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ORIGINAL ARTICLE

# Inclusion complex of usnic acid with β-cyclodextrin: characterization and nanoencapsulation into liposomes

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**Abstract** In this study β-cyclodextrin (β-CD) was used to improve usnic acid (UA) solubility and the inclusion complex (UA:β-CD) was incorporated into liposomes in order to produce a targeted drug delivery system for exploiting the antimycobacterial activity of UA. A phase-solubility assay of UA in β-CD at pH 7.4 was performed. An apparent stability constant of $K_{1:1} = 234.5\ M^{-1}$ and a complexation efficiency of 0.005 was calculated. In the presence of 16 mM of β-CD the solubility of UA (7.3 μg/mL) increased more than 5-fold. The UA:β-CD complex was prepared using the freeze-drying technique and characterized through infrared and $^1$HNMR spectroscopy, X-ray diffraction and thermal analyses. The UA:β-CD inclusion complex presented IR spectral modifications when compared with UA and β-CD spectra. $^1$HNMR spectrum of UA:β-CD inclusion complex showed significant chemical shifts in proton H5 located inside the cavity of β-CD ($\Delta\delta = 0.127$ ppm), suggesting that phenyl ring moiety of UA would be expected to be included within the β-CD cavity, interacting with the H-5 proton. A change in UA from its crystalline to amorphous form was observed on X-ray, suggesting the formation of a drug inclusion complex. DSC analysis showed the disappearance of the UA fusion peak UA:βCD complex. No differences between the antimicrobial activity of free UA and UA:βCD were found, supporting the hypothesis that the complexation with cyclodextrin did not interfere with drug activity. Liposomes containing UA:βCD were prepared using hydration of a thin lipid film method with subsequent sonication. Formulations of liposomes containing UA:βCD exhibited a drug encapsulation efficiency of 99.5% and remained stable for four months in a suspension form. Interestingly, the encapsulation of UA:βCD into the liposomes resulted in a modulation of in vitro kinetics of release of UA. Indeed, liposomes containing UA:β-CD presented a more prolonged release profile of free usnic acid compared to usnic acid-loaded liposomes.

**Keywords** β-cyclodextrin · Usnic acid · Inclusion drug complex · Liposomes · Antimicrobial activity

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## Introduction

Cyclodextrins (CDs) are cyclic oligomers formed by the action of certain enzymes on starch. They are oligomers of α-D-glucose, which are linked by glycosidic bonds between α-(1,4) carbon atoms [1]. The glucose chains form a cone-linked cavity into which hydrophobic compounds,

or part of them, may enter and form a water-soluble complex, thereby changing the physicochemical properties of the included drug [2]. The number of glucose units determines the name and size of the cone-linked cavity: α-cyclodextrin, β-cyclodextrin and γ-cyclodextrin, having six, seven and eight glucose units and an inner diameter cavity of 5.2, 6.6 and 8.4 Å, respectively. The most common pharmaceutical application of cyclodextrins is that of enhancers of solubility of hydrophobic drugs, but they can also increase the stability and bioavailability of drug molecules. In fact, it has been extensively reported in the literature that cyclodextrins can form inclusion complexes with a variety of hydrophobic compounds, changing the latter's physicochemical characteristics [3, 4]. The entrapment of drug inclusion complexes into drug delivery systems, such as liposomes [5–9] or nanoparticles [10], restrains their dissociation and contributes to altering the pharmacokinetics of the drugs in question.

Usnic acid (Fig. 1), a yellowish pigment, is a product of the secondary metabolism of lichens and exists in two enantiomeric forms (+)-usnic acid and (−)-usnic acid, which differ in orientation from the methyl group located in chiral carbon at the 9b position [11]. This dibenzofuran derivative is widely distributed in species of *Cladonia, Usnea, Lecanora, Ramalina, Evernia* and *Parmotrema* [12]. Usnic acid is one of the most extensively studied lichen derivatives because it exhibits, among others, antimicrobial [13] and antimycobacterial activities [14]. However, the therapeutic usefulness of usnic acid's potential benefits is limited by its unfavorable physicochemical properties, especially its very poor water solubility [15] and hepatotoxicity [16–18]. As a result, its use in a safe and efficient manner requires a suitable solubilizer agent and/or carrier system for improving the therapeutic index of this drug.

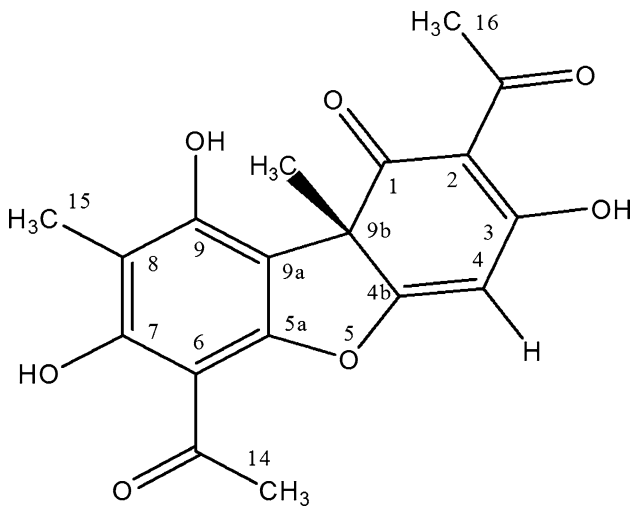


**Fig. 1** Chemical structure of usnic acid with atom assignments

Within this framework, the goal of this research was firstly to investigate the possibility of forming an inclusion complex of usnic acid with β-cyclodextrin and to prepare and characterize the usnic acid:β-cyclodextrin complex and, secondly, to encapsulate this complex into liposomes in order to produce a targeted drug delivery system for exploiting the antimycobacterial activity of usnic acid. The liposomal formulation containing the usnic acid:β-cyclodextrin complex was characterized and the in vitro kinetics of the drug evaluated.

## Materials and methods

### Reagents

(+)-usnic acid (98%), cholesterol (CH), octadecylamine (SA) and β-cyclodextrin were purchased from Sigma-Aldrich (St. Louis, USA); soya phosphatidylcholine (98% Epikuron 200®) was obtained from Lucas Meyer (Hamburg, Germany); and solvents and other chemicals were supplied by Merck (Darmstadt, Germany).

### Methodology

#### *Phase solubility study of usnic acid in β-cyclodextrin*

The phase solubility assay of usnic acid in β-CD in a phosphate buffer solution (pH 7.4) was performed according to Higuchi and Connors [19]. An excess amount of usnic acid (10 mg) was added to 1 mL of a phosphate buffer solution (pH 7.4) or β-cyclodextrin in buffer solutions at concentrations ranging from 0 to 16 mg/mL. The mixtures were shaken vigorously at 25 ± 1 °C until equilibrium was attained (about 72 h). An aliquot (250 μL) of the mixture was removed and filtered (millex® filter, Millipore, USA), and the supernatant analyzed for usnic acid content using spectrophotometry (Ultrospec® 300, Armshan Pharmaceutical) at 280 nm [20]. Assuming the formation of a complex with a 1:1 stoichiometric ratio, the apparent stability constant ($K_{1:1}$) was calculated from the linear relationship between the molar concentration of UA in the solution medium versus the β-CD molar concentration according to the equation: $K_{1:1} = \text{slope}/[S_0(1\text{-slope})]$, where $S_0$ is the solubility of usnic acid in the absence of β-cyclodextrin. The complexation efficiency (CE) was calculated from solubility data according to CE = slope/(1-slope) [21].

#### *Preparation of UA:β-CD inclusion complex*

The UA:β-CD solid inclusion complex was prepared using the freeze-drying technique in a 1:1 molar ratio

($1.45 \times 10^{-4}$ mol). The freeze-dried product was obtained by dissolving the β-cyclodextrin in water and slowly adding usnic acid in powder form. The mixture was stirred for 24 h, frozen in liquid nitrogen and lyophilized.

*Characterization of UA:β-CD inclusion complex*

The UA:β-CD inclusion complex was characterized using infrared and $^1$HNMR spectroscopy, X-ray powder diffraction and thermal analysis. Infrared spectra (IR) were recorded on an IR-TF Galaxy 3000 Mattson spectrometer in the 4,000–400 $cm^{-1}$ range using the KBr disc method. Proton nuclear magnetic resonance ($^1$H NMR) analysis was carried out using a Brucker DPX-200 Avance (200 MHz) equipment with samples diluted in deutered dimethyl sulfoxide ($d^6$-DMSO). Temperature-dependent X-ray powder diffraction data were gathered with Ni-filtered Cu Kα radiation ($\lambda = 1.50405$ nm) on a digitalized diffractometer (Rigaku Geiger-flex 2073). Differential scanning calorimetry (DSC) thermograms of the UA:β-CD inclusion complex, physical mixture and pure compounds (3 mg) were obtained with a DSC-50 thermal analyser (Shimadzu, Japan), using 50 mL/min nitrogen rate flow with temperatures ranging from 50 to 400 °C and a heating rate of 10 °C/min.

The content of usnic acid in the inclusion complex was assessed by dissolving an accurately weighed amount of UA:β-CD in a mixture of chloroform and methanol (1:1), sonicated for 5 min, and the volume filled to 5 mL with methanol. The suspension in which the β-cyclodextrin is insoluble but usnic acid is soluble was then centrifuged at 8.776 *g*, and the usnic acid solubilized in the supernatant was quantified by UV spectroscopy at 280 nm [20]. A standard curve of usnic acid was prepared in methanol at concentrations ranging from 1 to 15 μg/mL ([UA] = Abs—*a*/*b*; $a = 0.0746$; $b = 0.0111$; $r = 0.999$).

*Antimicrobial activity of UA:βCD complex*

The antimicrobial activity of UA:β-CD against three different microorganisms was evaluated to investigate the effect of complexation with β-cyclodextrin on usnic acid activity. The antimicrobial activity of usnic acid was tested on *Streptococcus mutans* (ATCC 25175), *Enterococcus faecalis* (ATCC 14 508) and *Actinobacillus actinomycetecomitans* (Y4-FDC, from the collection of the Federal University of Rio de Janeiro, Brazil), using the agar disc diffusion method. A sample of UA:β-CD (3 mg) was dissolved in sterile water to obtain 25, 50 and 75 μg/mL of drug concentrations. Usnic acid dissolved in DMSO (75 μg/mL) was used as a positive control. DMSO and βCD solutions were used as negative controls. The imbibed discs with sample solutions were placed on surface of Petri dishes containing culture medium inoculated with microorganism. Samples were tested on microorganism cultures 24 h previously seeded in their specific growing medium as follows: *E. faecalis* on agar medium, *S. mutans* on mitis salivarius agar and *A. actinomycetecomitans* on blood agar. The inhibitory zone diameters were determined after 24 h incubation.

*Entrapment of UA:βCD complex into liposomes*

Liposomes containing the UA:βCD complex (UA:βCD-Lipo) were prepared using the thin lipid film method [22]. Briefly, soya phosphatidylcholine, cholesterol and stearylamine (7:2:1) were dissolved in a mixture of $CHCl_3$:MeOH (3:1 v/v) under magnetic stirring. The solvents were removed under pressure for 60 min (37 ± 1 °C, 80 rpm), producing a thin lipid film. This film was then hydrated with 10 mL of an aqueous phase composed of UA:βCD complex dissolved in pH 7.4 phosphate buffer solution, resulting in the formation of multilamellar liposomes. The liposomal suspension was kept under magnetic stirring for 5 min and was then sonicated (Vibra Cell, BRANSON, USA) at 200 W and 40 Hz for 300 s to form small unilamellar liposomes. Finally, liposomes were frozen at –80 °C and lyophilized (EZ-DRY, FTSS System, New York, USA) at 200 bars for approximately 16 h. Liposomes containing usnic acid (UA-Lipo) were also prepared in the conditions described above by dissolving the drug into the organic phase.

*Characterization of UA:βCD-loaded liposomes*

The stability of usnic acid inclusion complex-loaded liposomes was evaluated using standard accelerated and long-term stability testing. After preparation, samples of liposomal suspension were submitted to centrifugation (1.300 *g* for 1 h at 25 °C), horizontal mechanical stirring (180 strokes/min for 48 h at 37 °C) and freeze-thaw cycles (−18 °C for 16 h and 25 °C for 8 h). For long-term stability evaluation, the macroscopic appearance and pH variation were monitored after preparation and at predetermined time intervals. Lyophilized liposomal samples (stored at 4 °C) were hydrated at predetermined time intervals and analyzed for determination of the usnic acid content. Liposomes were diluted with a mixture of chloroform/methanol (1:1) for a theoretical concentration of 9.6 μg/mL, and then sonicated for 5 min. Next, the volume was filled with methanol to 5 mL and the content of usnic acid determined at 280 nm as previously described.

Drug encapsulation efficiency was determined by the ultrafiltration/ultracentrifugation technique using Ultrafree® units (Millipore, USA). After centrifugation of the samples (Ultracentrifuge KT-20000, Kubota, Japan) at 8.776 *g* for 1 h at 4 °C, the usnic acid concentration in the

supernatant was measured by UV spectroscopy at 280 nm and the usnic acid encapsulation ratio calculated in relation to its initial content in the liposome formulation.

*Antimicrobial activity of UA:β CD inclusion complex*

The antimicrobial activity of usnic acid complexed with β-cyclodextrin encapsulated in liposomes (UA:β-CD-Lipo) evaluated was against ATCC and MRSA *Staphylococcus aureus* using the agar diffusion well method. The antimicrobial activity of pure usnic acid and usnic acid encapsulated in liposomes (UA-Lipo) were also evaluated for comparison purposes. ATCC 6538 and 6538P, and clinical isolated multiresistants strains of *S. aureus* (Brazilian pediatric clone AM 793 and pediatric clone AM 942, from the collection of the Federal University of Pernambuco, Brazil) were used to susceptibility tests. An aliquot of UA in DMSO solution (1,200 μg/mL) was diluted in sterile water to 600 and 300 μg/mL as well as liposomal formulations UA-Lipo and UA:β-CD-Lipo (1,200 μg/mL). Tetraciclin (300 μg/mL) was used as the antibiotic standard of sensibility/resistance of the tested microorganisms. DMSO and β-CD were used as negative controls. Microorganism suspensions were resuspended in sterile water using MacFarland scale ($10^8$ UFC). Agar plates were seed with bacterial suspension; 6 wells of 6 mm were performed and filled with testing solutions (100 μL). After 2 h of prediffusion, plates were incubated at 37 ± 1 °C for 24 h and inhibition halos were measured. Assays were carried out in triplicate and data analyzed at significant level ($p < 0.01$).

*In vitro kinetics of release of UA from UA:βCD-loaded liposomes*

The in vitro kinetics of release of usnic acid from UA:βCD-Lipo was assessed using the dialysis technique in comparison with UA-Lipo and UA:βCD. 1-mL aliquots of each formulation were placed separately inside dialysis bags (cellulose membrane, cut-off = 12,400 MW, Sartorius, Germany), which were sealed and immersed in a vessel containing 100 mL pH 7.4 phosphate buffer solution. The release system was maintained under magnetic stirring at 100 rpm and 37 ± 1 °C and at predetermined time intervals a 1 mL aliquot of dissolution medium was removed and the usnic acid content measured. After collection of the samples, the kinetic medium was refilled with an appropriate amount of phosphate buffer solution. The in vitro release profiles of usnic acid from liposomes were obtained by representing the percentage of the drug released in relation to the amount of the drug encapsulated. Results are expressed as the percentage of released drug as a function of time and values represent the mean ± standard deviation (SD) of three independent measurements.

The kinetic data of usnic acid from the inclusion complex and from liposomal formulations were fitted according to an exponential model using the following equation: $M_t/M_\infty = \left(1 - k_1.e^{-k_2 t}\right)$, where $M_t$ and $M_\infty$ are the mass of the drug released at a determined time ($t$) and at an infinite time ($t_\infty$) of the kinetic process, respectively; $k_1$ is a fitting constant, and $k_2$ is the kinetic rate constant.

## Results and discussion

Phase solubility of usnic acid in β-cyclodextrin

Figure 2 shows the phase solubility profiles of usnic acid in β-cyclodextrin in phosphate buffer solutions (pH 7.4) at different concentrations. A Type $A_L$ diagram [19] is displayed, where a linear relationship between the apparent drug solubility and β-cyclodextrin concentration can be seen, indicating the formation of soluble complexes. The fact that the solubility of usnic acid increased in the presence of β-cyclodextrin is a sign that one or more molecular interactions between them occurred to form distinct chemical species, which may be termed complexes. However, there is no guarantee that the complexes are first-order in the host. Since the slope of the $A_L$ diagram was close to unity, the stoichiometric 1:1 ratio was assumed for calculating the equilibrium constant. In this manner, the value of the binding equilibrium constant ($K_{1:1}$) of the usnic acid and β-cyclodextrin complex was 234.5 $M^{-1}$ (slope = 10.0049; $S_0$ (intercept) = 0.024; $r$ = 0.98701).

In the presence of 16 mM (1.8%) of βCD the solubility of usnic acid increased more than 5-fold at 37 °C, since the solubility of usnic acid in phosphate buffer solution at 25 °C was 7.32 μg/ml (0.02 mM). As reported, there was a 70-fold solubility increase in usnic acid in 10%

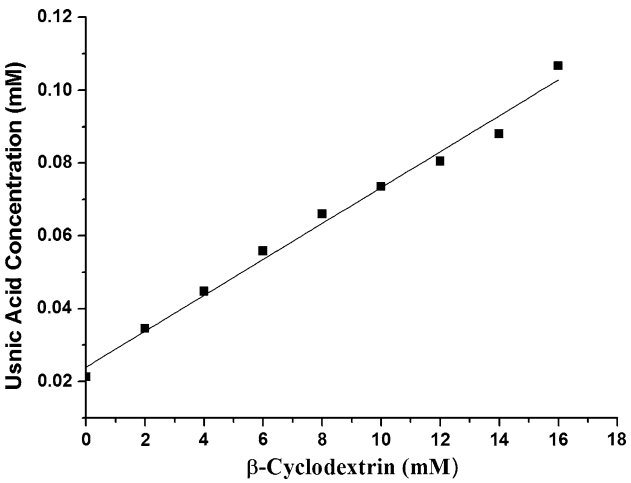


**Fig. 2** Phase solubility diagram of usnic acid (UA) in the presence of β-cyclodextrin (βCD) in phosphate buffer solution (pH 7.4) at 25 °C

hydroxypropyl-$\beta$-cyclodextrin (HP$\beta$CD) (350 μg/ml) at 25 °C in comparison with its water solubility (5 μg/ml) [23].

## Characterization of UA:$\beta$CD inclusion complex

There is still no consensus on the preparation of inclusion complexes, probably because each drug to be complexed is a special case and the optimal experimental conditions depend on the characteristics of both host and guest molecules [24]. In the case of usnic acid the freeze-drying method led to the formation of a stable complex, showing a significant interaction between the drug and $\beta$-cyclodextrin, as described below.

## Spectroscopic analysis

IR spectra of usnic acid, $\beta$-cyclodextrin, physical mixture of UA and $\beta$-CD, and UA:$\beta$-CD inclusion complex are presented in Fig. 3. The IR spectrum of UA showed characteristic peaks and vibrational assignments at 1,700 and 1,070 $cm^{-1}$ in agreement with data previously reported [25, 26]. Key vibrational assignments of the conjugated cyclic ketone group are found at 1,694 $cm^{-1}$. It is also possible to verify weak bands at 1,716 and 1,676 $cm^{-1}$ assigned to the $\nu(C = O)$ non-conjugated cyclic ketone and the non-conjugated methyl ketone, respectively. Furthermore, the antisymmetric and symmetric $\nu$(COC) aryl alkyl ether bands at approximately 1,288 and 1,070 $cm^{-1}$, respectively, are also presented.

A characteristic broad band at 3,700 and 3,100 $cm^{-1}$ corresponding to assignments of $\nu$(O–H) stretching is shown in the spectra of the $\beta$-CD. Characteristics peaks at 1,056–1,028 $cm^{-1}$ (C–O–C stretching) were also found [27, 28]. The spectra of UA:$\beta$-CD inclusion complex showed pronounced modification in the peaks intensity and shape at 1,750 and 1,250 $cm^{-1}$, indicating that interactions between UA and $\beta$-CD have taken place. Furthermore, a decrease in the intensity of bands around 1191, 1144 and 1118 $cm^{-1}$, corresponding to vibrational $\nu$(O–H) was observed, suggesting that an interaction between both molecules may have occurred.

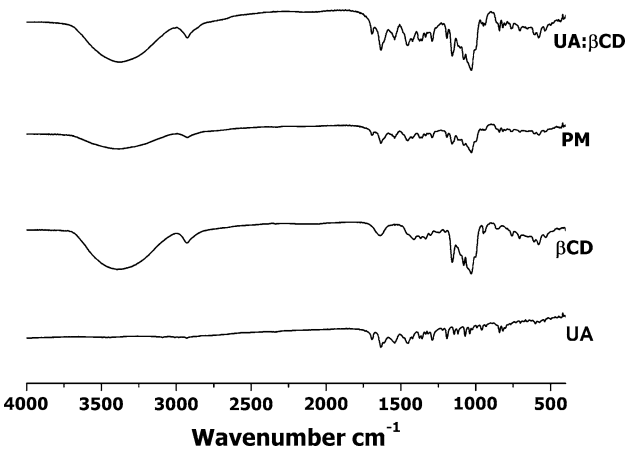


**Fig. 3** Infrared (IR) spectra of usnic acid (UA), $\beta$-cyclodextrin ($\beta$CD), physical mixture (PM) and inclusion complex (UA:$\beta$CD). Spectra were fitted using Origin 6.0 software

Tables 1 and 2 report the $^1$H NMR signals of UA and UA:$\beta$-CD inclusion complex as well as the variation in chemical shifts ($\Delta\delta$). $^1$H NMR spectrum of UA in deutered dimethyl sulfoxide showed signals at 13.406 and 11.396 ppm due to the protons of the hydroxyl-groups 7 and 9, respectively. A signal at 6.258 ppm due to the single aromatic proton in position 4 and the signals at 2.616, 2.046 and 2.506 ppm have been attributed to the 14, 15 and 16 methyl groups, respectively. The signals in the spectra of usnic acid and $\beta$-cyclodextrin were in agreement with data reported in the literature [26, 29]. A significant upfield shift for the resonance of the H-4 proton of usnic acid ($\Delta\delta = 0.177$ ppm) was observed in the presence of $\beta$-CD (Table 1). Changes in the signals of the H-4 proton located outside the cavity of $\beta$-CD ($\Delta\delta = 0.127$ ppm) and the H-5 proton located inside the cavity of $\beta$-CD ($\Delta\delta = 0.115$ ppm) were detected in the presence of UA (Table 2).

**Table 1** Proton NMR signals of usnic acid and $\beta$-CD complex (chemical shifts observed in signals of usnic acid)

| UA protons | $\delta_{UA}$ | $\delta_{UA:\beta CD}$ | $\Delta\delta$ (ppm) |
|---|---|---|---|
| 2 - $OCCH_3$ (16) | 2.506 | 2.511 | 0.005 |
| 3 - OH | – | – | – |
| 4 - H | 6.258 | 6.435 | 0.177 |
| 6 - $OCCH_3$ (14) | 2.616 | 2.669 | 0.053 |
| 7 - OH | 13.406 | 13.362 | −0.044 |
| 8 - $CH_3$ (15) | 2.046 | 2.002 | −0.044 |
| 9 - OH | 11.396 | 11.359 | −0.037 |
| 9b - $CH_3$ | 1.728 | 1.730 | 0.002 |

**Table 2** Proton NMR signals of usnic acid and $\beta$-CD complex (chemical shifts observed in signals of $\beta$-ciclodextrin)

| $\beta$-CD protons | $\delta_{\beta\text{-CD}}$ | $\delta_{UA:\beta\text{-CD}}$ | $\Delta\delta$ (ppm) |
|---|---|---|---|
| H1 | 4.82 | 4.833 | 0.013 |
| H2 | 3.31 | 3.341 | 0.031 |
| H3 | 3.61 | 3.566 | −0.044 |
| H4 | 3.28 | 3.407 | 0.127 |
| H5 | 3.55 | 3.435 | 0.115 |
| H6 | 3.66 | 3.637 | 0.023 |
| OH (2) | 5.71 | 5.699 | −0.011 |
| OH (3) | 5.66 | – | – |
| OH (6) | 4.57 | 4.457 | −0.113 |

Further, chemical shift of the 6-$OCCH_3$(14) phenolic group of UA in the presence of $\beta$-CD ($\Delta\delta = 0.053$ ppm), suggests that phenyl ring moiety of UA would be expected to be included within the $\beta$-CD cavity interacting with the H-5 proton. Generally, very low shifts in signals of $\beta$-CD protons are recordable for inclusion complexes, which means that the formation of an external associative complex between the drug and $\beta$-CD also may have occurred [29, 30]. From these findings, both 1:1 and 2:1 UA:$\beta$-CD complexes would be expected to be formed.

## X-ray powder diffraction analysis

The X-ray powder diffraction pattern (XRPD) provided further evidence for the formation of supramolecular species between usnic acid and $\beta$-cyclodextrin (Fig. 4). XRPD patterns of usnic acid and $\beta$-cyclodextrin exhibited several sharp diffraction peaks characteristic of these crystalline compounds, and the physical mixture showed peaks characteristic of usnic acid and $\beta$-cyclodextrin. The XRPD pattern of $\beta$-cyclodextrin is in agreement with the literature [3]. The diffractogram of physical mixture showed characteristic peaks of both pure components (UA and $\beta$-CD), supporting that there was no modification in the crystalline form of these compounds, confirming that no interaction occurred between them. On the other hand, XRPD patterns of the UA:$\beta$-CD inclusion complex differed considerably from those of the drug and the $\beta$-cyclodextrin alone with a completely diffuse diffraction pattern, which reveals the amorphous character of the sample. This difference represents a loss of crystallinity with the formation of a less organized system, indicating the existence of molecular interactions between usnic acid and $\beta$-cyclodextrin, resulting in an amorphous state.

## Differential scanning calorimetry

The DSC thermograms of pure usnic acid, $\beta$-cyclodextrin and UA:$\beta$-CD inclusion complex are plotted in Fig. 5. The usnic acid thermogram shows an endothermic peak at 200 °C, corresponding to the fusion peak of usnic acid, and an exothermic peak at 273 °C. These results are in accordance with the literature, where it is reported that usnic acid has a fusion peak at about 201.5 °C [31] or 204 °C and another around 273 °C [12], corresponding to its degradation.

The cyclodextrin exhibited a characteristic broad peak associated with water loss at around 60 °C [32], followed by a degradation peak at 321 °C. As regards the analysis of the UA:$\beta$-CD complex, the peaks of usnic acid and the peak of $\beta$-cyclodextrin at 325 °C are strongly reduced in intensity and almost disappeared from the thermogram, clearly indicating an interaction between both usnic acid and $\beta$-cyclodextrin molecules. These significant reducing was not observed in the physical mixture, where the characteristic peaks of usnic acid and $\beta$-cyclodextrin can be clearly observed in the thermogram.

## Antimicrobial activity of UA:$\beta$-CD inclusion complexes

The inhibitory zone diameters for the binary complex and free usnic acid (75 μg/mL) were, respectively, 11 ± 1.0 mm and 11.33 ± 0.5 mm for *S. mutans*; 13.33 ± 1.1 mm and 12.66 ± 2.3 mm for *E. faecalis* and

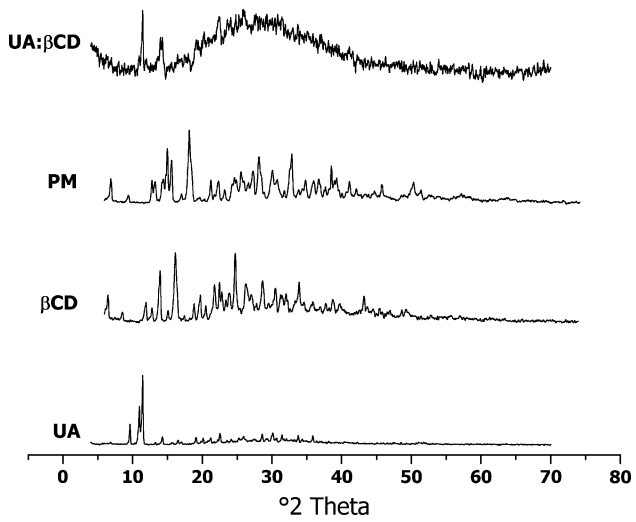


**Fig. 4** X-ray powder diffraction patterns of usnic acid (UA), $\beta$-cyclodextrin ($\beta$CD), physical mixture (PM) and inclusion complex (UA:$\beta$CD)

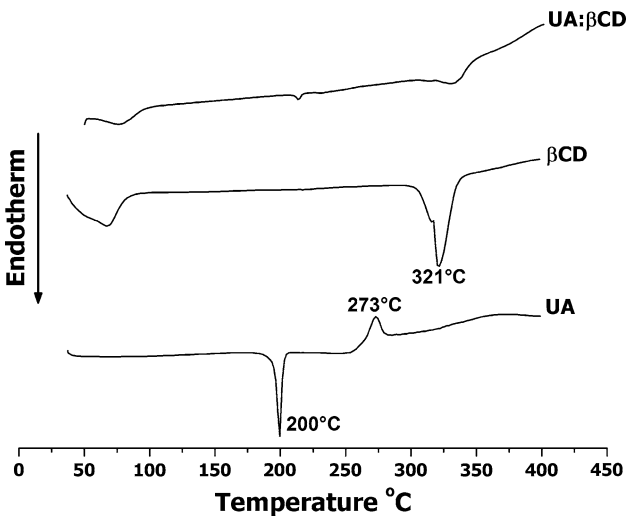


**Fig. 5** Differential scanning calorimetry (DSC) thermograms of usnic acid (UA), $\beta$-cyclodextrin ($\beta$CD) and usnic acid with $\beta$-cyclodextrin inclusion complex (UA:$\beta$CD)

11.33 ± 0.5 mm and 12.33 ± 2.5 mm for *A. actinomycetecomitans*. These results showed that no significant differences in antimicrobial activity between free usnic acid and UA:β-CD were observed, indicating that usnic acid activity was preserved after inclusion complex formation. The inclusion process therefore does not interfere with the antimicrobial activity of the drug.

## Characterization and stability of UA:β-CD-loaded liposomes

Small unilamellar vesicles (SUV) with a positively charged surface were prepared with 4.2 μmol lipids/mL of phosphate buffer encapsulating the equivalent of 1.2 mg/mL of usnic acid from the inclusion complex. A 1:12 drug/lipid molar ratio was thus achieved. After formulation, the macroscopic appearance of the liposomal suspension was observed. It exhibited a milky appearance with a typically bluish reflection (characteristic of small unilamellar vesicles). The liposomal formulations preserved their stability after accelerated stability testing with no significant changes to their initial characteristics after being submitted to centrifugation. However, after mechanical stress a redispersible creaming was observed after 48 h. The formulation remained stable after 27 freeze-thaw cycles and for approximately four months in suspension form stored at 4 °C. The pH of formulations after manufacturing was 7.4 and two months later their pH progressively decreased to 6.4. To overcome this problem, the liposomal formulations were lyophilized after manufacturing.

The content of usnic acid (3.49 UA μmol) corresponding to 1.2 mg/ml of liposomal suspension and encapsulation efficiency after preparation of liposomes was 100 ± 6% and 99.5 ± 0.2%, respectively, showing the maintenance of usnic acid stability during the manufacturing process but also the high efficiency of the encapsulation process. After five months the lyophilized liposomal formulations were hydrated and the content of usnic acid was determined, which remained at around 100%.

The encapsulation efficiency of usnic acid into liposomes was limited to 10 mM in the cyclodextrin complex. At high complex concentrations the presence of drug crystals was observed in the acid usnic liposomal preparation. This phenomenon may be explained by the destabilizing action of cyclodextrin on the liposomal membrane and the low drug solubility. Indeed, it is well known that cyclodextrins interact with various drugs and lipids of liposomal bilayers [33]. Similar results were found by Maestrelli and collaborators [34] for the encapsulation efficiency of ketoprofen into liposomes, which increases with a rise in the complex concentration up to 10 mM, whereas it decreases at higher complex concentrations.

## Antimicrobial activity of UA:β-CD-loaded liposomes

As shown in Table 3, usnic acid (600 μg/mL) was effective against MRSA *S. aureus* strains (AM 793 Brazilian pediatric clone and AM942 pediatric clone), whereas UA-lipo (Table 4) was more effective at higher concentrations (1200 μg/mL). UA:β-CD-lipo was ineffective against MRSA *S. aureus* strains (Table 5) even at higher concentration of UA (1200 μg/mL). These results can be explained by the time of incubation and the mechanism of drug diffusion from liposomes. Indeed, as the UA:β-CD

**Table 3** Antimicrobial activity of usnic acid against MRSA *Staphylococcus aureus* showed as zone of inhibition (mm)

| Samples (μg/mL) | *S. aureus* AM 103 ATCC 6538 | *S. aureus* AM 106 ATCC 6538P | *S. aureus* AM 632 MRSA | *S. aureus* AM 793 MRSA | *S. aureus* AM 942 MRSA |
|---|---|---|---|---|---|
| Tetraciclin (300) | 33 | 36 | 32 | 14 | 15 |
| UA (300) | 13 | 13 | 14 | 13 | 14 |
| UA (600) | 13 | 13 | 14 | 14 | 15 |
| UA (1200) | 14 | 14 | 14 | 14 | 15 |

*MRSA* methicilin resistant *Staphylococcus aureus, AM 793* Brazilian pediatric clone, *AM942* pediatric clone

**Table 4** Antimicrobial activity of usnic acid encapsulated in lipossomas (UA-lipo) against MRSA *Staphylococcus aureus* showed as zone of inhibition (mm)

| Samples (μg/mL) | *S. aureus* ATCC 6538 | *S. aureus* ATCC 6538P | *S. aureus* AM 632 MRSA | *S. aureus* AM 793 MRSA | *S. aureus* AM 942 MRSA |
|---|---|---|---|---|---|
| Tetraciclin (300) | 35 | 37 | 34 | 15 | 16 |
| UA-lipo (300) | 13 | 13 | 14 | 13 | 14 |
| UA-lipo (600) | 14 | 14 | 14 | 14 | 14 |
| UA-lipo (1200) | 15 | 13 | 14 | 15 | 15 |

*MRSA* methicilin resistant *Staphylococcus aureus, AM 793* Brazilian pediatric clone, *AM942* pediatric clone

**Table 5** Antimicrobial activity of UA:β-CD-loaded liposomes against MRSA *Staphylococcus aureus* showed as zone of inhibition (mm)

| Samples (μg/mL) | *S. aureus* AM 103 ATCC 6538 | *S. aureus* AM 106 ATCC 6538P | *S. aureus* AM 632 MRSA | *S. aureus* AM 793 MRSA | *S. aureus* AM 942 MRSA |
|---|---|---|---|---|---|
| Tetraciclin (300) | 34 | 36 | 34 | 15 | 16 |
| UA:β-CD-lipo (300) | 11 | 12 | 11 | 12 | 11 |
| UA:β-CD-lipo (600) | 11 | 12 | 12 | 12 | 12 |
| UA:β-CD-lipo (1200) | 12 | 12 | 12 | 13 | 12 |

*MRSA* Methicilin resistant *Staphylococcus aureus, AM 793* Brazilian pediatric clone, *AM942* Pediatric clone

inclusion complex was encapsulated in the aqueous intern cavity of liposomes, a longer time of incubation than 72 h may be required to drug diffusion firstly through the phospholipid bilayer of liposomes towards the medium and after diffusion through the membrane of bacteria.

All these findings proved that the encapsulation of UA in liposomes preserved its antimicrobial activity. Furthermore, they corroborated the encapsulation of a UA:β-CD inclusion complex in the aqueous cavity of liposomes controlling the drug diffusion.

## Kinetics of release of UA from UA:β-CD-loaded liposomes

Meanwhile, the potential use of cyclodextrin inclusion complexes encapsulated into liposomes in quest of an improvement in the pharmacokinetics and therapeutic efficacy of drugs has been reported in the literature [8]. Figure 6 shows the in vitro kinetic profile of usnic acid released from three different formulations: inclusion complex (UA:β-CD), usnic acid-loaded liposomes (UA-Lipo) and UA:β-CD complex-incorporated into liposomes (UA:β-CD-Lipo). UA:β-CD released practically 50% of usnic acid at 3 h, corresponding to the initial burst effect, followed by a linear release with 80% at 11 h, reaching 99.1% in 30 h. The release of usnic acid from liposomes reached 30% within 7 h and 55% in 24 h, achieving 70% in 72 h, whereas the release of usnic acid from UA:βCD-loaded liposomes was slightly slower (32.5% at 24 h) than from usnic acid-loaded liposomes (50% at 20.5 h and 65% at 33 h).

The kinetics of release of usnic acid from the liposomes (UA-lipo) was fitted according to an exponential model $M_t/M_\infty = \left(1 - k_1.e^{-k_2 t}\right)$, yielding $M_\infty = 831.73 \pm 11.41$ μg, $k_1 = 0.928 \pm 0.015$ and $k_2 = 0.067 \pm 0.004$ $h^{-1}$ with a correlation coefficient of $r^2 = 0.9943$. Conversely, the release of usnic acid from the inclusion complex encapsulated into liposomes give in $M_\infty = 423.26 \pm 6.50$, $k_1 = 1.019 \pm 0.025$ and $k_2 = 0.096 \pm 0.007$ with a correlation coefficient of $r^2 = 0.9903$.

The exponential decrease ($dM_t/dt \times t$) in the release rate of usnic acid from the liposomal formulations is shown in the inset of Fig. 5. The initial release rate was 52 μg/h for the usnic acid-loaded liposomes and 41 μg/h for UA:β − CD-liposomes. Their respective constants of time ($1/k_2$) are estimated at 15 h and 10 h, respectively. The elapsed time

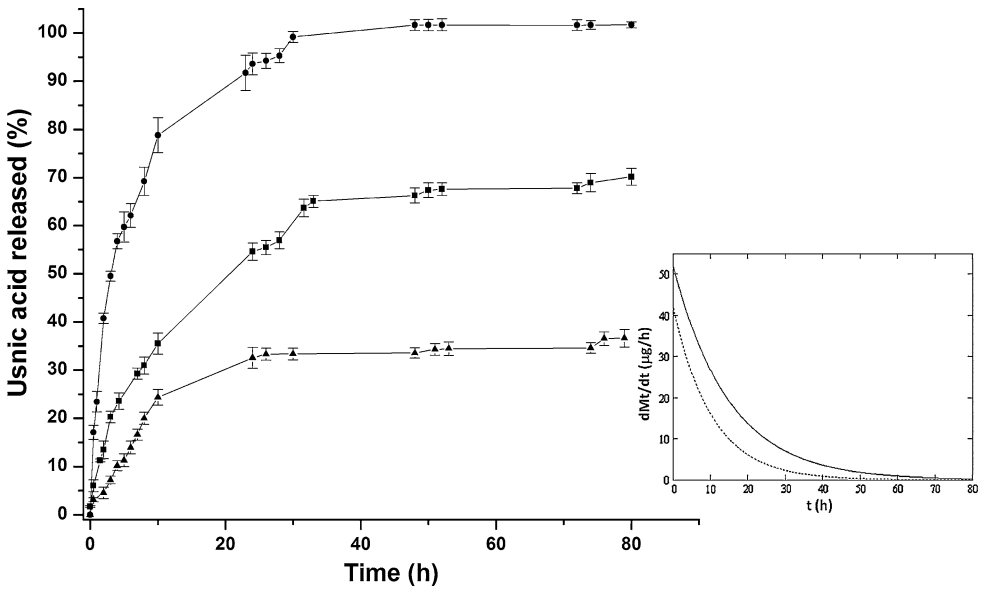


**Fig. 6** In vitro release profiles of usnic acid from inclusion complex-loaded liposomes (UA:βCD Lipo), usnic acid-loaded liposomes (UA-Lipo) and usnic acid with β-cyclodextrin inclusion complex (UA:βCD). The inset shows the release rate of usnic acid from the liposomal formulations

required to achieve the kinetics process (which can be assesses by computing $4/k_2$) was 60 h and 40 h, respectively. It can be verified thus that the complexation of usnic acid with $\beta$-cyclodextrin and its encapsulation into liposomes decreased the drug release rate, but a reduced amount of drug is released in a less time.

These results are in agreement with a previous study on doxorubicin (DOX) complexes with $\gamma$-cyclodextrin incorporated into liposomes [5]. A prompt release of 20% of DOX was observed for DOX-in-liposome, while only 10% was released from DOX complex-$\gamma$-cyclodextrin-in-liposome. It was demonstrated thus that the drug inclusion complex incorporated into liposomes presented a slower release in comparison with drug-loaded liposomes. The authors claimed that this behavior was due to the complexation of drug with cyclodextrin in liposomes and/or the stabilization of the liposomal membrane by cyclodextrin.

Furthermore, the results obtained from the in vitro release of betamethasone from liposomes containing inclusion complexes with $\gamma$-cyclodextrin at 10 mM concentration, which led to a slower release compared to betamethasone encapsulated into liposomes, reinforce the idea that cyclodextrins would improve the stability of liposomes [9]. The release kinetics of betamethasone was dependent only on the ability of cyclodextrin to complex the active substance and not on the interactions between cyclodextrins and the lipid components of the liposomal membrane. However, over time empty cyclodextrins produced by complex dissolution would interact more and more with cholesterol from the liposome bilayer. The extraction of cholesterol from the bilayer of liposomes will not immediately result in lysis and dissolution of the liposomes. Lipid bilayers losing cholesterol will largely remain intact but become more fluid and/or permeable for betamethasone release during the kinetic process.

In the light of these findings we believe that conventional liposomes containing usnic acid:$\beta$-cyclodextrin complexes are stable and provide a membrane diffusion barrier effect on the release kinetics of usnic acid, which may lead to further applications in therapy.

## Conclusions

Interactions between usnic acid and $\beta$-cyclodextrin were detected by spectroscopic analysis, indicating the formation of an inclusion complex with both molecules. A change from the crystalline to amorphous form of usnic acid was observed on X-ray powder diffraction analysis, confirming the formation of the drug inclusion complex. The complexation of usnic acid with $\beta$-cyclodextrin increased its solubility in water and did not affect its antimicrobial activity. Liposomes encapsulating the equivalent of 1.2 mg/mL of the usnic acid inclusion complex were obtained and this formulation maintained its stability for four months in a suspension form. This novel formulation released the drug more slowly than was the case of usnic acid-loaded liposomes. The encapsulation of the usnic acid inclusion complex with $\beta$-cyclodextrin into liposomes might be an alternative strategy for overcoming the low water solubility of usnic acid while maintaining its antimicrobial activity.

The complexation of usnic acid with $\beta$-cyclodextrin offers a way to improve its solubility and bioavailability. The successful incorporation of usnic acid inclusion complex into the aqueous cavity of liposomes encourages us to undertake further studies in the design of stealth and targeted nanocarrier systems for the treatment of tuberculosis.

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