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ORIGINAL ARTICLE

Physicochemical and thermodynamic characterization of hydroxy pentacyclic triterpenoic acid/γ-cyclodextrin inclusion complexes

Stephane Fontanay · Francine Kedzierewicz · Raphael Emmanuel Duval · Igor Clarot

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Abstract Hydroxy pentacyclic triterpenoic acids (HPTAs) have been described to exhibit numerous pharmacological properties. They also exhibit poor hydrosolubility, thus affecting their potential clinical interest. The association of these active substances with cyclodextrins could be employed to improve some properties such as bioavailability and activity. 1:1 Inclusion complexes of ursolic, oleanolic and betulinic acids with γ-cyclodextrin were evaluated by DSC and ^{1}H NMR spectroscopy. The apparent formation constants (K_f) of the formed complexes were determined using RP-HPLC. Thermodynamic parameters ΔG°, ΔH° and ΔS° were calculated with temperatures ranging from 25 to 45 °C to evaluate the complexation process. Finally the influence of γ-CD on the HPTA water solubility was investigated by phase-solubility studies.

Keywords HPTA · γ-Cyclodextrin · HPLC · NMR · DSC

Introduction

Ursolic acid (UA), Oleanolic acid (OA) and Betulinic acid (BA) are natural hydroxy pentacyclic triterpenoic acids (HPTAs) extracted from *Arctosphylos uvaursi, Syzygium aromaticum* and *Ziziphus mauritiana lam*, respectively (Fig. 1).

Due to their ability to interact with many enzymes [1], nucleic acids and biological membranes [2], HPTAs display numerous pharmacological activities including, anti-tumoral [3–5], anti-inflammatory [6, 7], hepatoprotective [8], anti-oxidant [9], anti-ulcer, anti-hypertensive [10], anti-diabetic [11], antiviral [12], anti-hyperlipidemic [13], anti-parasitical [7, 14], inhibitor of biofilm [15] and antimicrobial activities [7, 16–19]. Isolation and identification of HPTAs are usually and essentially obtained via methanolic or chloroformic extraction [20], and to date more than 50,000 pentacyclic triterpenes have been characterized.

Although of high interest, the development of HPTAs for therapeutic usages has been limited by their poor hydrosolubility and complexation with hydro-soluble shuttle, like cyclodextrins (CDs) has been suggested to potentially solve this issue.

CDs belong to a family of torus shaped, enzymatically synthesized and naturally occurring molecules. CDs are cyclic oligosaccharides composed, for the most, of six, seven or eight α-1,4 linked D-glucopyrannose units per molecule (respectively α-, β- and γ-CD) [21]. The exterior of the molecule is hydrophilic and its relative non polar central cavity [22] may selectively complex numerous organic hydrophobic molecules. The encapsulation of a solute inside the CD cavity can change the physico-chemical properties of the guest molecule to a great extent [23]. Hence CD complexation can be used to protect hydrophobic molecules from atmospheric oxidation, light degradation, heat induced transformations or evaporation [24]. This complexation can also increase the bioavailability of drugs. When using CDs, inclusion complexes were not always observed and a self-aggregation mechanism in the aqueous solutions could also lead to a solubilizing effect without penetration of the guest molecule in the CD cavity [25].

To characterize inclusion complexes, several physic, spectrometric and spectrophotometric methodologies may be used. HPLC has also been used to describe and characterize CD-guest complexes. Modifications of solute

S. Fontanay · F. Kedzierewicz · R. E. Duval · I. Clarot (✉)
Faculty of Pharmacy, SRSMC, Nancy-University, CNRS, 5 Rue Albert Lebrun, BP 80403, 54001 Nancy Cedex, France
e-mail: Igor.clarot@pharma.uhp-nancy.fr

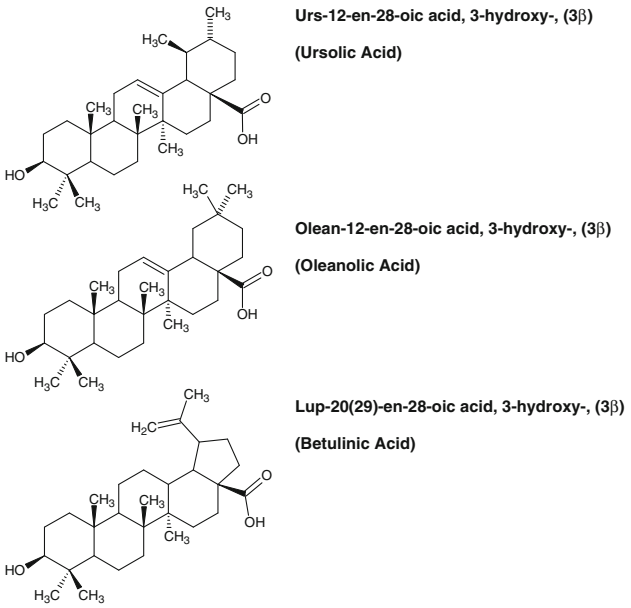

Fig. 1 Names and structures of the hydroxy pentacyclic triterpenoic acids investigated

retention with various CD concentrations in mobile phases were found to be related to the stoichiometry and stability of the complexes thus formed. In 2004, Claude et al. [26] have shown that hydroxy pentacyclic triterpenoic acids (UA, OA and BA) could be separated in RP-HPLC when using derivatized β and γ-cyclodextrins. They have observed that 1:1 complexes were obtained (apparent formation constant values ranging from 186 to 3469 M^{-1}) with acetonitrile/buffer mobile phases.

In this work, we studied and characterized γ-CD/HPTA complexes. We first evaluated the penetration of 3 HPTAs (UA, OA, BA) into the γ-CD cavity using differential scanning calorimetry (DSC) and nuclear magnetic resonance (NMR). Then, we described the retention of the three HPTAs on RP-18 chromatographic support using methanol-buffer mobile phases containing various concentrations of γ-CD and various temperatures. Apparent complex formation constant and thermodynamic data (enthalpy, entropy) were determined. Finally, the influence of γ-CD on the HPTA solubility was also investigated by phase-solubility studies.

Experimental

Reagents and chemicals

HPLC-grade methanol and HPLC-grade acetonitrile were commercially purchased from Prolabo (Paris, France) and water was freshly bidistilled. Na_2HPO_4, $2H_2O$ and H_3PO_4 were supplied by MERCK (France) and Riedel-de Haën (Sigma, France), respectively. Nylon filter membranes with 0.45 μm porosity were supplied from Macherey–Nagel (Germany).

γ-CD and D-glucose were supplied by Wacker (Werk Burghausen, Germany) and MERCK (France), respectively. UA ((3β)-3-hydroxyurs-12-en-28-oic acid; Acros Organic, France, ref. 290182500); OA (3β-hydroxyolean-12-en-28-oic acid; Sigma, France, ref. 093K0961) and BA (3β-hydroxylup-20(29)-en-28-oic acid; Aldrich, France, ref. 85,505–7) were commercially purchased.

Preparation of the complexes

The complexes were prepared by mixing (at 1:1 molar ratio) triterpene acid and γ-CD. Briefly, 100 mg of one of the HPTAs was dissolved in 160 μL of methanol and completed to 3 mL with water. A second solution containing 240 mg of γ-CD in 3 mL of water was prepared. Both solutions were left under stirring for 1 h at 37 °C (classical working temperature for biological purposes) in the dark under stirring conditions at 420 rpm in an Aerotron (Infors HT, Bottmingen, Switzerland). Preliminary studies on 24 h have showed that complexation process was completed in 1 h (when increasing the molar ratio to 1:2, half of the mass of the CD were lost during the filtration.) After reaction completeness, the complexes were filtered and air dried.

A physical mixture of HPTA and γ-CD (1:1 molar ratio) prepared by gently mixing the pulverized powders was used for analytical comparison.

HPLC analysis

The isocratic HPLC system consisted of a vacuum membrane degasser SCM100 (Thermo Electron Corporation, France), a HPLC Spectra System P100XR (Thermo Electron Corporation, France), a Rheodyne valve Model 8125 (Rheodyne, Cotati, CA, USA), fitted with a 20 μL sample loop and a UV detector Spectra System UV1000 (225 nm) model variable-wavelength monitor (Thermo Electron Corporation, France). A C18 silica Lichrospher® 100 RP-18 column (125 × 4 mm i.d., 5 μm particle size, MERCK, France) was used for all experiments. The column temperature was controlled with a column oven from Cluzeau (France) from 25 ± 1 to 45 ± 1 °C. The mobile phase flow-rate was set up at 1.00 ± 0.01 mL/min and systematically controlled throughout the experiments.

The phosphate buffer was composed of Na_2HPO_4, $2H_2O$ at 3.56 g/L, the pH was measured using a Tacussel PHM210 pHmeter (Radiometer, Netherland) and adjusted at 3.0 with the appropriate amount of H_3PO_4.

Mobile phases were prepared by mixing methanol, phosphate buffer (pH 3.0; 0.02 M) and phosphate buffer

(pH 3.0; 0.02 M) containing γ-CD to obtain the desired solvent and cyclodextrin concentrations (0–4 mM). Reasonable retentions of acids were obtained with mobile phases containing 88% of methanol. Lastly, these solutions were filtered through a 0.45 µm nylon filter membrane prior use. The column void volume, T_0, was determined using reagent grade copper sulphate solution (0.01 mg/mL) as described by Clarot et al. [27]. Sample solutions of HPTA (0.1 mg/mL) were prepared in pure MeOH without γ-CD and alpha D-glucose water solution (32.0 mM) was used as control probe.

Each solute was injected in triplicate and corresponding retention factors (k) were calculated (k ± SD). The validity of every linear relationship used in this report was systematically tested for linearity and non-linearity using classical statistical tests ($n = 3$, $\alpha = 5\%$). Tolerance curves leading to uncertainty scores on slopes and intercepts were used to describe apparent formation constant and thermodynamic parameters.

Differential scanning calorimetry

Thermal analysis was carried out with a Netzsch STA 409C (Geratebau, Germany) differential calorimeter calibrated with indium. All scans were performed between 30 and 350 °C with a heating rate of 10 °C/min in a dynamic argon atmosphere (50 mL min^{-1}). Samples (HPTAs, γ-CD, complexes and physical mixtures) were prepared weighing 5 mg of powder in aluminium open pans.

^{1}H NMR analysis

NMR sample solutions were prepared by dissolving excess amount of pure γ-CD or HPTA complexes in deuterium oxide. After filtration, ^{1}H-NMR spectroscopic experiments were performed at 300 K on a Bruker DRX-400 spectrophotometer (Wissembourg, France). Induced changes in the chemical shifts for γ-CD ($\Delta\delta$) by complexation were calculated. Spectra were acquired with number of scans = 32, number of dummy scans = 16, recycle delay = 1.5 s, acquisition size 1k × 512, processing size 2k × 2k, and mixing time 400 ms.

Solubility studies

Phase-solubility studies were carried out in water, according to Higuchi and Connors [28]. Briefly, excess amounts of HPTA were added to water containing increasing concentrations of γ-CD (2–8 mM) and suspensions were shaken at 30 °C for 7 days. After the equilibrium was reached, an aliquot of 100 µL was centrifuged and HPTA concentration was determined by HPLC analysis. Each experiment was carried out in triplicate (RSD < 5%).

Theory

Assuming that each HPTA forms a 1:1 complex with CD [26], the following equilibrium can occurred:

$$\text{HPTA} + \text{CD} \leftrightarrow \text{HPTA-CD} \quad (1)$$

When CD is added to the mobile phase, the solute retention factor, k, is affected as described by Eq. 2 for a 1:1 complex [29, 30]:

$$\frac{1}{k} = \frac{1}{k_0} + K_f \frac{[(CD)]}{k_0} \quad (2)$$

where k_0 is the retention factor without cyclodextrin, [(CD)] is the concentration of CD in the mobile phase, and K_f is the apparent formation constant of the formed complex.

As related by Matsui et al. [29], the complexation constant between cyclodextrin and methanol is related to the CD cavity size (0.93 M^{-1} for α-CD and 0.32 M^{-1} for β-CD). When considering a much larger γ-CD cavity, a much lower contact surface with methanol must lead to negligible apparent formation constant, as previously described for ethanolic mobile phases [31]. This weak interaction was not taken into account in the constant calculation.

As described by Eq. 2, when plotting 1/k versus [(CD)], a linear relationship reflects a solute-CD complex with a 1:1 stoichiometry and the (slope/intercept) ratio allows the determination of K_f.

To study the effect of the temperature on the complexation process of HPTAs by CD, the retention factors were determined for all CD concentrations tested at the following temperatures: 25, 30, 35, 40 and 45 °C. The well-known thermodynamic relationship shown in Eq. 3 was employed to determine the standard enthalpy (ΔH°) and the standard entropy (ΔS°) of transfer of HPTA from the mobile phase to the cyclodextrin cavity [27].

$$\text{Ln}K_f = \frac{-\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} \quad (3)$$

where K_f is the apparent formation constant of the inclusion complex, R is the gas constant and T the absolute temperature.

For a linear plot of ln K_f versus 1/T (Van't Hoff plot), the slope and intercept are respectively $-\Delta H^\circ/R$ and $\Delta S^\circ/R$.

For the determination of the Gibbs free energy which takes place during the inclusion process, Eq. 4 could be used.

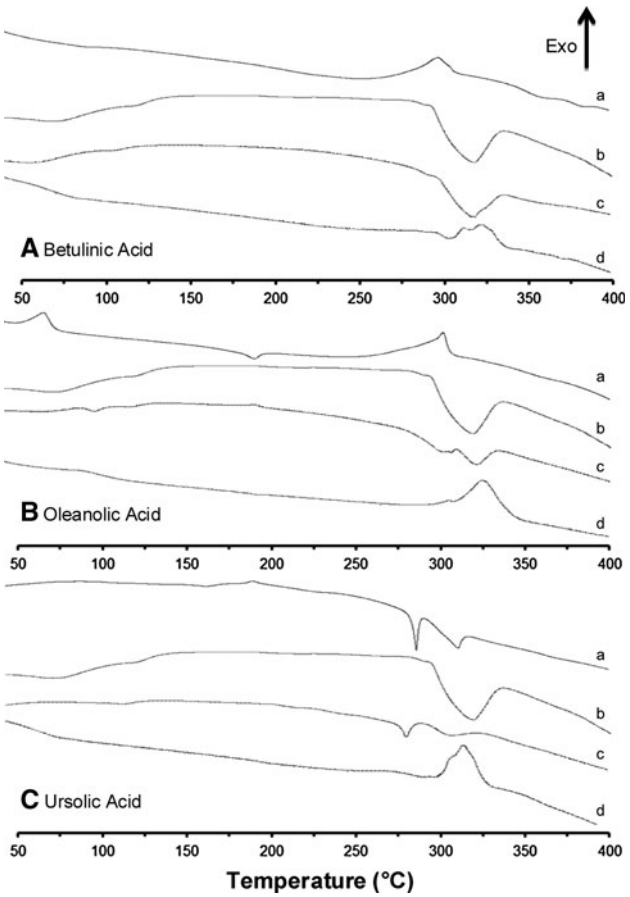

Fig. 2 DSC curves of betulinic (**A**), oleanolic (**B**) and ursolic acids (**C**): (*a*) acid alone, (*b*) γ-CD, (*c*) physical mixture (1:1) and (*d*) the complex

$$\Delta G^{\circ} = \Delta H^{\circ} - T\Delta S^{\circ} \quad (4)$$

Results and discussion

Differential scanning calorimetry studies

Thermal analysis has been reported to be a powerful analytical tool to characterize association complexes of drugs with cyclodextrins. DSC curves of HPTAs, γ-CD, their physical mixtures and the complexes are illustrated in Fig. 2. These thermograms revealed pronounced structural differences between pure substances, mixtures and association complexes.

For instance, the thermal profile of pure UA (Fig. 2Ca) showed an endothermic peak with a T_{max} temperature at about 283.5 °C, which was attributed to the melting of UA. The thermogram of the physical mixture (Fig. 2Cc) was similar to the superimposition of the thermograms of individual UA and γ-CD with the endothermic peak due to melting of UA. These curves indicated a lack of interactions in the physical mixture; suggesting that the complex was not obtained by simply mixing both powders. In the DSC curve of the inclusion complex (Fig. 2Cd), the melting peak of UA has disappeared. This may be attributed to a tight interaction between UA and γ-CD and suggests the formation of an inclusion complex [32].

^{1}H NMR studies

^{1}H NMR is a well-known technique to determine physicochemical interactions occurring in the formation of an inclusion complex [33].

The ^{1}H assignment of γ-CD is shown in Fig. 3 and the chemical shifts of the protons of free γ-CD or when complexed with HPTAs are given in Table 1.

Complexation of HPTAs with γ-CD, resulted in disappearing H-3 proton (due to peak overlap) and in a great shift of the H-5 proton, located inside the CD cavity. These results were consistent with the penetration of HPTAs inside the hydrophobic pocket of γ-CD indicating the formation of inclusion complexes.

Apparent formation constants K_f

The capacity factors of the three HPTAs (Table 2) on the C18 stationary phase were determined between 25 and 45 °C.

As expected by the classical theory of complexation applied to a reversed phase chromatographic system, we observed a decrease of retention factors paralleling an increased concentration of CD mobile phase. This phenomenon may be explained by the formation of a hydrophilic association complexes between cyclodextrin and triterpene acid. To examine the possible effect of γ-CD on solvent strength of the mobile phase, a 32.0 mM D-glucose methanol/phosphate buffer 88/12 (v/v) mobile phase was used (as γ-CD contains 8 glucoses equivalent). As no retention modifications were observed and with the hypothesis that no glucose-HPTAs complexes exist, the elution modifications observed in Table 2 in the presence of γ-CD cannot therefore be attributed to the solvent strength.

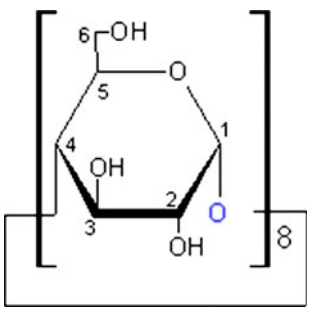

Fig. 3 Structure of γ-CD

Table 1 ^{1}H chemical shifts of the protons of γ-CD free or complexed with ursolic (UA), oleanolic (OA) or betulinic (BA) acids in D_2O

^{1}H assignment	δ γ-Cd_{free} (ppm)	Δ δ (ppm)		
		UA	OA	BA
H1	5.065	0.006	0.006	0.006
H3	3.890	*	*	*
H6	3.827	0.006	−0.008	0.000
H5	3.801	−0.024	−0.027	−0.023
H2	3.621	0.002	0.002	−0.003
H4	3.545	−0.006	−0.004	−0.012

* Not determined

The apparent formation constants of the different CD-HPTAs complexes formed were determined following the Eq. 1. Results are reported in Table 3. Good coefficients of correlation r were determined, thus reflecting the formation of 1:1 complexes independently of the HPTA derivatives tested.

Same order complexation constants were obtained for UA and OA (200–600 M^{-1}) compared to BA (600–1900 M^{-1}) due to their similar structures differing only by the position of two methyl groups (Fig. 1).

Thermodynamic parameters for the HPTA/γ-CD complexes

The retention factors, k, of the three HPTAs on the C18 stationary phase were determined between 25 and 45 °C (Table 2) in order to study mechanistic aspects of the affinity of HPTAs for γ-CD. When the temperature was increased, the retention factor decreased, as expected for a reversed phase system.

Linear Van't Hoff plots (Eq. 3) were obtained (data not shown) for all HPTAs tested, with good coefficients of correlation (r ranging between 0.95 and 0.99).

Free enthalpies (ΔG°), enthalpies (ΔH°) and entropies (ΔS°) of complex formation were calculated for each complex formed and described in Table 4. ΔG°, ΔH° and ΔS° were in the same range for UA and OA but increased for BA, the acid which allows for the most stable complex.

Negative enthalpies (ΔH°) values indicate that it is energetically more favorable for the solutes to be in the γ-CD cavity. These results suggested that the complexation process was essentially enthalpy-controlled ($\Delta H^\circ >> \Delta S^\circ$) and that dipolar and Van der Waals interactions between host and guest molecules are involved in the complex formation. As illustrated in Table 4, a higher exothermicity was obtained for BA (42585 kJ mol^{-1} vs. 21953 kJ mol^{-1} for UA and 23436 kJ mol^{-1} for OA). This result indicate that BA is more stronger included in the CD cavity and is consistent with K_f values shown in Table 3 (1881 M^{-1} vs. 584 M^{-1} for UA and 498 M^{-1} for OA). A contribution of hydrophobic interactions involves the breakdown and displacement of the highly ordered water molecules inside the cyclodextrin cavity. Whatever the triterpene acid under study, the binding mechanism is spontaneous (ΔG° from −15692 to −18435 kJ mol^{-1} at 298 K), whereas the negative entropy indicates greater order after complexation. It is mainly due to the loss of rotational and translational freedom degree of the molecules involved in the complexation process.

Table 2 Capacity factor k of UA, OA and BA for methanol/phosphate buffer (pH 3.0; 0.02 M) mobile phases (88/12 v/v) containing 0–4 mM of γ-CD and with temperature ranging from 25 °C to 45 °C

HPTA	Temp. (°C)	γ-CD concentration (mM)				
		0.0	1.0	2.0	3.0	4.0
UA	25.0	3.85 ± 0.03	3.71 ± 0.00	2.08 ± 0.02	1.72 ± 0.01	1.37 ± 0.00
	30.0	3.74 ± 0.12	3.71 ± 0.08	2.13 ± 0.05	1.89 ± 0.05	1.42 ± 0.05
	35.0	3.47 ± 0.01	2.87 ± 0.10	1.92 ± 0.01	1.71 ± 0.02	1.32 ± 0.05
	40.0	2.54 ± 0.01	2.39 ± 0.01	1.81 ± 0.01	1.64 ± 0.02	1.04 ± 0.02
	45.0	2.66 ± 0.00	2.40 ± 0.03	1.43 ± 0.01	1.46 ± 0.02	1.20 ± 0.02
OA	25.0	3.63 ± 0.01	3.59 ± 0.02	2.08 ± 0.03	1.76 ± 0.01	1.41 ± 0.01
	30.0	3.57 ± 0.13	3.66 ± 0.00	2.07 ± 0.01	1.99 ± 0.06	1.53 ± 0.03
	35.0	3.24 ± 0.01	2.74 ± 0.11	1.76 ± 0.06	1.69 ± 0.06	1.38 ± 0.03
	40.0	2.35 ± 0.02	2.20 ± 0.02	1.77 ± 0.02	1.65 ± 0.01	1.07 ± 0.01
	45.0	2.53 ± 0.04	2.26 ± 0.01	1.39 ± 0.01	1.43 ± 0.00	1.26 ± 0.02
BA	25.0	3.17 ± 0.05	2.36 ± 0.01	1.13 ± 0.03	0.78 ± 0.02	0.59 ± 0.00
	30.0	3.12 ± 0.04	2.44 ± 0.02	1.33 ± 0.04	0.92 ± 0.01	0.79 ± 0.05
	35.0	2.89 ± 0.03	1.96 ± 0.00	1.13 ± 0.02	0.92 ± 0.04	0.68 ± 0.02
	40.0	2.32 ± 0.02	1.99 ± 0.02	1.15 ± 0.00	0.82 ± 0.01	0.76 ± 0.00
	45.0	2.22 ± 0.03	1.67 ± 0.01	0.86 ± 0.01	0.80 ± 0.00	0.71 ± 0.02

Table 3 Apparent formation constant K_f (M^{-1}) and correlation coefficient r for HPTAs/γ-CD complexes of UA, OA and BA for methanol/phosphate buffer (pH 3.0; 0.02 M) mobile phases (88/12 v/v) containing 0–4 mM of γ-CD and with temperature ranging from 25 °C to 45 °C

Analyte	Temp. (°C)	K_f (M^{-1})	*r*
UA	25	584 ± 62	0.975
	30	512 ± 61	0.970
	35	418 ± 49	0.964
	40	413 ± 70	0.922
	45	324 ± 38	0.949
OA	25	498 ± 51	0.975
	30	400 ± 50	0.954
	35	351 ± 31	0.975
	40	323 ± 52	0.911
	45	264 ± 34	0.933
BA	25	1881 ± 418	0.985
	30	1010 ± 144	0.985
	35	966 ± 99	0.990
	40	670 ± 79	0.975
	45	557 ± 79	0.959

Table 4 Thermodynamic parameters for interaction of HPTA with γ-CD at 298 K

HPTA	ΔH° (J mol^{-1})	ΔS° (J $mol^{-1}.K^{-1}$)	ΔG° (J mol^{-1})
UA	−21953 ± 2812	−21 ± 9	−15692
OA	−23436 ± 1946	−27 ± 6	−15386
BA	−42585 ± 7690	−81 ± 25	−18435

Phase solubility study

HPTAs were practically insoluble in water. The phase solubility diagram of HPTAs with increasing γ-CD concentrations, is illustrated in Fig. 4. For each HPTA under study, a Bs curve type was observed [28]. From 0 to 1 mM γ-CD concentration, the apparent solubility of the three HPTAs is increased due to the formation of a soluble complex. The higher value with a five time coefficient was obtained for UA (from 0.015 mM without γ-CD to 0.090 mM with γ-CD). For OA and BA, the increase of solubility was limited to a two time coefficient. Further γ-CD addition results in the precipitation of the complex.

Conclusion

In this study, DSC and ^{1}H NMR experiments were used to demonstrate the formation of HPTAs/γ-cyclodextrin 1:1 inclusion complexes. Apparent formation constant K_f were determined with a C18 stationary phase and a thermodynamic study demonstrates an enthalpically driven mechanism and the spontaneity of the binding process, a step required for future medical applications. Finally, a non-negligible increase of the HPTAs water solubility using γ-CD was demonstrated by phase-solubility studies.

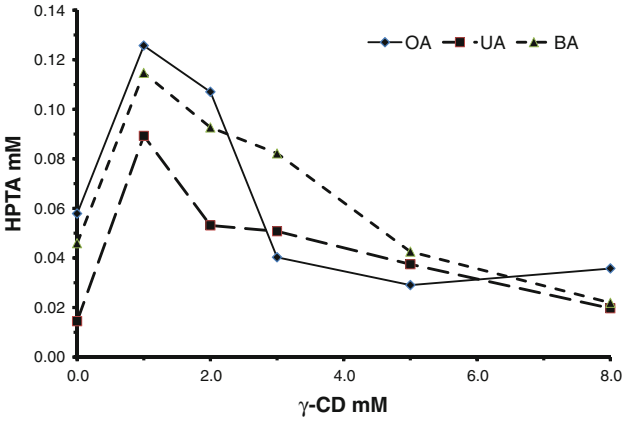

Fig. 4 Phase-solubility diagram of HPTA/γ-CD system

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References

1. Ali, Z., Khan, S.I., Ferreira, D., Khan, I.A.: Podocarpaside, a triterpenoid possessing a new backbone from *Actaea podocarpa*. Org. Lett. **8**, 5529–5532 (2006)
2. Han, S.K., Ko, Y.I., Park, S.J., Jin, I.J., Kim, Y.M.: Oleanolic acid and ursolic acid stabilize liposomal membranes. Lipids **32**, 769–773 (1997)
3. Li, J., Guo, W.J., Yang, Q.Y.: Effects of ursolic acid and oleanolic acid on human colon carcinoma cell line HCT15. World J. Gastroenterol. **8**, 493–495 (2002)
4. Murakami, S., Takashima, H., Sato-Watanabe, M., Chonan, S., Yamamoto, K., Saitoh, M., Saito, S., Yoshimura, H., Sugawara, K., Yang, J., Gao, N., Zhang, X.: Ursolic acid, an antagonist for transforming growth factor (TGF)-beta1. FEBS Lett. **566**, 55–59 (2004)
5. Tan, Y., Yu, R., Pezzuto, J.M.: Betulinic acid-induced programmed cell death in human melanoma cells involves mitogen-activated protein kinase activation. Clin. Cancer Res. **9**, 2866–2875 (2003)
6. Altinier, G., Sosa, S., Aquino, R.P., Mencherini, T., Della Loggia, R., Tubaro, A.: Characterization of topical antiinflammatory compounds in *Rosmarinus officinalis*. J. Agric. Food Chem. **55**, 1718–1723 (2007)
7. Chowdhury, A.R., Mandal, S., Mittra, B., Sharma, S., Mukhopadhyay, S., Majumder, H.K.: Betulinic acid, a potent inhibitor of eukaryotic topoisomerase I: identification of the inhibitory step, the major functional group responsible and development of more potent derivatives. Med. Sci. Monit. **8**, 254–265 (2002)
8. Saravanan, R., Pugalendi, V.: Impact of ursolic acid on chronic ethanol-induced oxidative stress in the rat heart. Pharmacol. Rep. **58**, 41–47 (2006)
9. Ismaili, H., Milella, L., Fkih-Tetouani, S., Ilidrissi, A., Camporese, A., Sosa, S., Altinier, G., Della Loggia, R., Aquino, R.:

In vivo topical anti-inflammatory and in vitro antioxidant activities of two extracts of *Thymus satureioides* leaves. J. Ethnopharmacol. **91**, 31–36 (2004)
10. Somova, L.I., Shode, F.O., Ramnanan, P., Nadar, A.: Antihypertensive, antiatherosclerotic and antioxidant activity of triterpenoids isolated from *Olea europaea*, subspecies *africana* leaves. J. Ethnopharmacol. **84**, 299–305 (2003)
11. Liu, J.: Pharmacology of oleanolic acid and ursolic acid. J. Ethnopharmacol. **49**, 57–68 (1995)
12. Xu, H.X., Zeng, F.Q., Wan, M., Sim, K.Y.: Anti-HIV triterpene acids from *Geum japonicum*. J. Nat. Prod. **59**, 643–645 (1996)
13. Somova, L.O., Nadar, A., Rammanan, P., Shode, F.O.: Cardiovascular, antihyperlipidemic and antioxidant effects of oleanolic and ursolic acids in experimental hypertension. Phytomedicine **10**, 115–121 (2003)
14. Germonprez, N., Maes, L., Van Puyvelde, L., Van Tri, M., Tuan, D.A., De Kimpe, N.: In vitro and in vivo anti-leishmanial activity of triterpenoid saponins isolated from *Maesa balansae* and some chemical derivatives. J. Med. Chem. **48**, 32–37 (2005)
15. Hu, J.F., Garo, E., Goering, M.G., Pasmore, M., Yoo, H.D., Esser, T., Sestrich, J., Cremin, P.A., Hough, G.W., Perrone, P., Lee, Y.S., Le, N.T., O'Neil-Johnson, M., Costerton, J.W., Eldridge, G.R.: Bacterial biofilm inhibitors from *Diospyros dendo*. J. Nat. Prod. **69**, 118–120 (2006)
16. He, Z.D., Ma, C.Y., Zhang, H.J., Tan, G.T., Tamez, P., Sydara, K., Bouamanivong, S., Southavong, B., Soejarto, D.D., Pezzuto, J.M., Fong, H.H.: Antimalarial constituents from *Nauclea orientalis* (L.). Chem. Biodivers. **2**, 1378–1386 (2005)
17. Kashiwada, Y., Wang, H.K., Nagao, T., Kitanaka, S., Yasuda, I., Fujioka, T., Yamagishi, T., Cosentino, L.M., Kozuka, M., Okabe, H., Ikeshiro, Y., Hu, C.Q., Yeh, E., Lee, K.H.: Anti-AIDS agents. 30. Anti-HIV activity of oleanolic acid, pomolic acid, and structurally related triterpenoides. J. Nat. Prod. **61**, 1090–1095 (1998)
18. Liu, J.: Oleanolic acid and ursolic acid: research perspectives. J. Ethnopharmacol. **100**, 92–94 (2005)
19. Setzer, W.N., Rozmus, G.F., Setzer, M.C., Schmidt, J.M., Vogler, B., Reeb, S., Jackes, B.R., Irvine, A.K.: Bioactive principles in the bark of *Pilidiostigma tropicum*. J. Mol. Model. **12**, 703–711 (2006)
20. Cowan, M.M.: Plant products as antimicrobial agents. Clin. Microbiol. Rev. **12**, 564–582 (1999)
21. Szejtli, J.: Cyclodextrin technology. Kluwer Academic Publishers, Dordrecht (1988)
22. Procyk, A.D., Bissell, M., Street Jr, K.W., Acree Jr, W.E.: Solubility in binary solvent systems: 8. Estimation of binary alkane plus p-dioxane solvent nonideality from measured anthracene solubilities. J. Pharm. Sci. **76**, 621–625 (1987)
23. Stella, V.J., He, Q.: Cyclodextrins. Toxicol. Pathol. **36**, 30–42 (2008)
24. Szejtli, J., Gerloczy, A., Szente, L., Benky-Elod, E., Sebestyen, G., Fonagy, A., Kurcz, M.: Enhancement of drug absorption with cyclodextrin inclusion complexes. Acta. Pharm. Hung. Acad. Sci. Hung. **49**, 207–221 (1979)
25. Jansook, P., Kurkov, S.V., Loftsson, T.: Cyclodextrins as solubilizers: formation of complex aggregates. J. Pharm. Sci. **99**, 719–729 (2010)
26. Claude, B., Morin, P., Lafosse, M., Andre, P.: Evaluation of apparent formation constants of pentacyclic triterpene acids complexes with derivatized beta- and gamma-cyclodextrins by reversed phase liquid chromatography. J. Chromatogr. A **1049**, 37–42 (2004)
27. Clarot, I., Clédat, D., Guillaume, Y.C., Cardot, P.J.P.: Chromatographic study of terpene-β-cyclodextrin complexes on porous graphitic carbon stationary phase. Chromatographia **54**, 447–453 (2001)
28. Higuchi, T., Connors, K.A.: Phase-solubility techniques. In: Reilly, C.N. (ed.) Advances in Analytical Chemistry Instrumentation, vol. 4, pp. 117–212. Wiley-Interscience, New York (1965)
29. Matsui, Y., Mochida, K.: Binding forces contributing to the association of cyclodextrin with alcohol in an aqueous solution. Bull. Chem. Soc. Jpn. **52**, 2808–2814 (1979)
30. Uekama, K., Hirayama, F., Irie, T.: New method for determination of the stability constants of cyclodextrin-prostaglandin inclusion complexes by liquid chromatography. Chem. Lett. **7**, 661–664 (1978)
31. Roussel, C., Favrou, A.: HPLC separation of the atropisomers of some substituted N-arylthiazoline-2-thiones with γ-cyclodextrin as a chiral mobile phase additive: size and lipophilicity effects of substituents. J. lncl. Phenom. Mol. **16**, 283–296 (1993)
32. Pralhad, T., Rajendrakumar, K.: Study of freeze-dried quercetin-cyclodextrin binary systems by DSC, FT-IR, X-ray diffraction and SEM analysis. J. Pharm. Biomed. Anal. **34**, 333–339 (2004)
33. Schneider, H.-J., Hacket, F., Rüdiger, V., Ikeda, H.: NMR studies of cyclodextrins and cyclodextrin complexes. Chem. Rev. **98**, 1755–1786 (1998)