical mixtures of UA with cyclodextrins and complexing agents (β-CD, HP β-CD and UA) were analyzed. The results of DSC analysis showed that, at around 200 °C, the endothermal peak in the complexes with cyclodextrins originating from the UA melting has disappeared. Complex diffractogram patterns do not contain peaks characteristic for the pure UA. They are more appropriate to cyclodextrin diffractogram. This fact points to the molecular encapsulation of UA in the cyclodextrin cavity. Chemical shifts in ^{1}H NMR spectra after the inclusion of UA into the cyclodextrin cavity, especially H-3 protons (0.0012 and 0.0102 ppm in the β-CD and HP β-CD, respectively) and H-5 and H-6 (0.0134 ppm) and hydrogen from CH_3 (0.0073 ppm) HP β-CD also points to the formation of molecular inclusion complexes. The improved solubility of UA in water was achieved by molecular incapsulation. In the complex with β-CD the solubility is 0.3 mg/cm^3, with HP β-CD 4.2 mg/cm^3 while the uncomplexed UA solubility is 0.06 mg/cm^3. The microbial activity of UA and both complexes was tested against eight bacteria and two fungi and during the test no reduced activity of UA in the complexes was observed.

Keywords Usnic acid · β-cyclodextrin · 2-hydroxypropyl β-cyclodextrin · Inclusion complex · Solubility · Antimicrobial activity

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Introduction

Usnea, also known as old man's beard, is not a plant but lichen, a symbiotic relationship between an algae and a fungus. The entire lichen is used. Usnea looks like long, fuzzy strings hanging from trees in North American and European forests, where it grows. Usnea lichens consists of the dried thallus of *Usnea* species, primarily *Usnea barbata* L. Of many studied, those of significance are lichens containing usnic acid (*Usneacceae*) as an active principle. Usnic acid (UA) was isolated from the *U. barbata* lichen. UA [$C_{18}H_{16}O_7$], a yellow crystal substance of natural origin is a dibenzofuran derivative [2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3(2H,9bH)-dibenzo-furandione]. The appearance of lichen and structural formula of UA are shown in Fig. 1a, b, respectively.

Constitutional components of many lichen species show some antibiotic activities [1]. UA, isolated from the lichen

Fig. 1 *Usnea barbata* lichen (**a**) and structure of UA (**b**)

U. barbata performs a significant antibacterial activity. UA shows the antimicrobial [2], antifungal (*Penicillium frequentas* and *Verticillium albo-atrium*, *Fusarium moniliforme*) [3], antiviral (*Herpes simplex*, *Polio virus*, *Epstein-Barr virus*) [4, 5], antiproliferative (cytotoxic, cytostatic activity against malignant cells K-562) [6, 7], anti-inflammatory and analgesic activities [8, 9]. However, all these activities have still not received the real clinical confirmation. Actually, the substance owns a pronounced antibacterial activity especially in the area of mouth cavity and upper respiratory organs.

The investigation of the extracts from 58 lichen acids of Swiss origin have shown that water pulps of 11 tested species are active against *Staphylococcus aureus* 114, but 27 tested species show insignificant activity [10]. It was found that both optical forms of UA, as well as the racemic mixture, act very efficiently upon *Streptococcaceae*, *Staphylococcus* and against the cause of tuberculosis in humans and animals. Both optical isomers act with equal success in antimicrobial activity against Gram-positive bacteria (*Enterococcus faecalis, Enterococcus faecium, S. aureus, Streptococcus mutans*, *Streptococcus pyogenes*) [11], anaerobic bacteria (*Bacteroides fragilis*, *Bacteroides ruminicola* ssp. *brevis*, *Bacteroides thetaiotaomicron*, *Bacteroides vulgatus*, *Clostridium perfringens*, *Propionibacterium acnes*) and mycobacteria (*Mycobacterium aurum*, *M. avium, M. smegmatis, M. tuberculosis var. bovis, M. tuberculosis var. hominis*). Having in mind certain pharmacodynamical activities (antiseptic and anesthetic effects) [12, 13] and the fact that UA can be taken, without any side effects, in daily doses of 3–6 g *per os*, the investigated substance finds its application in the production of pharmaceutical (septolete-lingualete, tooth paste) and cosmetic preparations (preservative in hydrated creams, inhibitor of Gram-positive microorganisms in deodorant sprays) [14]. However, from the technological point of view, there is a permanent problem of the lichen acids solubility. This is a limiting factor for the application of UA in pharmaceutical-cosmetic industry, so its sodium salt (Na-usninate) is used more often.

The extended number of patented and described procedures for the isolation and characterization of UA from various lichens are described in detail in literature [15–22]. These procedures are, for the most part, characterized by the insufficient consumption of reactants and solvents, longer reaction times and lower degree of purity of the obtained usniate. UA can be produced synthetically and a broad spectrum of derivatives and condensation products based on UA are also known [23].

The analysis and compatibility studies of the major natural origin active principles were performed for some new antiseptic products based on *Hypericum perforatum* L. and lichen *U. barbata*. Comparing the HPLC, FTIR and NMR spectroscopic data of the initial reference compounds (UA and hypericin) with their mixture it was found that the tested pharmaceutical product retained physicochemical properties qualitatively and quantitatively [24].

Inclusion complexes with cyclodextrin and their derivatives are increasingly used in order to improve the physical and chemical characteristics (solubility, evaporation, unpleasant smell, stability increase, etc.) of pharmacologically active substances. There is a number of scientific works showing the solubility and stability increase within many drugs such as atenolol [25–27], nifedipine [28–30], amlodipine [31, 32], allicin [33]. In order to improve the solubility of UA, a complex of usnic acid with β-cyclodextrin (βCD:UA), incorporated into the liposomes was prepared [34].

UA was under the active investigation for its broad spectrum antibiotic activities, prior to the discovery of penicillin. After the end of the World War II and until the late 1950s, most of 64 research publications on UA were related to its antimicrobial activity. After 1980s, the interest in UA was renewed because of the increasing experience of multi-drug resistance caused by the overuse of synthetic antibiotics [35]. The antibacterial activity of UA against *S. mutans* has been examined by Ghione et al. [36]. Lauterwein et al. [37] determined the in vitro activities of (+)-usnic acid, (D)-usnic acid, and vulpinic acid against aerobic and anaerobic microorganisms. They found that these lichen compounds did not inhibit Gram-negative rods or fungi at the concentrations lower than 32 μg/ml but were active against clinical isolates of *S. aureus*, *E. faecalis* and *E. faecium*. Ingólfsdóttir's [38] review lists the antimicrobial activity of (+)- and (D)-usnic acid in the table against Gram-positive, Gram-negative, anaerobic bacteria, mycobacteria, and yeast/fungi with the relevant references. According to the data, Hippocrates used some of these lichens to treat urinary problems. It is generally believed that the production of UA is exclusively restricted to lichens.

The aim of this paper was to prepare inclusion complexes of usnic acid with β-cyclodextrin (βCD:UA) and 2-hydroxypropyl β-cyclodextrin (HP β-CD:UA), describe complexes with suitable methods, determine solubility and examine their microbiological activity.

Materials and methods

Materials

UA was isolated from the *U. barbata* lichen and re-crystallized twice from boiling ethanol (of about 90 % purity). β-CD, 98 %, was supplied from Merck, Darmstadt while HP β-CD (97 %) was supplied from Sigma-Aldrich, Wisconsin. Other solvents and reagents used were of p.a. purity.

Experimental

The preparation of inclusion complex by co-precipitation

UA (1 mmol, 344 mg) and β-CD (1 mmol, 1,135 mg) or HP β-CD (1 mmol, 1,540 mg) were mixed and dissolved in 150 cm^3 of water. The solutions were mixed at room temperature for 72 h, evaporated in a vacuum evaporator at 50 °C to volume of $\sim$20 cm^3, and then dried in a desiccator above the concentrated sulfuric acid at 25 °C. After drying, a yellow crystalline complex was obtained and used for further investigations in this work as such.

The preparation of the physical mixture

Physical mixtures were prepared by simple mixing of UA with complex agents, β-CD or HP β-CD in mole ratio of 1:1.

Infrared Fourier transformation (FTIR)

FTIR spectra of the samples were recorded in KBr tablet (0.4 mg of sample, 140 mg of KBr) in wavelengths from 4,000 to 400 cm^{-1} on the FTIR spectrophotometer of Bomem Hartmann & Braun MB-series.

X-ray crystallography

X-ray diffraction was performed on a Phillips X'Pert powder diffractometer under the following conditions: the samples were exposed to monochrome CuK_α radiation and analyzed under the angle 2θ between 7 and 47° with 0.05° increments and recording time $\tau = 5$ s. The voltage and the strength of the electric current were 40 kV and 20 mA, respectively.

^{1}H-NMR spectrometry

^{1}H-NMR spectra of the samples were made on a Bruker AC 250 NMR spectrometer with operating frequencies of 250 MHz, in a 5 mm dia. glass cuvette at room temperature, by the pulse method with multiple pulse repetitions. D_2O was used as the solvent for UA:β-CD complex and UA:HP β-CD, and $CDCl_3$ for UA.

Differential scanning calorimetry (DSC)

DSC curves of the samples were recorded on a DuPont DSC differential scanning calorimeter with the scanning rate of 10 °C/min, with the temperature range of 20–360 °C. Thermal properties were studied by heating of about 5 mg of the sample in closed aluminum containers in the nitrogen atmosphere.

The solubility test

The content of UA liberated in aqueous solutions of inclusion complexes with cyclodextrin was determined by measuring UV absorbance at 282 nm on a Varian Cary UV–VIS-100 Conc. Spectrophotometer device. Distilled water was used as blind control trial.

Antimicrobial activity

Antimicrobial activity of UA, inclusion complexes of βCD:UA and HP β-CD:UA were tested against eight bacteria: *S. aureus, Staphylococcus epidermidis*, *Micrococcus luteus*, *E. faecalis*,

Echerichia coli, *Bacillus subtilis*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* and two fungi *Candida albicans* 10259 and *C. albicans* 10231. Minimum inhibitory concentrations (MICs) determination was performed by the serial dilution technique using 96-well microtitre plates. The investigated compounds were dissolved in 5 % DMSO (1 mg/ml) and added in broth medium with inoculum. The microplates were incubated for 48 h at 37 °C for bacteria and or 72 h at 28 °C, for fungi. The lowest concentrations without the visible growth were defined as MICs. The minimum bactericidal concentrations (MBCs) and fungicidal concentrations (MFCs) were determined by serial subcultivation of 2 ml into the microtitre plates containing 100 ml of broth per well and further incubation for 48 h at 37 °C or 72 h at 28 °C, respectively. The lowest concentration with no visible growth was defined as MBC/MFC respectively, indicating the killing of 99.5 % of the original inoculum. All experiments were performed in duplicate and replicated three times.

Results and discussion

Figure 2 shows FTIR spectra of UA (A), β-CD (B), the inclusion complex of β-CD with UA (C), HP β-CD (D) and the inclusion complex of HP β-CD with UA (E).

By analyzing FTIR spectra, the absence of the peaks characteristic of UA can be observed in the spectra of the UA complex with cyclodextrin. Those bands are specific for C–H stretching vibrations of the UA aromatic part of the structure which are present in the FTIR spectrum of the UA at 2,916 cm^{-1}, as the complex band of low intensity was not observed in the spectra of the complex. Also, C–H deformation vibrations out of the plane that are very indicative of the aromatic structure occur in the spectrum of the UA at 820 cm^{-1} as a band of high intensity, which was again not present in the spectra of the complex. This fact points to the inclusion of UA in the cavities of β-CD and HP β-CD. A wide band of low intensity at 3,427 cm^{-1} is the result of stretching vibrations of phenol OH groups located in the structure of UA. A band in this area of the complex and complex agents (β-CD and HP β-CD) is also present and corresponds to stretching OH vibrations of the hydroxyl groups present in these structures. What is characteristic is the appearance of moving the band centroid from the stretching OH vibrations in the complexes towards lower values of wave numbers compared to the status bar of the same vibration in the spectrum of UA.

Differences between OH stretching vibrations frequency of cyclodextrin complexes with UA and OH stretching vibrations of pure UA are 31 cm^{-1} (Table 1). The differences between OH stretching vibrations frequency of cyclodextrins and cyclodextrin complexes are 11 cm^{-1} and 35 cm^{-1} for cyclodextrin and HP β-CD, respectively (Table 1). Shifts towards higher wave number values occur at OH deformation vibrations in both complexes (Table 1).

These displacements are indicative of the establishment of hydrogen bonds between the proton donor group (the OH group of UA in this case) and the proton acceptor of O atoms from the glucopyranose units of cyclodextrin. Carbonyl C=O groups usually give the band at around 1,700 cm^{-1}; in the UA spectrum the band of these vibration occurs at 1,691 cm^{-1}. The reason for this shift towards the lower frequency is the weakening of C=O connections due to delocalization of π-electrons in the UA. In the complexes, this band additionally shifts towards lower frequences, even 5 cm^{-1} in comparing to the UA (Table 1).

This shift may be the reason for the existence of hydrogen bonds in the inclusion complexes which were acquired between the guest and host molecules.

Bands originating from the stretching C–O–C vibrations that occur in the FTIR spectrum of β-CD at 1,030 cm^{-1} and at 1,080 cm^{-1} do not change their frequency in the UA complex. In the FTIR spectrum of HPBCD bands of these vibrations occur at 1,031 cm^{-1} and 1,081 cm^{-1} and also remain unchanged in complex. This indicates that the C–O–C groups from the host molecules were not involved in interaction with the UA in forming the inclusion complexes.

Due to further investigations of inclusion complexes, ^{1}H NMR spectra of UA, complex agents and complexes with β-CD and HP β-CD are made. Figure 3 shows the ^{1}H NMR spectrum of UA.

Signal δ at 1.72, which appears in ^{1}H NMR spectrum of UA as a singlet is indicative of methyl group protons in the position C9b, while the singlet at 2.11 comes from the methyl group protons in C8 position. Methyl groups from –C=OCH_3 in the positions 2 and 6 of UA molecules give singlets at 2.67 and 2.68, respectively. The aromatic proton gives a signal at 5.98. Singlets at 11.05 and 13.33 correspond to the protons from aromatic OH groups in positions 7 and 9, respectively.

The comparative analysis of the ^{1}HNMR spectrum of complex agents with corresponding complexes recorded in D_2O at 25 °C shows that in the complexes some of the proton signals from the UA are absent, while others are moved. This is the first indicator that UA was included into the cyclodextrin cavities. In the UA complex with β-CD, there is a signal shift of H-3 proton in β-CD for 0.0012 ppm, while the signal position of H-5 remains unchanged. Both H-3 and H-5 protons are located in the inner part of cyclodextrin cavity, H-3 is gravitating towards the wider part of the cone and H-5 towards the narrower part of the cone. Since shifting of the signal for H-3 proton in the complex was observed, it indicates that the inclusion of UA went with the wider part of the cone.

In the case of the UA complex with HP β-CD shiftings of the signal from H-3 proton are even higher, amounting to 0.102 ppm. H-5 and H-6 protons have a shift of

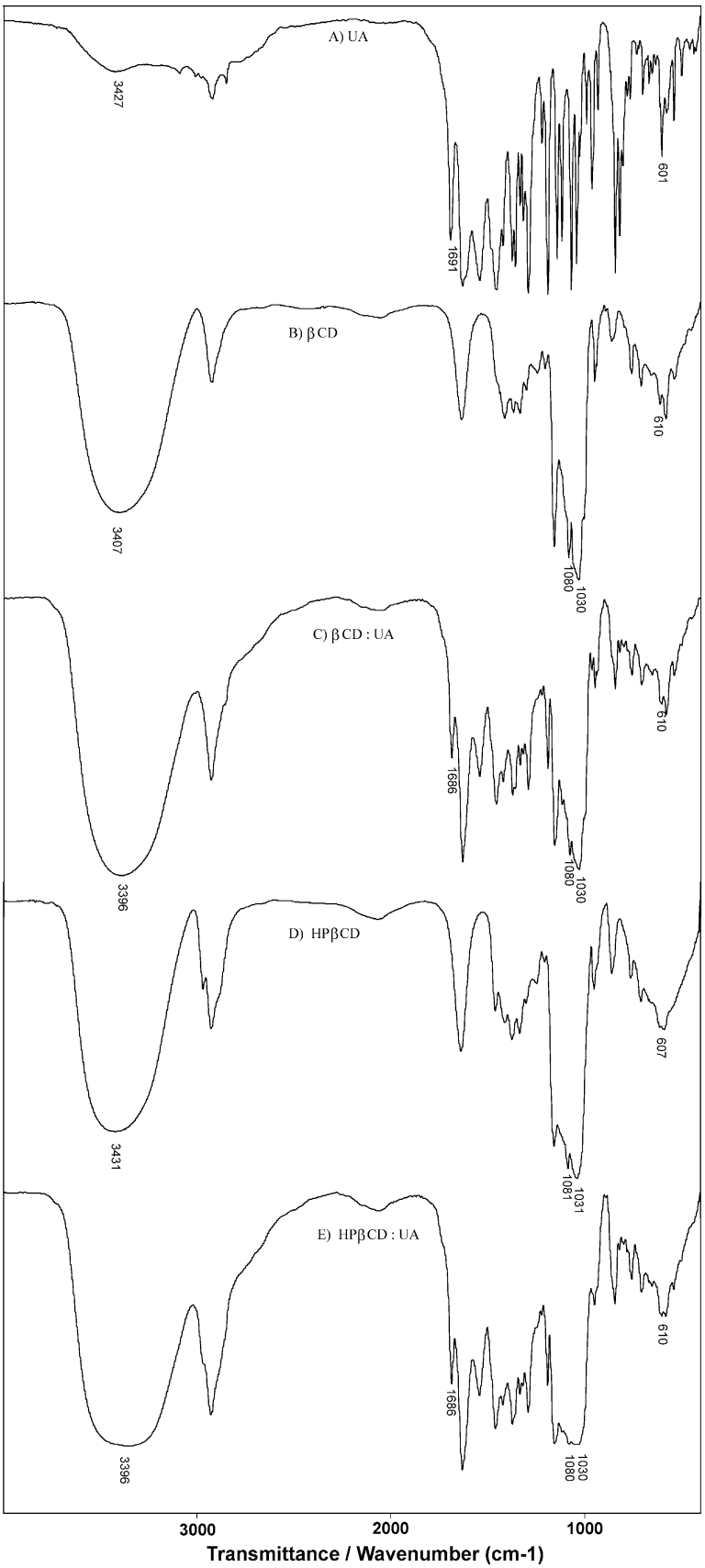

Fig. 2 FTIR spectra of UA (**A**), β-CD (**B**), the inclusion complex of β-CD with UA (**C**), HP β-CD (**D**), the inclusion complex of HP β-CD with UA (**E**)

Table 1 Frequency bands and their shifts in the FTIR spectra of UA, cyclodextrins and inclusion complexes indicating the forming of complexes

	UA	Δ^1	Δ^2	βCD	Δ^3	βCD:UA	HP βCD	Δ^4	HP βCD:UA
Frequency bands and their shifts, cm^{-1}									
$\nu_{(OH)}$	3,427	−31	−31	3,407	−11	3,396	3,431	−35	3,396
$\gamma_{(OH)}$	601	+9	+9	610	0	610	607	+3	610
$\nu_{(C=O)}$	1,691	−5	−5	–	–	1,686	–	–	1,686
$\nu_{(C–O–C)}$	–	–	–	1,030	0	1,030	1,031	−1	1,030
	–	–	–	1,080	0	1,080	1,081	−1	1,080

$\Delta^1 = \nu$ (or $\gamma)_{\beta CD:UA}$ - ν (or $\gamma)_{UA}$

$\Delta^2 = \nu$ (or $\gamma)_{HP\beta CD:UA}$ - ν (or $\gamma)_{UA}$

$\Delta^3 = \nu$ (or $\gamma)_{\beta CD:UA}$ - ν (or $\gamma)_{\beta CD}$

$\Delta^4 = \nu$ (or $\gamma)_{HP\beta CD:UA}$ - ν (or $\gamma)_{HP\beta CD}$

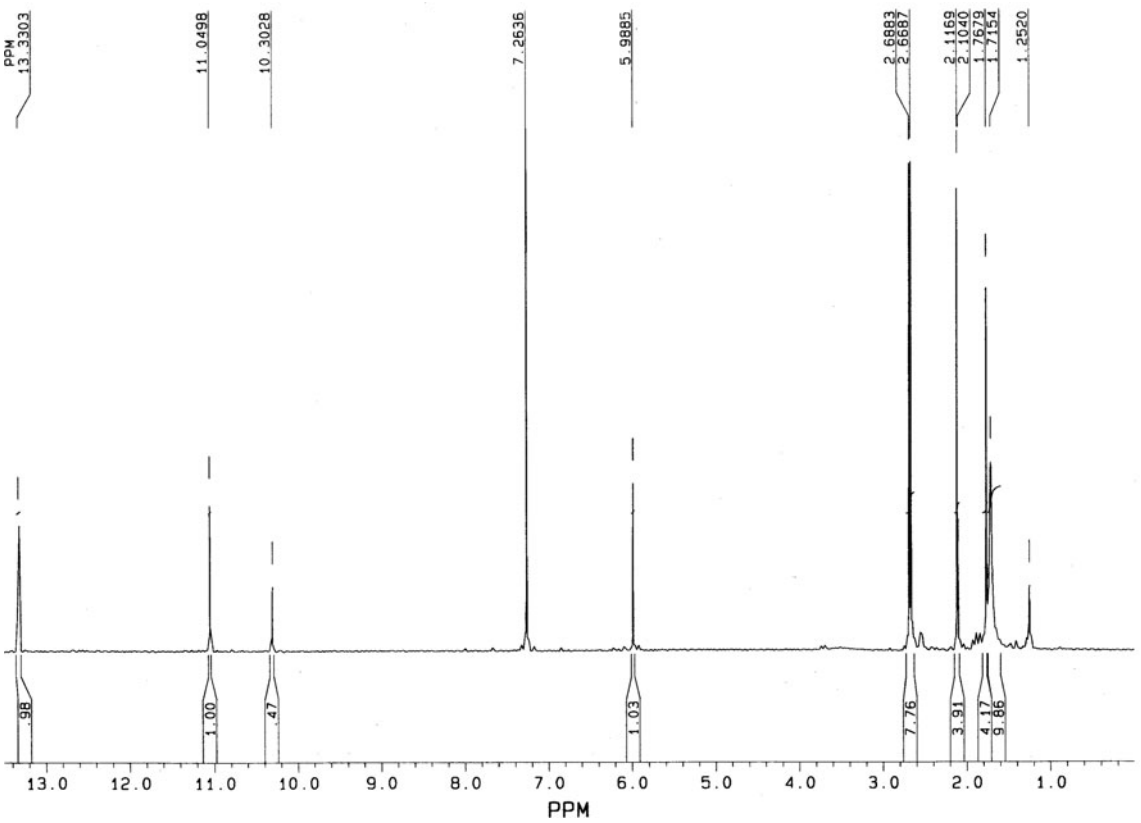

Fig. 3 ^{1}H NMR spectrum of UA in $CDCl_3$

0.0134 ppm, while protons from the methyl group of HP β-CD have a shift of 0.0073 ppm. These shift from the protons given indicate the formation of the corresponding inclusion complexes.

These low values of chemical shifts indicate a non-covalent interactions between guest and host molecules, which is in agreement with the NMR analysis of cyclodextrin inclusion complexes with other compounds such as atenolol, nifedipine, amlodipine, allicin [25–33].

Important chemical shifts for the β-CD, HP β-CD and the corresponding inclusion complexes with UA are shown in Table 2.

Figure 4 illustrates the most likely way of UA inclusion in the cyclodextrin cavities.

Table 2 Important chemical shifts (δ) for the βCD signal, βCD:UA inclusion complex, HPβCD and HPβCD:UA inclusion complex

	δ (ppm)			
	βCD	βCD complex	HPβCD	HPβCD complex
H-3 of βCD	3.9533	3.9521		
H-6 of βCD	3.8654	3.8654		
H-5 of βCD	3.8337	3.8337		
H-3 of HPβCD			3.9472	3.9370
H-5, H-6 of HPβCD			3.8569	3.8435
CH_3 of HPβCD			1.1313	1.1240

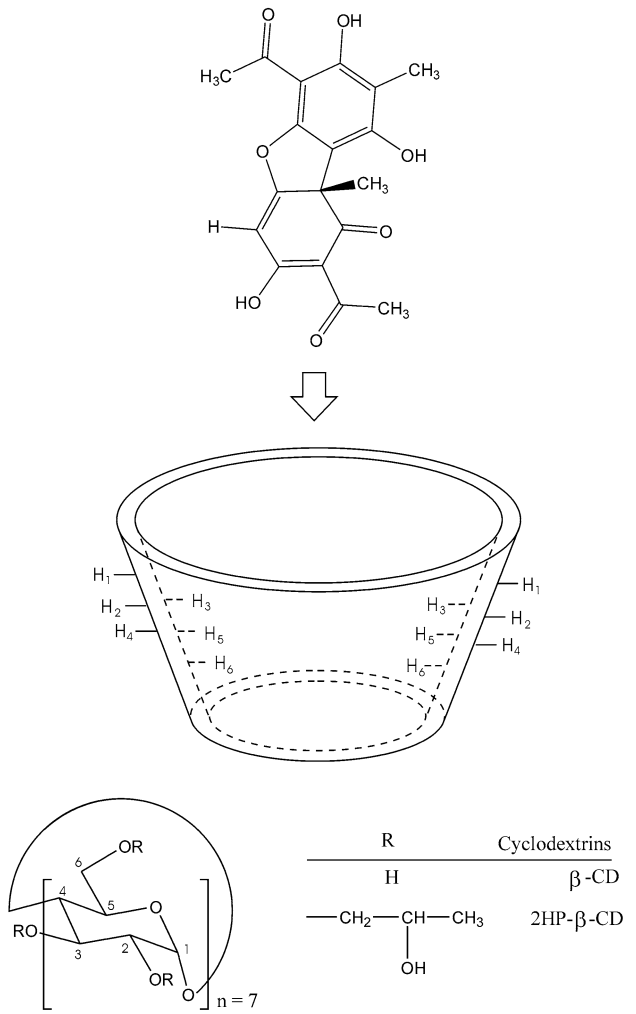

Fig. 4 Schematic presentation of possible UA inclusion in the cyclodextrin cavity

Diffraction patterns of UA inclusion complexes with the corresponding cyclodextrins and complex agent (host and guest molecules) are shown in Fig. 5a, b.

In Figure 5a, b a high similarity between the diffraction patterns of the complex and the ones of the complex agents of β-CD and HP β-CD is observed. Since the UA was included in the cyclodextrin cavities, it is protected from the X-rays so there are no characteristic peaks present in the diffraction patterns. The diffraction pattern of HP β-CD shows a wide diffraction peak which is not structured and points to the disorder of the crystal structure at large distances, while the β-CD is more "crystalic", i.e., it contains more pronounced diffraction peaks. In both cases diffraction patterns of the physical mixture show a simple collection of guest and host components, i.e., they contain UA and cyclodextrin peaks, which is not the case with the corresponding complexes.

DSC diagrams of UA, β-CD, HP β-CD, the corresponding inclusion complexes and physical mixtures are shown in the Fig. 6a, b.

DSC curves of both complexes show temperature changes that differ from both host and guest molecules. UA has a strong endothermic peak in a narrow range of about 200 °C, which in fact represents its melting point. In DSC curves of both complexes this peak is absent. In the UA complex with β-CD peak appears at 166 °C and in the complex with HP β-CD at 213 °C. Such changes in DSC diagrams are the result of new supramolecular structures formation in which the thermal change of molecules included is altered.

The solubility test

The improved solubility of UA in water was achieved by molecular incapsulation. In the complex with βCD the solubility is 0.3 mg/cm^3 and 4.2 mg/cm^3 with HP βCD, while the uncomplexed UA solubility is 0.06 mg/cm^3. Solubility profiles of pure UA, βCD:UA and HP βCD:UA during 80 h are shown in Fig. 7.

The results of the microbial activity of UA, βCD:UA and HP βCD:UA tested against eight bacteria: *S. aureus, S. epidermidis, M. luteus, E. faecalis, E. coli, B. subtilis, K. pneumoniae* and *P. aeruginosa* and two fungi *C. albicans* 10259 and *C. albicans* 10231 are shown in Table 3.

The presented results indicate that the antimicrobial activity of inclusion complexes is not reduced as compared to the antimicrobial activity of pure UA. Specifically,

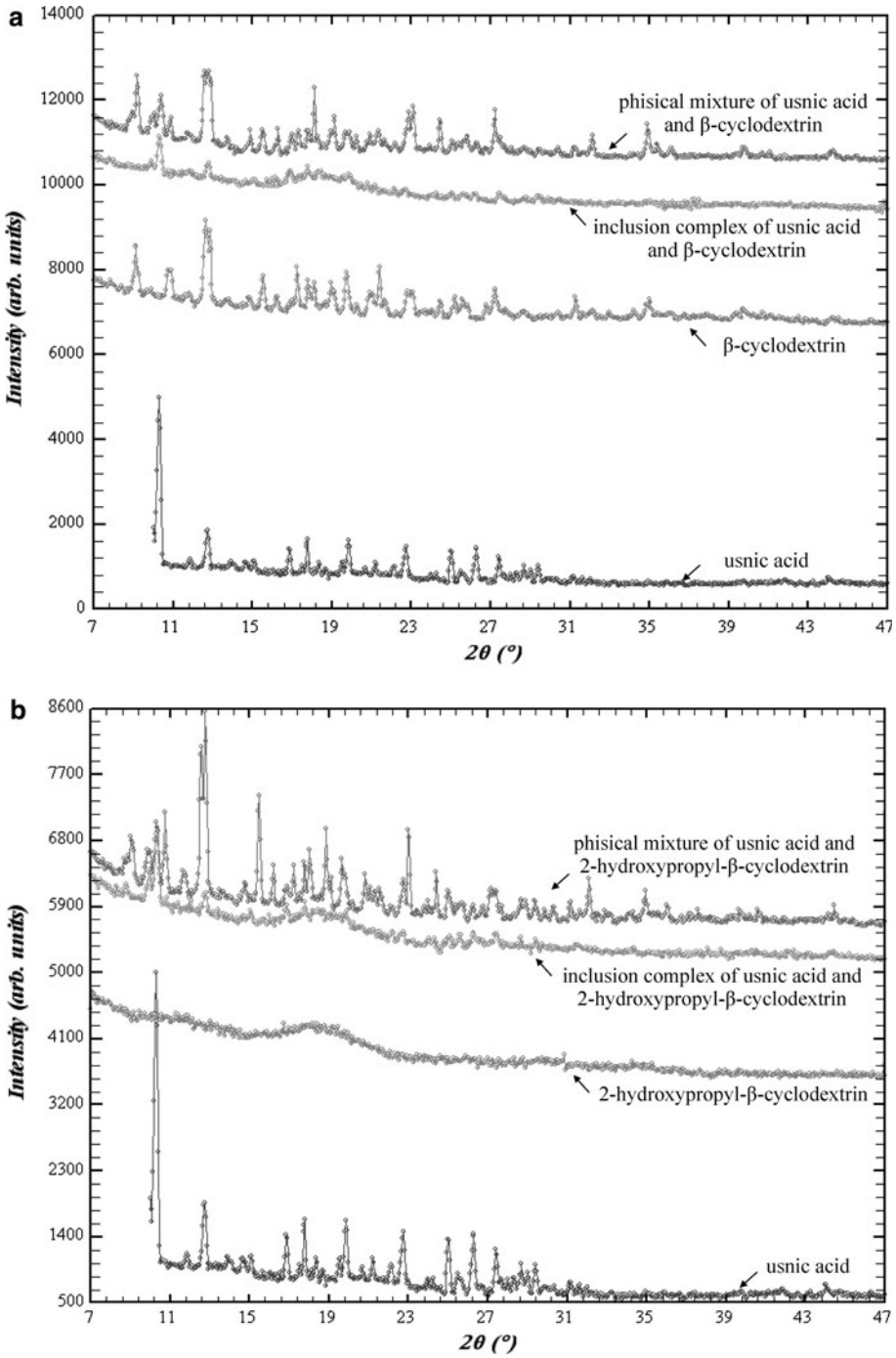

Fig. 5 XR diffraction patterns: **a** UA, β-CD, β-CD:UA complex, physical mixture of UA with β-CD; **b** UA, HP β-CD, HP β-CD:UA complex, physical mixture of UA with HP β-CD

inclusion complexes have shown a much better microbial activity in individual cases compared to pure UA. The MIC that both HP βCD:UA and βCD:UA inclusion complexes show against the bacteria of *S. epidermidis, M. luteus, E. faecalis* and against the tested fungi are four times higher than the MIC of pure UA. MIC of HP βCD:UA shows four times greater activity while MIC of βCD:UA shows two times greater activity compared to the pure UA against the *S. aureus* bacteria. Both complexes, along with UA, show the identical MIC against the bacteria of *E. coli, B. subtilis, K. pneumoniae* and *P. aeruginosa.*

Conclusion

By applying the appropriate methods it is shown that UA can be obtained as the inclusion complex with β-CD and its HP β-CD derivative, giving supramolecular systems that

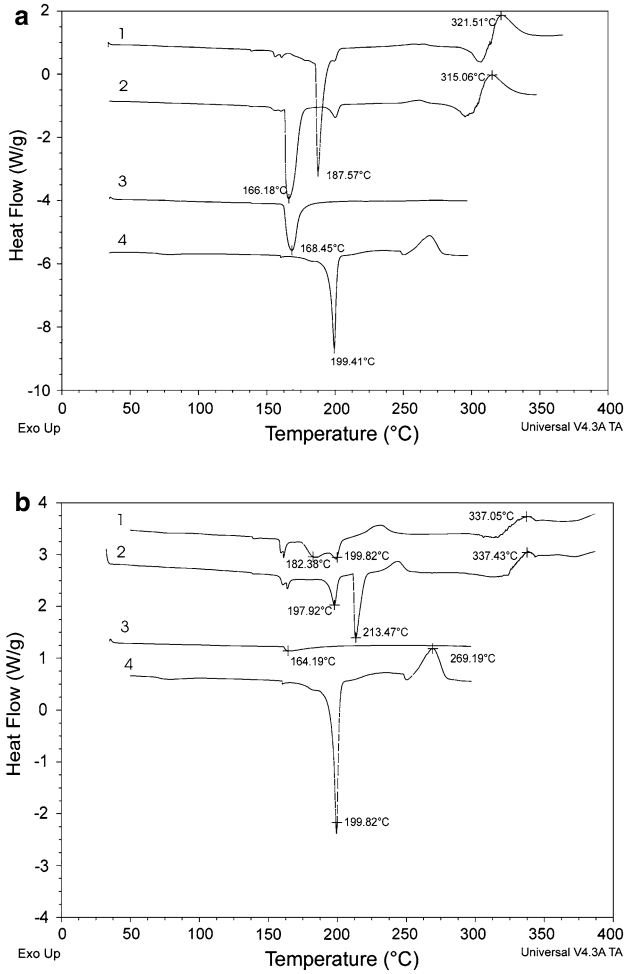

Fig. 6 DSC diagrams: **a** the physical mixture of βCD:UA (*1*), β-CD:UA complex (*2*), β-CD (*3*) and UA (*4*); **b** the physical mixture of UA with HP β-CD (*1*), HP β-CD:UA complex (*2*), HP β-CD (*3*) and UA (*4*)

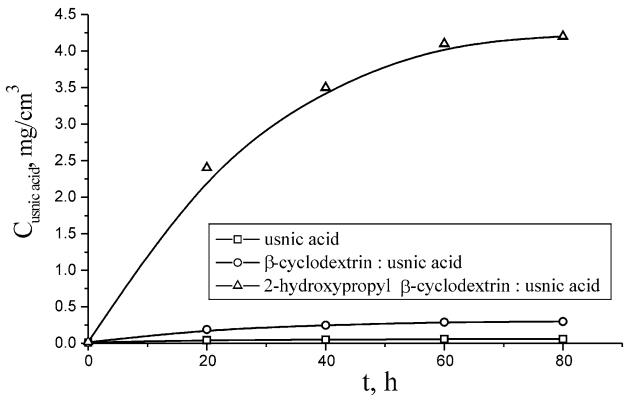

Fig. 7 Solubility profiles of pure UA, βCD:UA and HP βCD:UA over time The microbial activity

could be of interest for the development of pharmaceutical formulations. In both complexes a much higher degree of UA solubility was achieved, along with the increased antimicrobial activity. These inclusion complexes can provide a satisfactory bioavailability and application of UA as a safer pharmacological agent with a wide range of effects.

Table 3 The microbial activity of UA, βCD:UA and HP βCD:UA against bacteria and fungi

	UA	HP βCD:UA	βCD:UA
The MIC (μg/ml)			
S. aureus	31.25	125	62.5
S. epidermidis	31.25	125	125
M. luteus	31.25	125	125
E. faecalis	31.25	125	125
E. coli	62.5	62.5	62.5
B. subtilis	62.5	62.5	62.5
K. pneumoniae	62.5	62.5	62.5
P. aeruginosa	62.5	62.5	62.5
C. albicans 10259	31.25	125	125
C. albicans 10231	62.5	125	125

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