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ORIGINAL ARTICLE

Understanding the interactions between artemisinin and cyclodextrins: spectroscopic studies and molecular modeling

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Abstract Artemisinin extracted from *Artemisia annua* L. proved to be currently, with its derivatives, the most effective drugs against simple and severe malaria, and is also effective on the chloroquine-resistant forms. The advantageous effect of some cyclodextrins (CDs) on artemisinin solubilization was demonstrated by different authors. The present work aims to confirm the effect of several CDs on artemisinin solubilization and to analyse the complexes formed between these CDs and artemisinin in order to understand their solubilization capacities. In this context, solubility studies, liquid-state NMR spectroscopy (^{1}H NMR studies and ROESY experiments) as well as theoretical studies (molecular modeling) have been performed. Randomly methylated-βCD, Crysmeb® and hydroxypropylated-γCD were also found to improve the aqueous solubilization of artemisinin as well as βCD, γCD and hydroxypropylated-βCD whose effects were already demonstrated. The best solubilization ability was found with Crysmeb®. The spectroscopic studies showed a lot of interactions between artemisinin and all the CDs studied, but mainly outside the cavity. Molecular modeling confirmed that artemisinin and CDs formed non-inclusion complexes.

Keywords Artemisinin · Cyclodextrins · Non-inclusion complexes · Solubilization · Molecular modeling

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Introduction

Artemisinin (Fig. 1), a molecule extracted from *Artemisia annua* L., was isolated for the first time in 1972 in China [1]. In the last decades, artemisinin and its derivatives were proven to be actually the most effective drugs against simple and severe malaria, and is also effective on multi-drug resistant forms [2–4]. The low aqueous solubility and high first-pass metabolism of artemisinin induce a poor and variable bioavailability [5]. This may create problems of recrudescence and resistance. So, improving aqueous solubility of artemisinin may lead to a better bioavailability and cyclodextrins (CDs) could be used in this topic.

CDs are cyclic oligosaccharides made of six, seven, or eight (for α-, β- and γ-cyclodextrin, respectively) glucopyranose units bound by α-1,4-ether linkages containing an hydrophobic central cavity and an hydrophilic outer surface. By exchange with the water molecules present in their cavity in the crystalline state, CDs are able to include large organic molecules with low aqueous solubility by noncovalent interactions forces [6] leading to an increase of aqueous

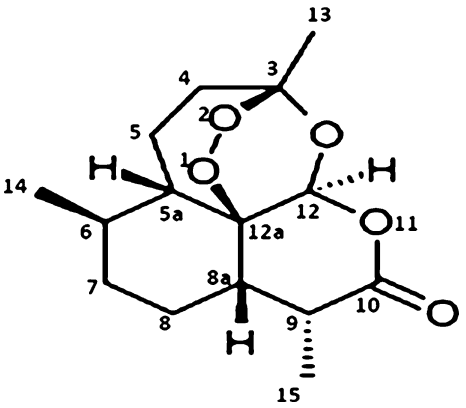

Fig. 1 Chemical structure of artemisinin and numbering system of the molecule according to Blasko et al. (1988) [32]

solubility and stability of these molecules [7]. However, this phenomenon is not always verified for all the substances. The complexation ability depends on the geometry of the guest molecule inside the CD cavity and also its charge and polarity criteria [6]. CDs are also known to form non-inclusion complexes [8, 9].

Different publications have shown an increase in the solubility of artemisinin thanks to the combination with CDs [10–12]. The authors of these various publications did not test all the CDs, notably the Crysmeb, a new generation cyclodextrin. Other studies carried out by Wong et al. [13, 14] prove that this improved solubility involve a better bioavailability of artemisinin combined with CDs. In these studies, they suggested that there is inclusion but, the types of interactions existing between the CDs and artemisinin were not yet clearly established.

The complexes formed with CDs and molecules can be of several types (Fig. 2): inclusion complexes (a–f) and non specific complexes also called non-inclusion complexes (g) [15].

Some authors tried to demonstrate formation of inclusion complexes [12, 16, 17] but the methods used were not adequate for this kind of conclusion.

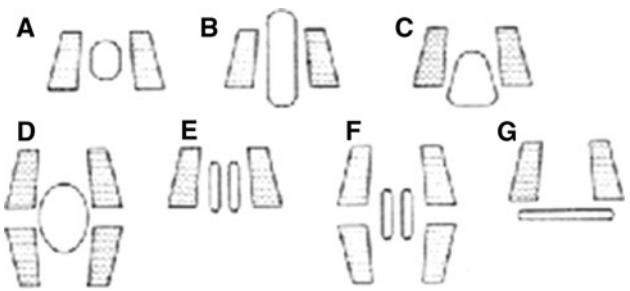

Fig. 2 Various structures of cyclodextrin-guests complexes, in aqueous solution, described in the literature **a** complete inclusion; **b** " axial " inclusion; **c** partial inclusion; **d** complexes 2:1; **e** complex 1:2; **f** complex 2:2; **g** non-inclusion complex [15]

The aim of the present work is to compare the increase in solubility of artemisinin in association with the majority of CDs including Crysmeb and to study the nature of the interactions between artemisinin and the different CDs by the use of both NMR studies and molecular modeling. Molecular modeling with AM1 calculations have already been used by various authors [18–22] to study different complexes of several drugs with CDs.

Materials and methods

Material

Artemisinin (ARTE, 98%) was purchased from Sigma-Aldrich (Darmstadt, Germany).

β-cyclodextrin (βCD, 12.76% H_2O), γ-cyclodextrin (γCD, 7.34% H_2O), hydroxypropylated β-cyclodextrin (HPβCD, DS: 0.8, 3.9% H_2O), randomly methylated β-cyclodextrin (Rameb, DS: 1.8, 3.4% H_2O), hydroxypropylated γ-cyclodextrin (HPγCD, DS: 0.6, 6.8% H_2O) were provided by Wacker-Chemie GmbH (Munich, Germany). Kleptose® Crysmeb (Crysmeb, DS: 0.50, 3.99% H_2O) was obtained from Roquette Frères (Lestrem, France).

Ethanol (Eur Ph 96%) was acquired at VWR (Fontenay-sous-Bois, France). HPLC-Grade methanol was purchased, with KOH from VWR (Fontenay-sous-Bois, France). All the other reagents were of analytical grade and acquired at Merck (Darmstadt, Germany) and Alfa Aesar (Karlsruhe, Germany), respectively. Deuterated water was purchased from Euriso-top (Gif-sur-Yvette, France).

Methods

Solubility studies

Complexation capacity of six CDs (natural CDs: β and γCDs and derivatives: HPβCD, Rameb, Crysmeb, HPγCD) with artemisinin in aqueous solution was studied by using the solubility method [23]. Excess amount of artemisinin were added to increasing concentrations of aqueous CDs solutions. The concentrations tested were 20, 50, 75, 100 and 150 mM except for βCD (2, 4, 8, 10 and 12 mM). The flasks were then shaken continuously in a thermostatically controlled water bath set at 37 °C for 48 h. This equilibrium time was determined experimentally by testing different times from 12 to 96 h. All the conditions were realized in triplicate. After equilibrium was attained, suspensions of the flasks were filtered through 0.45 µm PVDF membrane. These filtrates were used for HPLC analysis.

The stability constants of complexation Ks for each CD were then calculated from the phase solubility diagrams which were all linear in this case. So, we have a

stoichiometric ratio complex of 1:1. We applied the following relationship:

$$Ks = \frac{Slope}{S_0(1 - slope)}$$

where S_0 is the intrinsic solubility of artemisinin in the absence of CD and the slope refers to the gradient plot of artemisinin solubility (mM) versus CD concentration (mM).

Quantification of artemisinin (validation–selectivity)

UV detection of artemisinin is not easy, due to the lack of chromophore. So, it must be pre- or post- derivatized. We used in this study the prederivatization technique previously published [24].

For the prederivatization, 1 ml of sample (standard solution with artemisinin only or solution containing artemisinin complexes) was transferred into a 10 ml measuring flask. 4 ml of 0.2% (m/v) NaOH solution was added in the flask, and then left to react at 50 °C for 30 min. After cooling during 10 min, 1 ml of ethanol was added. Finally the flask was filled with acetic acid 0.2 N.

All solutions were filtered on a PTFE 0.45 µm membrane before HPLC analysis.

The HPLC apparatus used for artemisinin quantification was a Hitachi Alliance from VWR with LaChrom Elite software for data acquisition.

Chromatographic separation was performed with a reversed phase RP-18 LiChroCART column (250 × 4 mm I.D.; particle size: 5 µm). Mobile phase consisted in a mixture of methanol and phosphate buffer (0,005 M; pH: 6.3) (45/55, v/v). A flow rate of 1 ml/min and detection at 260 nm were used. The column was maintained at 35 °C and the injection volume was 20 µl.

This method was completely validated in presence of CDs (we choose Crysmeb as prototype) according to the accuracy profile approach [25].

The selectivity of the method with the other CDs was verified by the calibration curves.

NMR spectroscopy

^{1}H NMR studies One-dimensional ^{1}H NMR spectra were recorded at 20 °C on a Bruker Avance 500 operating at a proton NMR frequency of 500.13 MHz using a 5 mm probe and a simple pulse-acquire sequence. Acquisition parameters consisted of a spectral width of 10,333.6 Hz, a 30° pulse, an acquisition time of 3.17 s and a relaxation delay of 1 s. For each sample, 128 scans were recorded. FIDs were Fourier transformed with LB = 0.3 Hz and GB = 0. The resonance due to residual solvent (HOD) was used as internal reference. CD reference solutions were prepared by dissolving appropriate amounts of βCD, γCDs, HPβCD, Rameb, Crysmeb and HPγCD directly in 700 µl D_2O in order to obtain concentrations around 50 mM (except for βCD for which the concentration was 10 mM).

Sample solutions were prepared by adding excess amounts of artemisinin to the CD solutions. The resulting suspensions were shaken at 20 °C during 24 h and then centrifugated for 5 min at 12,000 rpm with the temperature set at 20 °C. The artemisinin reference solution (around 0.297 mM) was prepared in D_2O. Another reference solution was done in MeOD (around 8 mM) to be sure to have a good NMR signal, as the solubility in D_2O is low. Variation of ^{1}H NMR chemical shifts of CD protons ($\Delta\delta$) caused upon complexation were calculated to confirm the interaction with artemisinin.

ROESY experiments Rotating-frame Overhauser Effect SpectroscopY (2D-ROESY) spectra were acquired in the phase sensitive mode using the same spectrophotometer and Bruker standard parameters (pulse program roesyph). Each spectrum consisted of a matrix of 2K (F2) by 256 (F1) points covering a spectral width of 5122.9 Hz. Spectra were obtained from the same sample solutions prepared for the ^{1}H NMR studies, using a spin-lock mixing time of 350 ms. Before Fourier transformation, the sine apodization function was applied in both dimensions. 32 scans were collected for each of the experiments.

Molecular modeling

The complexes between artemisinin and several CDs have been studied by quantum chemistry method using the B3LYP functional energy [26] and the extended basis set 6-31G (d). βCD, Rameb and Crysmeb have been build as described in the [22].

The geometries were fully optimized without constraint by minimisation of the analytical gradient of the energy function using the Gaussian 03 suite of programs [27]. Starting from the optimized geometry of each complex, artemisinin and CD were separately reoptimized in order to derive the relative deformation energy of each partner. It can be obtained by comparison of the energy of the molecule in the complex and the energy of the molecule optimized alone.

Results

Analytical method validation

The HPLC–UV method for artemisinin quantification in solution containing artemisinin–Crysmeb complex was successfully validated as shown on Table 1 and Fig. 3.

Table 1 Validation results for artemisinin HPLC–UV quantification in solutions containing artemisinin–Crysmeb complexes

Validation criteria	Artemisinin	
Response function	Linear regression through 0 fitted with the level 60.0 only; Calibration range (3 points) 20.00–60.0 μg/ml	
Trueness		
Concentration (μg/ml)	Relative bias (%)	Recovery (%)
20.0	−5.314	94.69
40.0	−4.663	95.34
60.0	−2.672	97.33
Precision		
Concentration (μg/ml)	Repeatability (RSD %)	Intermediate precision (RSD%)
20.0	3.161	3.619
40.0	3.418	4.232
60.0	2.752	3.739
Accuracy		
Concentration (μg/ml)	90% β-expectation tolerance interval (%)	
20.0	[−12.88, 2.249]	
40.0	[−13.87, 4.544]	
60.0	[−11.21, 5.867]	
Linearity		
Slope	0.9865	
Intercept	−0.9699	
r^2	0.9912	

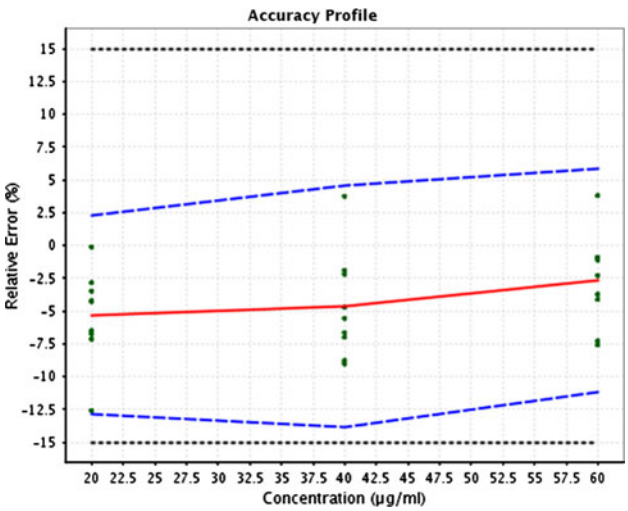

Fig. 3 Accuracy profile obtained by considering linear regression through zero fitted using the highest level only

The Fig. 3 shows a plain line which is the relative bias. The dashed lines are the β-expectation tolerance limits and the dotted curves represent the acceptance limits (15%). The green dots represent the relative back-calculated concentrations and are plotted with respect to their targeted concentration.

Accuracy implies evaluating total error, the sum of systematic and random errors of the analytical procedure [28–30]. As illustrated in Fig. 3, the accuracy profile shows that the relative upper and lower 90% β-expectation tolerance limits are totally included inside the acceptance limits set at ±15%. The method can thus be considered as accurate between 20.0 and 60.0 μg/ml.

The limit of detection was 2.7 μg/ml. These results were estimated using the mean intercept of the calibration model and the residual variance of the regression. The limit of detection was determined with the accuracy profile as the smallest concentration levels where the 90% β-expectation tolerance limits remain inside the ±15% acceptance limits [29–31]. As shown in Fig. 3, the LOQ is the smallest concentration level of the validation standards: 20.0 μg/ml.

The calibration curves obtained with the others CDs were similar to those obtained with Crysmeb.

Solubility studies

Figures 4 and 5 show phase solubility diagrams of artemisinin with, respectively, natural CDs (β and γCDs) and their derivatives (HPβCD, Rameb, Crysmeb, HPγCD). In all cases, linear solubility curves were obtained and could be classified as type A_L which indicates that the molar ratio of artemisinin and the CDs studied is 1:1 [23].

The stability constants (Ks) observed on Table 2 increase in the order: γCD < HPγCD < HPβCD < Rameb < Crysmeb < βCD. All the Ks were <1,000 M^{-1} which suggested a relative lability of the complexes formed with artemisinin [23].

The Table 3 below shows the effect of natural CDs (10 mM) and derivatives ones (50 mM) on artemisinin aqueous solubility at 37 °C. Without CDs, aqueous

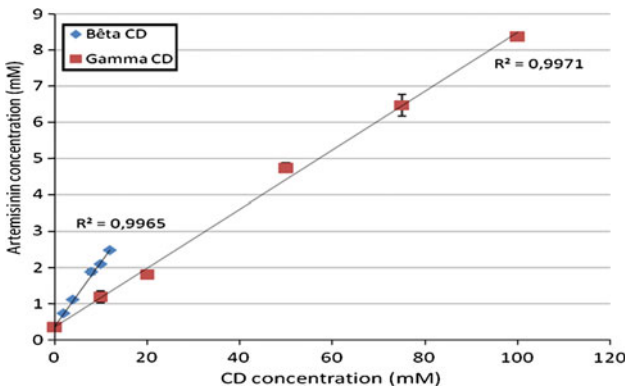

Fig. 4 Phase solubility diagrams of artemisinin-natural CDs in water 37 °C: β and γCDs

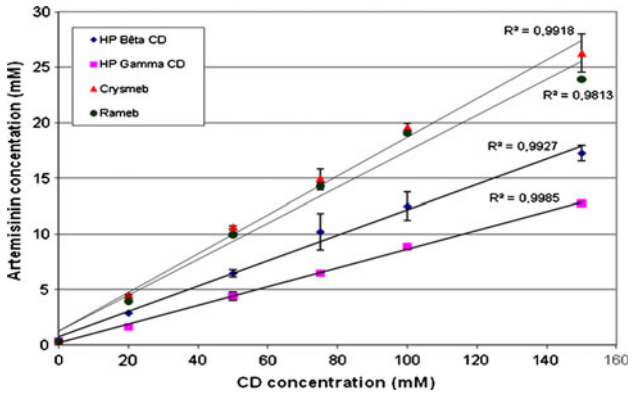

Fig. 5 Phase solubility diagrams of artemisinin-derivatives CDs in water at 37 °C: HPγCD, HPβCD, Rameb and Crysmeb, respectively, from down to up ($n = 3$)

Table 2 Apparent stability constants Ks and type of solubility curves of Artemisinin–CDs complexes determined by solubility method in water at 37 °C

CDs	Ks (M^{-1})	Type
βCD	608.53	A_L
γCD	222.64	A_L
HPβCD	367.48	A_L
HPγCD	262.58	A_L
Crysmeb	602.53	A_L
Rameb	551.28	A_L

Table 3 Effect of natural CDs and derivatives on artemisinin solubility in water at 37 °C

	CD Concentration (mM)	Artemisinin concentration (mg/ml)
Without CD	0	0.098
βCD	10	0.59
γCD	10	0.33
HPβCD	50	1.83
HPγCD	50	1.24
Rameb	50	2.81
Crysmeb	50	3

solubility of artemisinin at 37 °C was 0.098 mg/ml. A comparable result was previously observed by Usuda et al. [10] with 0.084 mg/ml at 25 °C. With a low concentration of Crysmeb (50 mM), a 30-fold increase of artemisinin solubility was obtained and when a 150 mM concentration is used, a 75-fold increase can be reached.

In our study, the methylated βCDs allowed to achieve the best results in terms of increasing artemisinin aqueous solubility. Crysmeb characterized by a low degree of substitution (DS) and less methylated than Dimeb (Di-méthyl βCD) was shown to be less hydrophobic and more polar than Dimeb [31].

We can also consider βCD as a good candidate. In fact, despite its low solubility, a sevenfold increase can be reached and it is the most available CD in term of accessibility and cost (especially for emergent countries with limited resources for drug development). Besides βCD has the best stability constant among the CDs studied. So, we selected Arte/Crysmeb, Arte/Rameb and also Arte/βCD complexes to continue the investigations, notably for molecular modeling studies while all the CDs selected and their corresponding complexes with artemisinin were evaluated for NMR studies.

NMR-studies

The ^{1}H assignment of artemisinin in MeOD and D_2O, which was not available in the literature, is given in Table 4. These values are close to those found in $CDCl_3$ by Blasko et al. [32].

The slight shifts variations calculated upon complexation for artemisinin and reported in Table 5 proved that there are several interactions with the CDs. These interactions of artemisinin protons seem more accentuated for H-7α, H-8α, H-8β, H-8a, H-9, 14-CH_3, 15-CH_3 with βCD and H-4α, H-7β, H-12 and 14-CH_3 with γCD (Table 5).

Observation of the effect of artemisinin on the CDs is very important to understand the nature of interactions. The ^{1}H NMR spectra between 3 and 5 ppm of the different CDs in D_2O alone or associated in the complex with artemisinin

Table 4 ^{1}H assignment of artemisinin in MeOD and D_2O comparatively to $CDCl_3$

Proton	$CDCl_3$ (Blasko et al. 1988 [32])	MeOD	D_2O
4α	2.43	2.39	2.34
4β	2.05	2.07	2.12
5α	2.01	2.03	2.02
5β	1.47	1.49	1.43
5a	1.37	1.37	1.38
6α	1.42	1.49	1.43
7α	1.08	1.15	1.04
7β	1.79	1.75	1.85
8α	1.87	1.80	1.87
8β	1.12	1.18	1.13
8a	1.75	1.73	1.70
9	3.40	3.31	3.34
12	5.87	6.03	6.14
13-CH_3	1.44	1.38	1.40
14-CH_3	0.99	1.00	0.92
15-CH_3	1.21	1.17	1.12

Table 5 Chemical shift variations (ppm) for the protons of artemisinin: difference between the free and complexed states

Protons	Δδ Bêta CD (ppm)	Δδ Gamma CD (ppm)
4α	0.02	0.06
4β	0	0
5α	0.03	0
5β	−0.02	0.03
5a	−0.02	0.03
6	−0.02	0
7α	0.06	0.01
7β	0.03	−0.05
8α	0.05	0
8β	0.05	0.03
8a	0.08	0.02
9	0.06	0.01
12	−0.02	−0.15
13-CH_3	−0.02	0.01
14-CH_3	0.13	0.13
15-CH_3	0.05	0.03

$\Delta\delta = \delta$(complexed state) − δ(free state)

Table 6 Variation of ^{1}H NMR chemical shifts (ppm) of βCD, γCD, Crysmeb, Rameb, HPβCD and HPγCD protons in the presence of artemisinin

Protons	Δδ βCD	Δδ γCD	Δδ Crysmeb	Δδ Rameb	Δδ HPβCD	Δδ HPγCD
H-1	−0.01	0	−0.01	0	0	0
H-2	0	0	0	0	0	−0.02
H-3	**−0.02**	**−0.02**	**0.02**	**0.02**	**−0.01**	**−0.01**
H-4	−0.01	0	−0.06	−0.03	0	−0.06
H-5	**0.01**	**−0.02**	**0.02**	**0.05**	**0.01**	**0.01**
H-6	−0.01	−0.01	−0.04	0.03	−0.02	0.03

$\Delta\delta = \delta$(complexed state) − δ(free state)

Chemical shift values of internal protons are in bold

were analysed (data not shown) and the different CDs were found to be practically superimposable whether they are alone or in the complex. The phenomenon is clearly viewed in Table 6 with the calculation of differences between ^{1}H NMR chemical shifts values of the CDs protons in the complexed and free states.

It is known that large shifts in the resonance of protons H-3 and H-5 located inside the CD cavity induced by the presence of the guest molecule indicate inclusion complexation [33, 34]. The shifts variation observed here for protons H-3 and H-5 are very weak. It suggests that artemisinin don't enter in the CDs cavities.

Then we realized 2D ROESY experiments to see whether the interactions between artemisinin and CDs protons could be detected and if they are located inside the cavity of CDs (H-3 or H-5) or outside (H-2 and H-4).

These data indicated that several parts of artemisinin interacted with the protons located inside the CDs, but also interacted with outside protons of CDs. In fact, protons 4α, 6, 7β, 13-CH_3, 14-CH_3 and 15-CH_3, according to Fig. 6a, interacted simultaneously with protons inside and outside of the cavity of βCD. The same remark can be done for protons 6, 13-CH_3, 14-CH_3 and 15-CH_3 in the case of γCD, for protons 4α, 6, 7β, 8a, 13-CH_3, 14-CH_3 and 15-CH_3 with Crysmeb and for protons 5β and 13-CH_3 with HPβCD (Fig. 6b, c, e). These observations are in favor of the establishment of electrostatic interactions between the CDs and artemisinin in these complexes. For Rameb and HPγCD, the interactions with H-2 and H-4 are not clearly viewed (Fig. 6d, f).

Molecular modeling studies

In order to understand interactions between artemisinin and CDs, molecular modeling studies were performed. Two types of complexes have been obtained depending on the orientation of artemisinin towards the primary or the secondary CDs alcohols.

The results on Table 7 are presented as energetic outcomes expressed as interaction, deformation and complexation energies. A schematic representation of this calculation method is given on Fig. 7. The interaction energy is defined as the difference between the energy of the complex and the sum of the energies of the both partners at their complex geometry. The deformation energy is determined by the difference between the energy of one of the partners of the complex at its equilibrium geometry (which is obtained by reoptimization of the one found in the complex) and its energy at the complex geometry. The complexation energy is the difference between the energy of the complex and the sum of the energy of each partner at their respective equilibrium geometry.

The interaction energies (Table 7) are always favorable. But these values are relatively low compared to the other complexes studied with this theoretical method like miconazole [18, 21] and tagitinin C [20]. Let us mention that for these molecules, the authors had demonstrated existence of inclusion complexes. The low interaction energies obtained in our study are in favor of the formation of electrostatic interactions between the components of these complexes. This weakness of interaction energies is also in agreement with the relative lability of the complexes suggested by the results of solubility studies (Table 2).

The complexation energies values are negative for all the complexes. It means that their formation is energetically favorable.

The deformation of artemisinin (Table 7) is weaker than the deformation of the studied CDs. This can be explained

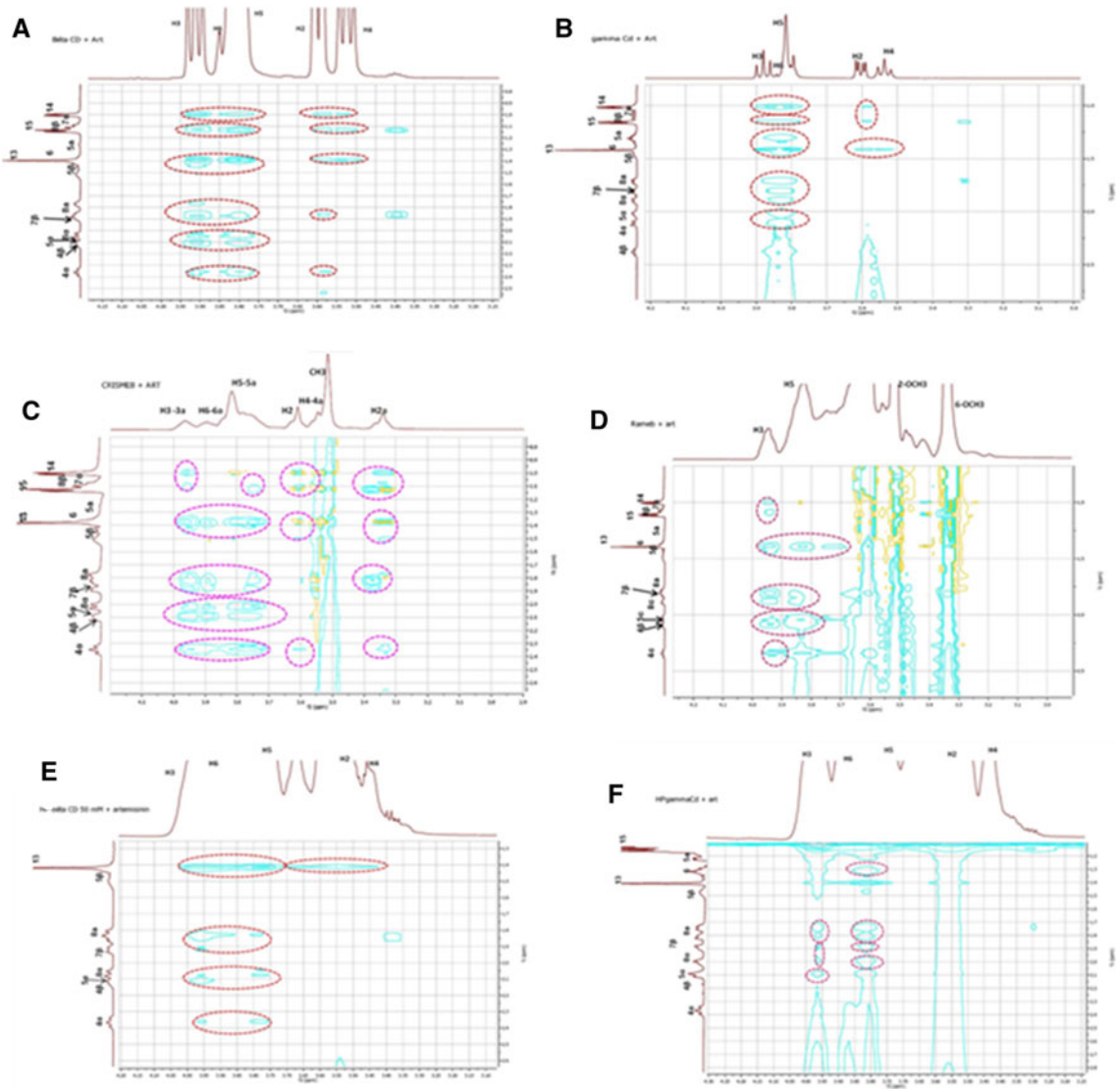

Fig. 6 Two-dimensional ROESY spectra of artemisinin in presence of, respectively, βCD (**a** 10 mM), γCD (**b** 50 mM), Crysmeb (**c** 50 mM), Rameb (**d** 50 mM), HPβCD (**e** 50 mM) and HPγCD (**f** 50 mM) in D_2O. Interactions are *circled*

by the rigidity of artemisinin with its three rings. The highest deformation energy for artemisinin is obtained with Crysmeb in the conformation A (with artemisinin peroxide towards the primary alcohols). This suggests that the complex formation in this conformation should be difficult. Moreover, we can see that in this conformation, the deformation of Crysmeb is higher than the one in the conformation B (with artemisinin peroxide towards the secondary alcohols). So, the conformation B is the most probable for the complex Crysmeb/artemisinin.

In Fig. 8, the structures of the different complexes are represented. All the representations show that there is not inclusion of artemisinin in the CDs; except for the complex Crysmeb/artemisinin in the conformation A where we can see an inclusion of the 13-CH_3 (partial inclusion of the molecule). But the examination of the energies values previously done on Table 7 clearly demonstrates that this conformation A was less probable. For β-CD and Rameb, both conformations A and B are possible and can be present simultaneously in solution.

In all cases, the driving force of the interaction between artemisinin and the CDs is electrostatic interaction. Non-inclusion complexes can be located in accordance with the NMR results.

Discussion

Solubility studies

The study of the solubility of artemisinin in association with the different CDs shows that molar ratio of the

Table 7 Interaction, deformation and complexation energies in Kcal/mole for artemisinin–CDs complexes by reference to reoptimized isolated CDs and artemisinin molecules (B3LYP/6-31G(d) calculations)

Conformation	Peroxyde towards the primary alcohols (A)	Peroxyde towards the secondary alcohols (B)
Complexe β-CD/artemisinin		
Interaction energy	−17.34	−19.06
Complexation energy	−9	−14.39
Deformation energy of β-CD	8.02	4.21
Deformation energy of artemisinin	0.32	0.46
Complexe Rameb/artemisinin		
Interaction energy	−13.47	−6.55
Complexation energy	−7.26	−5.09
Deformation energy of Rameb	5.86	1.32
Deformation energy of artemisinin	0.35	0.14
Complexe Crysmeb/artemisinin		
Interaction energy	−26.45	−15.82
Complexation energy	−10.68	−11.00
Deformation energy of Crysmeb	14.82	4.40
Deformation energy of artemisinin	0.95	0.42

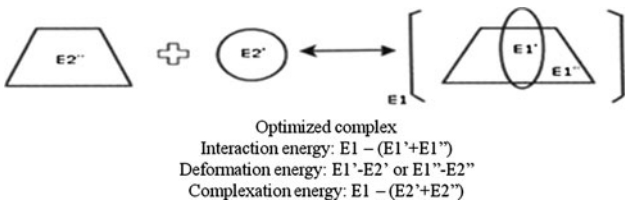

Fig. 7 Schematic representation of the interaction, deformation and complexation energies

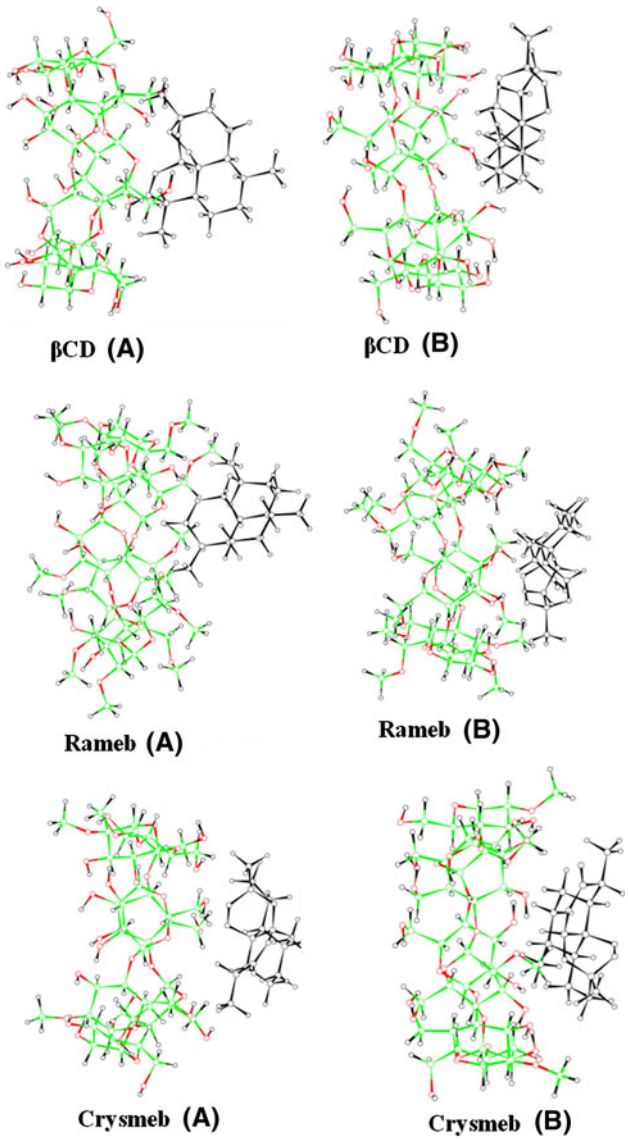

Fig. 8 3D-model of the complexes with the orientation of the peroxyde towards the primary alcohols (*A*) or the secondary alcohols (*B*) of the CDs; respectively, from up to down artemisinin–βCD complex, artemisinin–Rameb complex and artemisinin–Crysmeb complex

complexes is 1:1 (Figs 3, 4). According to the Ks values on Table 2, the complexes formed with γCDs are less stable than those formed with βCDs. These results confirmed the data published by Wong and Yuen in 2003 [11]. They compared natural CDs for their ability to increase artemisinin solubility and found that the complexation capability of artemisinin with CDs increased in the order α- < γ- < βCD.

When we considered the βCDs, the natural one has the highest Ks value, but its solubilization capacity on artemisinin is limited due to its low aqueous solubility (1.85 g/100 ml) while for the derivatives we can go up to 40 g/100 ml (HPβCD). Among the βCDs derivatives, there is a distinction between the hydroxypropyl—derivatives and the methylated ones which were found to have a higher affinity for artemisinin with a better solubilization effect.

Solubility studies alone don't constitute a proof of inclusion complexes formation. Non-inclusion complexes of active substances with cyclodextrin have been already described [8, 9] and they can also increase solubility of this substance.

NMR and molecular modeling studies

The T-ROESY results (Fig. 6) associated to the very low variations observed in spectra when they are in complexed state (Table 6) are not in favor of the existence of an inclusion of artemisinin in the CDs of this study.

The theoretical studies of molecular modeling (Table 7 and Fig. 8) confirmed the results obtained with NMR, and gave the proof that artemisinin does not form inclusion

complexes with the CDs but it establishes electrostatic interactions with oxygen atoms and alcoholic functions for the CDs studied.

These conclusions are different from those of Illaparkurthy et al. [16]. In fact, they conclude on the existence of inclusion complexes between artemisinin, dihydroartemisinin, arteether and HPβCD by means of solubilisation studies and molecular modeling. Their molecular modeling studies were performed by three different docking methods (SYBYL DOCK, FlexiDock, and DOCK 4.0.1) but their DOCK 4.0.1 results suggest that the polar portion of artemisinin (oxygen atoms) is oriented outside the cavity while FlexiDOCK 4.0 and DOCK SYBYL results show that this portion is inside the cavity. They conclude that DOCK 4.0.1 offers a better correlation in terms of orientation of molecules inside the cyclodextrin's cavity and also in terms of docking scores and finally added that experimental thermodynamic and nuclear magnetic resonance studies might help in the development of a better explanation of the inclusion process itself.

Another study of Illaparkurthy et al. [35] on the thermodynamic parameters reveals involvement of hydrophobic bonding in the cyclodextrin complexes studied.

Ansari et al. [12] also speak of an inclusion of dihydroartemisinin in HPβCD cavity on the basis of differential scanning calorimetry (DSC) results, whereas it is known that DSC studies just give informations on the amorphous or crystalline state of the complexes. This method can prove interaction between the CD and the molecule but not an inclusion into the cavity of the CD.

Recently, Chadha et al. [36] have studied the complexes of arteether with CDs. They concluded on the formation of inclusion complexes on the basis of DSC studies, NMR analysis and molecular modeling. In their NMR studies, they have just considered the ^{1}H chemical shifts of arteether and not the ^{1}H chemical shifts of the CDs, while it is the variation of H-3 and H-5 protons of the CDs which could be the proof of inclusion complexation. The variation of the drug protons can just indicate interactions with the CDs. However, the phenomenon with arteether can be different from one observed with artemisinin.

It is generally accepted that in aqueous solutions CDs form what is called "inclusion complexes" where water molecules located within the lipophilic central cavity are replaced by a lipophilic guest molecule or a lipophilic moiety as, for example, a drug molecule. But this is not the only possibility. "Non-inclusion complexes" might also occur.

The existence of non-inclusion complexes has been the subject of a patent application publication by Loftsson in 2003 [8] and he also went back on this statement in others publications [9, 37]. He assumed that the hydroxy groups on the outer surface of the cyclodextrin molecule are able to form hydrogen bonds with other molecules and CDs can, like non-cyclic oligosaccharides and polysaccharides, form water soluble complexes with lipophilic water-insoluble compounds.

Moreover, it has been shown that α-CD forms both inclusion and non-inclusion complexes with dicarboxylic acids and that the two types of complexes coexist in aqueous solutions [38].

In a recent study, Messner et al. [39] tested eleven different molecules with HPβCD and found that they self assembled to form aggregates with increase of solubility. In comparison to simple drug/CD complexes, aggregation of CD complexes will not affect therapeutic efficacy of the drugs, kinetics of drug release from CD complexes or drugs pharmacokinetics.

Circular dichroism spectroscopy studies of artemisinin–βCD complexes were realized by Marconi et al. [17]. In their conclusions they said on one side that out of the many energetically suitable structures of the complex, just a few are able to justify the sequence of signs of the spectrum and on another side that the reduction of the O–O peroxidic group of artemisinin with subsequent cleavage seems to be unaffected by complexation with βCD. It means that there is not an inclusion of this part of the molecule. The statements of this work are in agreement with our results and are a demonstration of the conformations calculated at the more elaborated level (B3LYP) used in our study.

The NMR results obtained here clearly shown that there are not important shifts of H-3 and H-5 protons which are inside the CDs cavities (Fig. 5), excluding the possibilities of inclusion in the studied CDs and this is corroborated by the molecular modeling results (Table 7).

All this confirm that there is not a real inclusion of artemisinin in CDs but only interactions especially on the outer surface of the CDs.

Conclusion

Phase-solubility studies were conducted for artemisinin in aqueous solutions of βCD, γCD, Crysmeb, Rameb, HPβCD, HPγCD. All the CDs were shown to enhance the solubility of artemisinin to a significant extent. The association constant (K_S) was calculated for each of the complexes. The stoechiometry of all the artemisinin–CD complexes was found to be 1:1.

NMR studies and molecular-modeling calculations showed that artemisinin and the different CDs formed non-inclusion complexes.

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References

1. Anonym, Qinghaosu antimalaria coordinating research group: antimalarial studies on qinghaosu. Chin. Med. J. **92**, 811 (1979)
2. Chang, H.M., But, P.P.H.: Pharmacology and applications of chinese materia media. 1. World Scientific Publishing, Singapore (1986)
3. Duke, S.O., Paul, R.N.: Development and fine structure of the glandular trichomes of *Artemisia annua* L. Int. J. Plant Sci. **154**, 107–118 (1993). doi:10.1086/297096
4. Ferreira, J.F.S., Janick, J.: Floral morphology of *Artemisia annua* with special reference to trichomes. Int. J. Plant Sci. **156**, 807–815 (1995). doi:10.1086/297304
5. Titulaer, H.A.C., Zuidema, J., Kager, P.A., Wetsteyn, J.C.F.M., Lugt, C.H.B., Merkus, F.W.H.M.: The pharmacokinetics of artemisinin after oral, intramuscular and rectal administration to volunteers. J. Pharm. Pharmacol. **42**, 810–813 (1990)
6. Frömming, K.H., Szejtli, J.: Cyclodextrins in pharmacy. Kluwer Academic Publishers, Dordrecht (1994)
7. Loftsson, T., Brewster, M.: Pharmaceutical applications of cyclodextrins. 1. Drug solubilisation and stabilization. J. Pharm. Sci. **85**, 1017–1025 (1996)
8. Loftsson, T.: Non-inclusion cyclodextrin complexes. Pub No US 2003/0109492 A1. (2003)
9. Loftsson, T., Duchêne, D.: Cyclodextrins and their pharmaceutical applications. Int. J. Pharm. **329**, 1–11 (2007)
10. Usuda, M., Endo, T., Nagase, H., Tomono, K., Ueda, H.: Interaction of antimalarial agent artemisinin with cyclodextrins. Drug. Dev. Ind. Pharm. **26**, 613–619 (2000)
11. Wong, J.W., Yuen, K.H.: Inclusion complexation of artemisinin with α-, β- and γ-cyclodextrins. Drug. Dev. Ind. Pharm. **29**, 1035–1044 (2003)
12. Ansari, M.T., Iqbal, I., Sunderland, V.B.: Dihydroartemisinin–cyclodextrin complexation: solubility and stability. Arch. Pharm. Res. **32**, 155–165 (2009). doi:10.1007/s12272-009-1130-4
13. Wong, J.W., Yuen, K.H.: Improved oral bioavailability of artemisinin through inclusion complexation with beta- and gamma-cyclodextrins. Int. J. Pharm. **227**, 177–185 (2001)
14. Wong, J.W., Yuen, K.H., Nagappan, S., Shahul, W.S., Ho, S.S., Gan, E.K., Toh, W.T.: Therapeutic equivalence of a low dose artemisinin formulation in falciparum malaria patients. J. Pharm. Pharmacol. **55**, 193–198 (2003)
15. Wenz, G.: Cyclodextrins as building blocks for supramolecular structures and functional units. Angew. Chem. Int. Ed. Engl. **33**, 803–822 (1994)
16. Illapakurthy, A.C., Sabnis, Y.A., Avery, B.A., Avery, M.A., Wyandt, C.M.: Interaction of artemisinin and its related compounds with hydroxypropyl-β-cyclodextrin in solution state: experimental and molecular-modeling studies. J. Pharm. Sci. **92**, 649–655 (2003)
17. Marconi, G., Monti, S., Manoli, F., Degli Esposti, A., Mayer, B.: A circular dichroism and structural study of the inclusion complex artemisinin-β-cyclodextrin. Chem. Phys. Lett. **383**, 566–571 (2004)
18. Piel, G., Dive, G., Evrard, B., Van Hees, T., Henry de Hassonville, S., Delattre, L.: Molecular modeling study of β- and γ-cyclodextrin complexes with miconazole. Eur. J. Pharm. Sci. **13**, 271–279 (2001)
19. Henry de Hassonville, S., Perly, B., Piel, G., Van Hees, T., Barillaro, V., Bertholet, P., Delattre, L., Evrard, B.: Inclusion complexes of cyproterone acetate with cyclodextrins in aqueous solution. J. Incl. Phenom. Macrocycl. Chem. **44**, 289–292 (2002)
20. Ziémons, E., Dive, G., Debrus, B., Barillaro, V., Frédérich, M., Lejeune, R., Angenot, L., Delattre, L., Thunus, L., Hubert, P.: Study of the physicochemical properties in aqueous medium and molecular modeling of tagitinin C/cyclodextrin complexes. J. Pharm. Biomed. Anal. **43**, 910–919 (2007)
21. Barillaro, V., Dive, G., Bertholet, P., Evrard, B., Delattre, L., Ziémons, E., Piel, G.: Theoretical and experimental investigations on miconazole/cyclodextrin/acid complexes: molecular modeling studies. Int. J. Pharm. **342**, 152–160 (2007)
22. Castagne, D., Dive, G., Evrard, B., Frédérich, M., Piel, G.: Spectroscopic studies and molecular modeling for understanding the interactions between cholesterol and cyclodextrins. J. Pharm. Pharmaceut. Sci. **13**, 362–377 (2010)
23. Higuchi, T., Connors, K.A.: Phase solubility techniques. Adv. Anal. Chem. Instru. **4**, 117–212 (1965)
24. Zime-Diawara, H., Gbaguidi, F., Evrard, B., Quetin-Leclercq, J., Moudachirou, M., Debrus, B., Hubert, Ph., Rozet, E.: Validation, transfer and measurement uncertainty estimation of an HPLC–UV method for the quantification of artemisinin in hydro alcoholic extracts of *Artemisia annua* L. J. Pharm. Biomed. Anal. **56**, 7–15 (2011)
25. Analytical methods committee. Analyst. **120**, 2303–2308 (1995)
26. Becke, A.D.: Density-functional thermochemistry. III. The role of exact exchange. J. Chem. Phys. **98**, 5648–5652 (1993)
27. Frisch M.J., Trucks G.W., Schlegel H.B., Scuseria G.E., Robb M.A., Cheeseman J.R., Montgomery J.A. Jr., Vreven T., Kudin K.N., Burant J.C., Millam J.M., Iyengar S.S., Tomasi J., Barone V., Mennucci B., Cossi M., Scalmani G., Rega N., Petersson G.A., Nakatsuji H., Hada M., Ehara M., Toyota K., Fukuda R., Hasegawa J., Ishida M., Nakajima T., Honda Y., Kitao O., Nakai H., Klene M., Li X., Knox J.E., Hratchian H.P., Cross J.B., Adamo C., Jaramillo J., Gomperts R., Stratmann R.E., Yazyev O., Austin A.J., Cammi R., Pomelli C., Ochterski J.W., Ayala P.Y., Morokuma K., Voth G.A., Salvador P., Dannenberg J.J., Zakrzewski V.G., Dapprich S., Daniels A.D., Strain M.C., Farkas O., Malick D.K., Rabuck A.D., Raghavachari K., Foresman J.B., Ortiz J.V., Cui Q., Baboul A.G., Clifford S., Cioslowski J., Stefanov B.B., Liu G., Liashenko A., Piskorz P., Komaromi I., Martin R.L., Fox D.J., Keith T., Al- Laham M.A., Peng C.Y., Nanayakkara A., Challacombe M., Gill P.M.W., Johnson B, Chen W, Wong M.W., Gonzalez C., Pople J.A.: Gaussian, Inc., Wallingford CT, Gaussian 03, Revision D.02 (2004)
28. Rozet, E., Hubert, C., Ceccato, A., Dewé, W., Ziemons, E., Moonen, F., Michail, K., Wintersteiger, R., Streel, B., Boulanger, B., Hubert, Ph.: Using tolerance intervals in pre-study validation of analytical methods to predict in-study results. The fit-for-future purpose concept. J. Chromatogr. A. **1158**, 126–137 (2007) http://hdl.handle.net/2268/1810
29. Hubert, Ph., Nguyen-Huu, J.J., Boulanger, B., Chapuzet, E., Chiap, P., Cohen, N., Compagnon, P.A., Dewe, W., Feinberg, M., Lallier, M., Laurentie, M., Mercier, N., Luzard, G.M., Nivet, C., Valet, L.: Harmonization of strategies for the validation of quantitative analytical procedures. A SFSTP Proposal? Part I. J. Pharm. Biomed. Anal. **36**, 579–586 (2004) http://hdl.handle.net/2268/6169
30. Rozet, E., Ceccato, A., Hubert, C., Ziemons, E., Oprean, R., Rudaz, S., Boulanger, B., Hubert, Ph.: An analysis of recent pharmaceutical regulatory documents on analytical method validation. J.Chromatogr. A. **1158**, 111–125 (2007) http://hdl.handle.net/2268/3646
31. Jacquet, R., Favetta, P., Elfakir, C., Lafosse, M.: Charaterization of a new methylated βCD with a low degree of substitution by

matrix-assisted laser desorption/ionization mass spectrometry and liquid chromatography using evaporative light scattering detection. J. Chromatogr. A **2005**, 106–112 (1083)
32. Blasko, G., Cordell, G.A.: Definitive 1H- and 13C-NMR assignments of artemisinin (Qinghaosu). J. Nat. Prod. **51**, 1273–1276 (1988)
33. Inoue, Y.: NMR studies on cyclodextrins and inclusion complexes. Annu. Rep. NMR Spectrosc. **27**, 59–101 (1993)
34. Djedaïni, F., Perly, B.: Nuclear magnetic resonance of cyclodextrins, derivatives and inclusion compounds. In: new trends in cyclodextrins and derivatives, chap 6, Paris (1990)
35. Illapakurthy, A.C., Wyandt, C.M., Stodghill, S.P.: Isothermal titration calorimetry method for determination of cyclodextrin complexation thermodynamics between artemisinin and naproxen under varying environmental conditions. Eur J Pharm Biopharm. **59**, 325–332 (2005)
36. Chadha, R., Gupta, S., Pathak, N., Shukla, G., Jain, D.V.S., Pissurlenkar, R.R.S., Coutinho, E.C.: Binary and ternary complexes of arteether β-CD—characterization, molecular modeling and in vivo studies. Pharmacol. Pharm. **2**, 212–225 (2011)
37. Brewster, M.E., Loftsson, T.: Cyclodextrins as pharmaceutical solubilizers. Adv. Drug Deliv. Rev. **59**, 645–666 (2007)
38. Gabelica, V., Galic, N., De Pauw, E.: On the specificity of cyclodextrin complexes detected by electrospray mass spectrometry. J. Am. Soc. Mass Spectrom. **13**, 946–953 (2002)
39. Messner, M., Kurkov, S.V., Flavià-Piera, R., Brewster, M.E., Loftsson, T.: Self-assembly of cyclodextrins: the effect of the guest molecule. Int. J. Pharm. **408**, 235–247 (2011)