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Assessment of the complexation degree of camptothecin derivatives and cyclodextrins using spectroscopic and separative methodologies

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ABSTRACT

The complexation of camptothecin and homocamptothecin derivatives, topoisomerase I inhibitors, with two cyclodextrins (CDs) of pharmaceutical interest (native and hydroxypropylated β-CD) was studied at pH 3.5 and 6. In a first step, the affinity order of the six compounds studied for the β-CD and HP-β-CD was evaluated in HPLC using immobilized stationary phases [Cyclobond I 2000 (β-CD) and Cyclobond I 2000 RSP (HP-β-CD)]. In a second step, the apparent binding constants of the 12 complexes studied were determined at both pH by HPLC using Scott's method with CD as a chiral additive. The 1:1 stoichiometry of the complex formed between HP-β-CD and the homocamptothecin derivative elomotecan (*R*)-6 was established by fluorescence spectroscopy using the continuous variation method developed by Job and ESI-MS. Complementary investigations were achieved for topotecan (*S*)-3 and elomotecan (*R*)-6 using CE. Further studies provided similar conclusions concerning affinity of all the derivatives studied for both CDs: that is, a slightly larger affinity was observed for HP-β-CD with respect to β-CD, except for (*S*)-3. For (*S*)-3, this affinity increase with pH, in the range studied.

1. Introduction

Camptothecin (*S*)-**1** (Fig. 1) is a natural alkaloid, which is relatively insoluble in aqueous solutions, produced by two Asian trees, Camptotheca accuminata[1] and Mappia foetida.[2] Despite its promising anti-cancer potential, CPT has not been developed as an anti-cancer drug due to its poor solubility and stability in any pharmaceutical solvent and the severe side effects were observed, including poorly predictable hemorrhagic cystitis, gastrointestinal toxicities, and myelosuppression.[3] In 1985, the identification of topoisomerase I as the target for antineoplastic treatment, and elucidation of its inhibition by a camptothecin-dependent mechanism, led to renewed efforts to develop analogs of this series.[4]

Several approaches have been investigated to overcome the solubility and/or the stability of the camptothecin. Figure 1 presents a part of the new molecules that were studied here after. First, the development of a camptothecin series led to the discovery of irinotecan [a prodrug of SN38 (*S*)-**2**] and topotecan (*S*)-**3**, both compounds which are used for clinical therapies against colorectal and ovarian tumors, respectively.[5,6] Additionally, a new class of camptothecin derivatives, named homocamptothecins (hCPTs), as new inhibitors of topoisomerase I emerged from the drug design strategies of the E-ring. The hCPTs, including (*R*)-**4,** are characterized by the expanded seven-membered lactone E-ring and a β-hydroxylactone group which enhances the plasma stability.[7] Diflomotecan (*R*)-**5**, an hCPT derivative with a difluoro substitution of the A ring was the first clinical candidate in the homocamptothecin series.[8] Recently, clinical trials have been carried out with a second compound, elomotecan, (*R*)-**6**, which bears a methylpiperidine substituent at the 12-position of the B-ring in addition to a chloro and a methyl group on the adjacent A ring.[9] More recently, new hexacyclic CPT analogs with a dipeptide ester group at the C-20 position have been described as water-soluble prodrugs.[10] Among the prodrugs synthesized, the lead compound, namely TP300 with a glycylsarcosyl ester, is highly water soluble (>10 mg/mL).[11]

The second approach for the solubility and/or stability improvement of camptothecin and its derivatives concerns the interaction with macromolecules including liposomes,[12–15] microspheres,[16] lipids,[17] polymeric nanoparticules,[18–20] polymer-conjugated CPT,[21] and cyclodextrins.[22–25]

CDs and their derivatives have been extensively used to improve the solubility, stability, or bioavailability of a variety of poorly soluble and labile drugs including anti-cancer drugs and have been applied in various pharmaceutical formulations.[26] Inclusion complexes between CPT and CDs have been characterized by computational and experimental studies.[22–24,27] For example, Kang et al.[22] reported a linear increase in the solubility of camptothecin with increasing concentration of cyclodextrins and especially with the randomly substituted dimethyl-β-cyclodextrin, RDM-β-CD, allowing a maximum solubility 171-fold higher than that without CD that is, 228.45 μg/mL versus 1.34 μg/mL in an acid medium.

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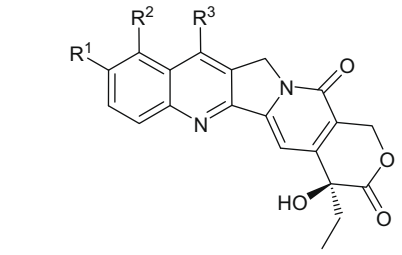
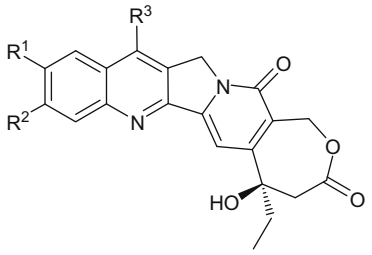
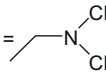
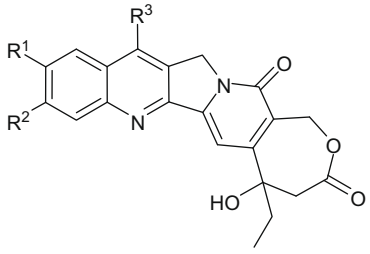


(*S*)-**1**: camptothecin: $R^1 = R^2 = R^3 = H$
(*S*)-**2**: SN-38: $R^1 = OH$, $R^2 = H$, $R^3 = C_2H_5$



(*R*)-**4**: homocamptohecin: $R^1 = R^2 = R^3 = H$

(*S*)-**3**: topotecan: $R^1 = OH$, $R^2 =$  $R^3 = H$



(*S*)-**5** and (*R*)-**5**: diflomotecan: $R^1 = R^2 = F$, $R^3 = H$

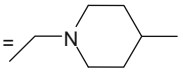
(*S*)-**6** and (*R*)-**6**: elomotecan: $R^1 = CH_3$, $R^2 = Cl$, $R^3 =$ 

Figure 1. Structures of camptothecin and homocamptothecin derivatives studied.

Additionally, the lactone-form stability is reported in this study with a 10-fold reduced hydrolysis rate constant. The HP-β-CD has been used by Saetern et al.[23] to describe the CPT solubility/or stability enhancement at a physiologically pH range (5.5–7.0). For example, in water at pH 7.0, the solubility increased from 4 μg/mL to 96 μg/mL using HP-β-CD. Cyclodextrin·CPT complexes have been prepared in a synthetic polymer using polyethylene glycol. The cyclosert-camptothecin (IT-101) is a linear beta-cyclodextrin-based polymer and CPT conjugate with solubility value greater than 200 mg/ml (equivalent to a 12–20 mg/mL of CPT) in aqueous solution. For the sake of comparison, the solubility of CPT (lactone form) is 4 μg/mL at the same experimental conditions.[28]

A recent study described the interactions between silatecan and sulfobutyl ether or 2-hydroxypropyl substituted β-cyclodextrins,[27] but no additional study has yet been reported in the literature for the complexation of homocamptothecin derivatives with cyclodextrins. We have previously reported the enantiomeric separations of homocamptothecin derivatives with highly sulfated cyclodextrins[29] but without characterization of the interaction; to the best of our knowledge, no additional studies have been reported in the literature. Herein we report the interaction between CPT or its derivatives (Fig. 1) and natural or substituted cyclodextrins using spectroscopic and separative methodologies. Stoichiometries and apparent binding constants (*K*) were therefore determined.

2. Results and discussion

2.1. Stoichiometry study

2.1.1. Fluorescence study

The continuous variation method, Job's method,[30,31] based on the fluorescence intensity measurements, was used to determine the stoichiometry of the inclusion complexes between (*R*)-**6** and HP-β-CD at pH 3.5 and 6. At both pH values, the maximum of polynomial plots ($\Delta F[(R)\text{-}\mathbf{6}] = f(r)$) corresponds to the molar fraction $r = 0.5$, supporting the formation of inclusion complex with *n*:*n* $[(R)\text{-}\mathbf{6}]_n{\cdot}(\text{HP-}\beta\text{-CD})_n$ stoichiometry.

2.1.2. Mass spectrometry study

The stoichiometry of the inclusion complexes of (*R*)-**6** and β-CD was studied by ESI-MS using solutions containing 1/10 and 1/100 molar ratios of (*R*)-**6** versus β-CD with different concentrations of (*R*)-**6** (22 μM, 50 μM, and 220 μM); the spectra obtained at a molar ratio of 1/10 with (*R*)-**6** concentrations of 220 μM and 22 μM are presented in Figure 2a and b, respectively. In the first spectrum (Fig. 2a), the lowest remarkable *m/z* ratio corresponds to $[(R)\text{-}\mathbf{6}{+}H]^+$ detected at 522.0. In addition, (*R*)-**6** appears at 1043.8 *m/z* ratio as a dimeric form $[((R)\text{-}\mathbf{6})_2{+}H]^+$, favored by high concentration of the solute, as underlined in the literature,[32] and by the ionization mode. β-CD was observed at 1135.7 and 1157.9 *m/z* ratios as a monocationic $[\beta\text{-CD}{+}H]^+$ form and as a natrium adduct $[\beta\text{-CD}{+}Na]^+$ (major form), respectively. All these characteristic peaks were observed for (*R*)-**6** and β-CD studied alone at 220 μM. The highest *m/z* ratio detected at 1658.3 corresponds to the $[(R)\text{-}\mathbf{6}{\cdot}\beta\text{-CD}{+}H]^+$ complex. It is noteworthy that a 1:1 complex between (*R*)-**6** and **β**-CD complex appears at 840.4 too, corresponding to $[(R)\text{-}\mathbf{6}{\cdot}\beta\text{-CD}{+}Na{+}H]^{2+}$ (dicationic form as confirmed by isotopic repartition). This complex is the predominant form with respect to $[(R)\text{-}\mathbf{6}{\cdot}\beta\text{-CD}{+}H]^+$ as the cyclodextrin is essentially on the $[\beta\text{-CD}{+}Na]^+$ form. According to the apparatus performances, a maximal *m/z* ratio of 3000 can be analyzed. If a 2:2 complex was formed in solution, in MS using electrospray, it would lead to multicharged ions and would then be detected at a smaller *m/z* ratio that is, *m*/4, *m*/3, or *m*/2 ratio. However, no additional peak characteris-

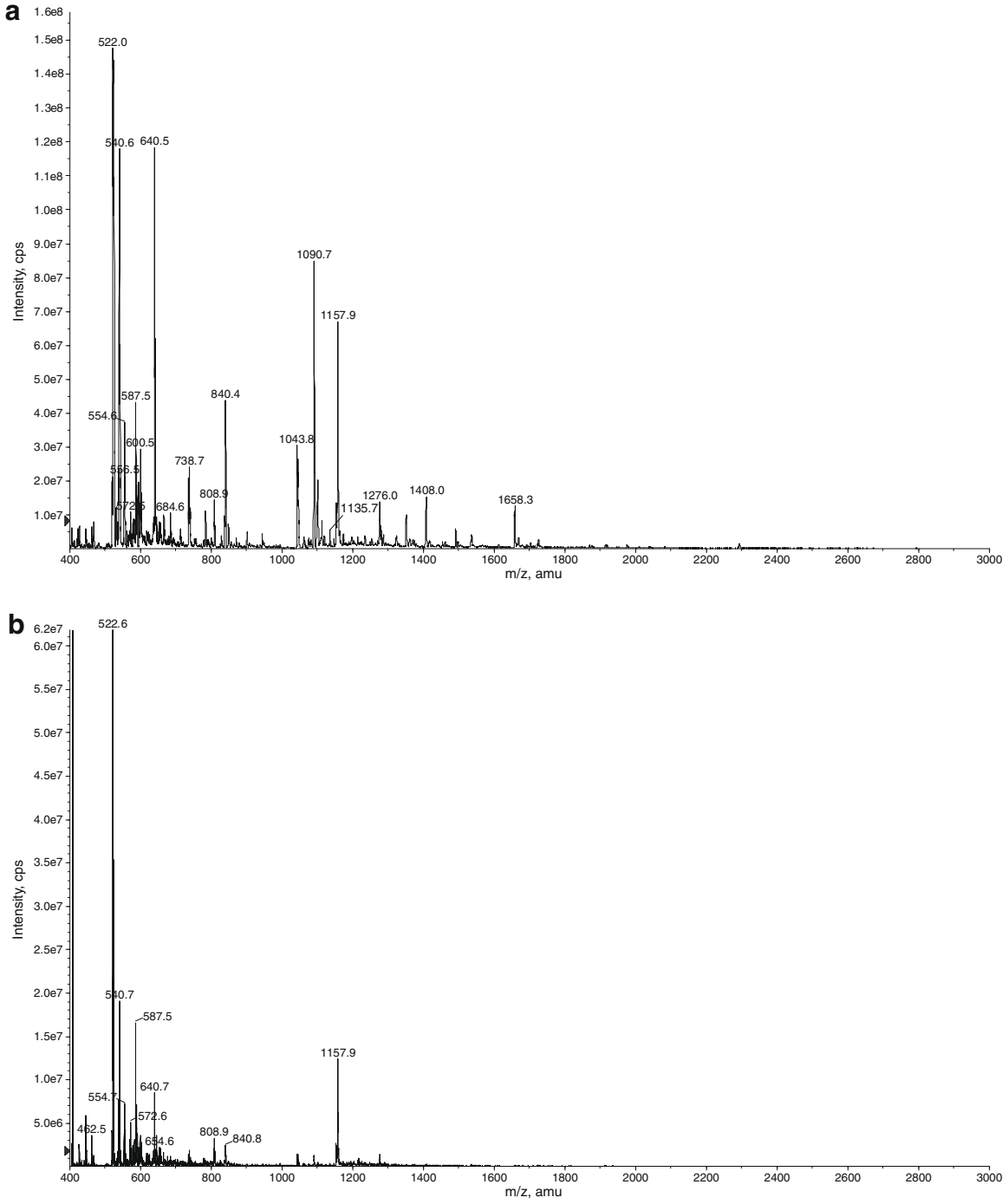


Figure 2. ESI-MS spectra of solutions containing a 1/10 molar ratio of (*R*)-**6** versus β-CD with (a) 220 μM of (*R*)-**6** and (b) 22 μM of (*R*)-**6**.

tic of a 2:2 multi-charged complex, involving either $[\beta\text{-CD+H}]^+$ or $[\beta\text{-CD+Na}]^+$, was detected. Nevertheless the presence of a peak at 1090.7, characteristic of a dicationic form reveals the presence of $[((R)\text{-}\mathbf{6})_2\cdot\beta\text{-CD+2H}]^{2+}$ complex, resulting probably from the dimer of (*R*)-**6** in solution. In order to determine if the occurrence of this complex is linked to the high concentration of (*R*)-**6**, diluted solutions, at 50 and 22 μM were studied. The spectrum at 22 μM of (*R*)-**6** is shown in Figure 2b. Whatever the concentration used, (*R*)-**6** appears at an *m/z* ratio of 522.6. The intensity ratios of $[(R)\text{-}\mathbf{6}\text{+H}]^+$ versus $[((R)\text{-}\mathbf{6})_2\text{+H}]^+$ and of $[(R)\text{-}\mathbf{6}\cdot\beta\text{-CD+H+Na}]^{2+}$ versus $[((R)\text{-}\mathbf{6})_2\cdot\beta\text{-CD+2H}]^{2+}$ increase gradually when solutions are diluted. The peak corresponding to a 1:1 complex $[(R)\text{-}\mathbf{6}\cdot\beta\text{-CD+H+Na}]^{2+}$ at the 840.8 *m/z* ratio became very small. In the same time, a peak at 1657.8 corresponding to the $((R)\text{-}\mathbf{6}\cdot\beta\text{-CD+H})^+$ complex, gradually disappears when the concentration decreases. Nevertheless, the 1:1 complex, even at a small concentration, appears to be predominant in dilute solution that better reflects experimental conditions accounted during fluorescence spectroscopy. Studies carried out at a 1/100 molar ratio led to the same conclusions.

The synthesis of results obtained by fluorescence spectroscopy (*n*:*n* complex) and by mass spectrometry allowed us to attribute the 1:1 stoichiometry ($n = 1$), in accordance with the stoichiometry proposed for the complexes formed between (*S*)-**1** and native (α, β and γ) or substituted (HP-α, HP-β, HP-γ) cyclodextrins.[22–24]

2.2. Interactions study

2.2.1. HPLC study

In a first step, chromatographic experiments with both Cyclobond columns (β-CD and HP-β-CD chiral selectors) using a water/methanol—80/20 mobile phase, were achieved in order to rank the affinity of the six solutes for these CDs. The results obtained with both of these columns are summarized in Table 1. Whatever the compound considered, a greater retention can be observed on Cyclobond I 2000 RSP (HP-β-CD) than on Cyclobond I 2000 (β-CD). Moreover, compounds can be classified in three groups depending on their retention that is, their affinity for the stationary phases: the first group includes compound (*S*)-**3** that is the less retained (tr <10 min or tr <20 min for β-CD or HP-β-CD, respectively); the second group is relative to compounds (*S*)-**1,** (*S*)-**2**, (*R*)-**4,** and **5** with retention times close to 13 min or 40 min for β-CD or HP-β-CD, respectively; the third group includes compound **6** which was not eluted after 100 min whatever the stationary phase used.

Table 1
HPLC retention parameters on Cyclobond I 2000 (immobilized β-CD) and Cyclobond I 2000 RSP (immobilized HP-β-CD): retention time (t_r) and retention factor (k)

Compound	Chiral selector	t_r (min)	k
(*S*)-**1**	β-CD	12.82	2.88
	HP-β-CD	41.99	13.48
(*S*)-**2**	β-CD	10.34	2.13
	HP-β-CD	37.83	12.04
(*S*)-**3**	β-CD	8.51	1.58
	HP-β-CD	17.67	5.09
(*R*)-**4**	β-CD	13.97	3.23
	HP-β-CD	43.40	13.97
(*S*)-**5**	β-CD	11.15[a]	2.38
	HP-β-CD	34.16[b]	10.78
(*R*)-**5**	β-CD	12.69[a]	2.85
	HP-β-CD	36.76[b]	11.68
6	β-CD	>100	>29
	HP-β-CD	>140	>41

[a] R_s = 0.7.
[b] R_s = 0.5.

In a second step, chromatographic experiments with LiChrospher RP-18 using β-CD or HP-β-CD as chiral additives at different concentrations in the mobile phase were performed. To control the ionization state of the derivatives studied, it is necessary to set the pH of the mobile phase by using a buffer solution. A study at the physiological pH 7.4 was not performed because, unfortunately at this pH, the hydrolysis of the E-ring leads to the conversion of the lactone into the carboxylate form[33] of the camptothecin derivatives. We then chose to study the complexation of compounds **1**–**6** with β-CD and HP-β-CD at pH 6 near the neutrality where the lactone form is predominant[23] and at pH 3.5 to further compare with the capillary electrophoresis results. Moreover, in order to improve the solubility of the β-CD in the mobile phase, ethanol was used instead of methanol. Finally, after an optimization step to determine the ethanol percentage that permits the elution of all the compounds **1**–**6** with β-CD and HP-β-CD under the same analytical conditions, a phosphate buffer (pH 3.5 or 6)/ethanol—82/18 (v/v) mixture was selected as mobile phase. The results obtained in term of retention for the compounds (*S*)-**1**, (*S*)-**3** and **6** with β-CD and HP-β-CD at pH 3.5 are illustrated in Table 2. Retention order obtained without cyclodextrin in the mobile phase is the following: $t_{r(S)\text{-}\mathbf{3}} < t_{r(S)\text{-}\mathbf{1}} < t_{r\mathbf{6}}$. This order may be the result of the difference between the hydrophobic character and the ionization state of these compounds. According to their acid–base properties, (*S*)-**3** and **6** are dicationic (protonation at the N4 atom on the D ring[32] and at the extra-cyclic nitrogen atom[34]) under the experimental conditions (pH 3.5), whereas (*S*)-**1** is monocationic. This ionization should result in a decrease in the retention, as observed for (*S*)-**3** versus (*S*)-**1**. For **6**, a greater retention with respect to (*S*)-**1** may be the result of two counter balanced phenomena: ionization state and hydrophobicity. Indeed, the difference in the homologation of the E-ring and the substituent's nature on the A and B-rings for **6** contributes to an increase in its hydrophobicity with respect to (*S*)-**3**, leading to a greater retention of **6**. In addition, a decrease in the retention is observed by increasing the concentration of the cyclodextrin in the mobile phase. For example, the retention time of camptothecin (*S*)-**1** using B mobile phase (pH 3.5) decrease from 26.56 to 21.11 min as the β-CD concentration increases from 0 to 10 mM. This trend previously described[35–39] indicates that the solutes form complexes with the CD, leading to a decrease in the solute—stationary phase interactions. Indeed, the retention behavior of the solute in HPLC is based on the partitioning of the solute between the mobile and the stationary phase. When CD is added to the mobile phase, the solute retention is split into two main physicochemical processes, that is, solute complexation by cyclodextrin and transfer of a free (uncomplexed) solute from the mobile phase to the stationary phase.

2.3. Apparent binding constants determination

The apparent binding constant K between the compound and CD can be determined using the following relation developed by Uekama et al.:[40]

$$\frac{1}{k} = \frac{1}{k_0} + \frac{K}{k_0}[\mathrm{CD}]^n \quad (1)$$

where k is the solute retention factor, k_0 the solute retention factor without cyclodextrin in the mobile phase, [CD] the cyclodextrin concentration and n the stoichiometry of the complex. For an inclusion complex of 1:1 stoichiometry ($n = 1$) a linear plot of $1/k$ versus [CD] must be obtained; the ratio of the slope to the intercept with the y-axis of this plot permits to determine the apparent binding constant K. This model was then applied to the determination of the apparent binding constant of solutes **1**–**6** with β-CD and HP-β-CD at pH 3.5 or pH 6. For compound **6** the K values were only calculated at pH 3.5 as large retention times were observed at pH 6 whatever the nature and the concentration of the CD used (t_r >280 min). Linear plots were obtained for all the complexes studied, with the r^2 values greater than 0.94, in accordance to a 1:1 stoichiometry demonstrated by spectroscopic studies. Figure 3 shows the plot obtained for (*S*)-**1** with HP-β-CD at pH 3.5. The apparent and averaged binding constants of the complexes formed between **1** and **6** and both cyclodextrins are reported in Table 3.

Compounds can be classified in three groups depending on the magnitude of the apparent binding constant K (M^{-1}) obtained: the first group includes compound (*S*)-**3** with apparent binding constant K always low ($K \leqslant 14\ M^{-1}$); the second group is relative to compounds (*S*)-**1,** (*S*)-**2**, and (*R*)-**4** with the apparent binding constant K between 21 and 34 M^{-1} despite a smaller complexation of (*S*)-**2** with β-CD; the third group includes compounds **5** and **6**, with apparent binding constants in the range 34–60 M^{-1}. It is noteworthy that the magnitude of the apparent binding constants, directly linked to the affinity of the solutes for the cyclodextrins, can be correlated to the retention order obtained using Cyclobond phases for all the compounds classified in three groups. Moreover, whatever the pH chosen, a slightly greater affinity for HP-β-CD than for β-CD is observed with compounds (*S*)-**1**, (*S*)-**2**, (*R*)-**4, 5,** and **6** whereas for (*S*)-**3** it seems equivalent (apparent binding constants of the same magnitude). A twofold complexation of (*S*)-**2**

Table 2
HPLC retention parameters of compounds (*S*)-**1**, (*S*)-**3,** and **6** on LiCrospher RP-18 using β-CD and HP-β-CD at different concentrations in the mobile phase (phosphate buffer pH 3.5/ethanol—82/18 (v/v)): retention time (t_r) and retention factor (k)

Compound	CD	[CD] (mM)	t_{r1} (min)	t_{r2} (min)	k_1	k_2
(*S*)-**1**		0	26.56	—	16.71	—
	β-CD	5	24.02	—	12.34	—
	β-CD	10	21.11	—	9.66	—
	HP-β-CD	5	22.81	—	14.20	—
	HP-β-CD	10	20.01	—	1.11	—
(*S*)-**3**		0	3.16	—	1.07	—
	β-CD	5	3.10	—	1.05	—
	β-CD	10	3.05	—	0.88	—
	HP-β-CD	5	3.11	—	1.08	—
	HP-β-CD	10	3.09	—	1.06	—
6[a]		0	76.56	76.56	51.81	51.81
	β-CD	5	60.13	63.02[b]	39.09	41.01
	β-CD	10	48.42	52.81[c]	31.28	34.21
	HP-β-CD	5	56.05	58.12[b]	36.36	37.76
	HP-β-CD	10	43.20	46.25[b]	27.85	27.80

[a] First eluted enantiomer: (*R*)-**6**; second eluted enantiomer: (*S*)-**6**.
[b] *Rs* <0.5.
[c] *Rs* = 0.7.

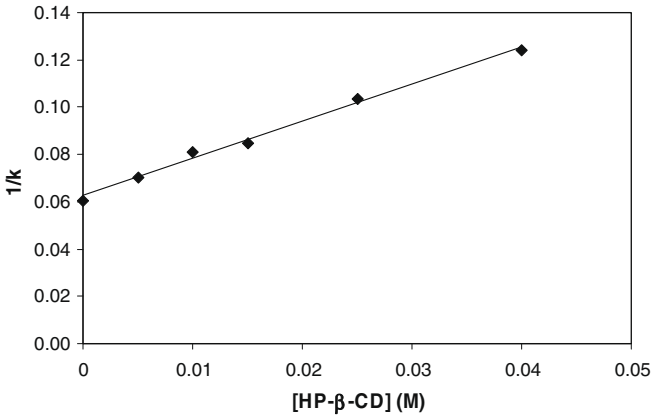


Figure 3. Variations of 1/*k* versus [HP-β-CD] obtained from HPLC experiments for (*S*)-**1** using HP-β-CD in the mobile phase (phosphate buffer pH 3.5/ethanol—82/18 (v/v)), at 298 K.

with HP-β-CD with respect to β-CD was observed under our experimental conditions. The influence of the pH on the complex formation can be examined too. While no variation in the apparent binding constant appears when changing the pH from 3.5 to 6 for compounds (*S*)-**1**, (*S*)-**2**, (*R*)-**4,** and **5,** a slight increase was observed for compound (*S*)-**3**. For all compounds, this pH increase leads to the deprotonation at the N4 atom of the D ring (pK_a 3.6[34]). Additionally, according to published results of topotecan **(*S*)-3**,[27] at pH 6 a partial deprotonation at the phenolic group (pK_a 6.5) and protonation at the extra-cyclic nitrogen atom on the B ring (pK_a 10.7) lead to a molecular structure including an intra-molecular bond. A greater percentage of the neutral form at pH 6 than at pH 3.5, more hydrophobic and then more complexed by the CDs, explains its distinct behavior. For compound **6**, the apparent binding constants were not determined as a large retention time was observed at pH 6 (t_r >280 min without CD).

Table 3
Apparent and averaged binding constants K (M^{-1}) determined by HPLC for the complexes formed between **1**–**6** and CDs (β-CD and HP-β-CD) at pH 3.5 and 6, in a buffer containing 18% of ethanol

Compound	pH	CD	$K \pm \Delta K$ (M^{-1})
(*S*)-**1**	3.5	HP-β-CD	28 ± 2
	6	HP-β-CD	33 ± 2
	3.5	β-CD	21 ± 2
	6	β-CD	24 ± 2
(*S*)-**2**	3.5	HP-β-CD	27 ± 2
	6	HP-β-CD	25 ± 2
	3.5	β-CD	12 ± 2
	6	β-CD	<10
(*S*)-**3**	3.5	HP-β-CD	<7
	6	HP-β-CD	14 ± 1
	3.5	β-CD	<7
	6	β-CD	13 ± 1
(*R*)-**4**	3.5	HP-β-CD	34 ± 2
	6	HP-β-CD	32 ± 1
	3.5	β-CD	23 ± 1
	6	β-CD	24 ± 2
(*R*)-**5**	3.5	HP-β-CD	60 ± 2
	6	HP-β-CD	60 ± 2
	3.5	β-CD	52 ± 2
	6	β-CD	48 ± 2
(*S*)-**5**	3.5	HP-β-CD	50 ± 2
	6	HP-β-CD	52 ± 2
	3.5	β-CD	38 ± 2
	6	β-CD	34 ± 2
(*R*)-**6**	3.5	HP-β-CD	80 ± 3
	6	HP-β-CD	n.d.
	3.5	β-CD	60 ± 2
	6	β-CD	n.d.
(*S*)-6**1**	3.5	HP-β-CD	68 ± 3
	6	HP-β-CD	n.d.
	3.5	β-CD	46 ± 2
	6	β-CD	n.d.

n.d.: not determined.

2.3.1. CE study

According to the pK_a values of the studied compounds,[34] almost 50% of cationic forms are present at pH 3.5. Use of CE for the determination of apparent binding constant with neutral CDs was then proposed for all the compounds. Nevertheless, preliminary experiments revealed that for compounds (*S*)-**1**, (*S*)-**2**, (*R*)-**4,** and (*R*)-**5**, this ionization was insufficient for a distinct migration of the solute with respect to DMSO, neutral compound used as marker substance of the electroosomotic flow. This study was then limited to compounds (*S*)-**3** and (*R*)-**6**. Migration times of (*S*)-**3** and (*R*)-**6** were measured using 0, 1, 2.5, 4, 5.5, 7, and 8.5 mM of β-CD or HP-β-CD in the BGE. With HP-β-CD they were varied from 5.55

to 6.11 min and from 6.09 to 8.16 min, respectively in this concentration range. The apparent binding constants were calculated from the mobilities of the analytes using double-reciprocal equation according to Benesi–Hildebrand's method:[41]

$$\frac{[CD]}{\mu_i - \mu_f} = \frac{1}{\mu_c - \mu_f}[CD] + \frac{1}{(\mu_c - \mu_f)}K \quad (2)$$

where K is the apparent binding constant, [CD] is considered to be the total concentration of the cyclodextrin since the complexed CD concentration must be insignificant, μ_i is the experimental electrophoretic mobility observed for the studied solute, μ_f and μ_c are the electrophoretic mobilities of the solute in the free and complexed forms, respectively. The electrophoretic mobility μ_i can be calculated from the apparent mobility according to:

$$\mu_{app} = \mu_i + \mu_{eof} = \frac{l \cdot L}{t \cdot V} \quad (3)$$

where l and L correspond to the capillary length to the detector and total capillary length, t to the migration time of the analyte, V to the applied voltage and μ_{eof} to the electroosmotic flow. This latest was calculated from the migration time of the neutral marker substance (DMSO). Variations of $[CD]/(\mu_i - \mu_f)$ versus [CD] were studied: the r^2 values for the linear fit were greater than 0.99; linear plots were in accordance with a 1:1 stoichiometry demonstrated by spectroscopic studies. Even if the addition of the CD induces an increase in the BGE viscosity, this variation was too weak to influence the K values as similar results were obtained after correction by the usual ratio i/i_0 according to Wren and Rowe[42] (i and i_0 are the current in presence and in absence of CD, respectively). The apparent binding constants K are $97 \pm 30\ M^{-1}$ and $135 \pm 25\ M^{-1}$ for (*S*)-**3**·β-CD and (*S*)-**3**·HP-β-CD, respectively. For (*R*)-**6**·β-CD and (*R*)-**6**·HP-β-CD they are $230 \pm 15\ M^{-1}$ and $280 \pm 15\ M^{-1}$, respectively. According to experimental precision, complexation of (*S*)-**3** with β-CD and HP-β-CD can be considered of the same magnitude, whereas for (*R*)-**6** complexation is slight greater for HP-β-CD. Moreover, a twofold greater complexation of (*R*)-**6** versus (*S*)-**3** is involved with both CDs.

3. Conclusion

The results presented in this study show the interactions between campthotecin or homocamptothecin derivatives and cyclodextrins by using complementary spectroscopic and separatives techniques. Firstly, fluorescence and mass spectrometry allow us to determine the 1:1 stoichiometry ratio illustrated by the (*R*)-**6**·β-CD complex study. Secondly, separatives methods, that is, capillary electrophoresis and high performance liquid chromatography, confirm the general 1:1 stoichiometry characterized by linear representations ($1/k = f([CD])$ for HPLC experiments and $[CD]/(\mu_i - \mu_l) = f([CD])$ for CE experiments) observed during the calculation of the apparent binding constants, K. The HPLC technique using reverse stationary phase and a mobile phase including the cyclodextrin, has been performed at both pH 3.5 and 6.0 to calculate the apparent binding constant of all the derivatives. For all compounds, no significant differences between the K values were determined from acidic to neutral mobile phases except for (*S*)-**3** where a slight increase is observed. From HPLC data, whatever the pH, higher apparent binding constant is determined with HP-β-CD than with β-CD for (*S*)-**1**, (*S*)-**2**, (*R*)-**4**, (*R*)-**5,** and (*R*)-**6**. In CE, similar behavior of (*R*)-**6** was underlined, whereas no significant difference of the K values has been exhibited for (*S*)-**3**. The higher K values calculated with CE than with HPLC experiments should be explained by the higher percentage of ethanol used in the HPLC mobile phases than in the CE electrolyte. Both separative techniques demonstrated that the complexation constant calculated for the (*R*)-**6**·HP-β-CD complex is higher than K values of the β-CD complex. Similar K values have been measured for CPT (*S*)-**1** and hCPT (*R*)-**4** which differ only at the E-ring. Moreover, different K values have been measured in each series, where the substitution of the A and/or B-rings occurs. These results could be in favor of an inclusion of the solutes by the quinoline part. Finally, the HP-β-CD complexation abilities and its higher solubility versus β-CD, could lead to select this CD for the further development of pharmaceutical formulations allowing the improvement of the bioavailability of this chemical series.

4. Experimental

4.1. Chemicals

Racemic mixtures or pure enantiomers of camptothecin and homocamptothecin derivatives **1**–**6** used in this study (Fig. 1) were synthesized as previously described.[8,43]

Methanol and ethanol of HPLC grade were obtained from Merck (Nogent sur Marne, France) or Baker (Noisy le Sec, France). Deionized (DI) water was obtained from Milli-Q system (Millipore, Saint Quentin en Yvelines). Phosphoric acid (85%, w/w), NaOH 0.1 M was obtained from Beckman (Beckman coulter France, Villepinte, France). Triethylamine (TEA) of analytical grade was purchased from Merck (Nogent sur Marne, France). β-CD ($M_W = 1135$) and HP-β-CD ($M_W = 1395$) were a gift from Roquette Laboratories (Lestrem, France). The 2-hydroxypropylated CD represents a multicomponent mixture with an average molecular substitution (MS) equal to 0.63.

4.2. Fluorescence spectroscopy

For fluorescence study, a phosphate buffer (25 mM, pH 3.5 or pH 6.0) with 5% of ethanol was prepared from a phosphoric acid solution adjusted to a convenient pH by the addition of TEA.

All the spectrofluorimetric measurements were carried out on a Fluorolog (Horiba-Jobin Yvon, Longjumeau, France) spectrofluorimeter, equipped with a xenon lamp and 10 mm quartz cells. Excitation and emission slits were both set at 10 nm. Data were acquired at a 600 nm min^{-1} scan rate. The excitation and the emission wavelengths were 340 and 471 nm, respectively. The sample solution containing fluorescent dye and host were kept at 298 K for spectroscopic measurements by a circulating thermostated water-jacket.

The stoichiometry of (*R*)-**6**/HP-β-CD complex was determined by the continuous variation method,[30,31] based on the difference in the fluorescence intensity ΔF ($\Delta F = F - F_0$) of (*R*)-**6** observed in the presence (F) and in the absence (F_0) of HP-β-CD. Equimolar solutions of the guest, (*R*)-**6**, and of the host HP-β-CD were prepared in the above described phosphate buffer and were mixed to standard volumes and proportions in order to remain the total concentration constant ([**6**(***R***)] + [HP-β-CD] = 10 μM), with the molar fraction r ($r = [HP\text{-}\beta\text{-}CD]/([(R)\text{-}\mathbf{6}] + [HP\text{-}\beta\text{-}CD])$) varying in the range 0–1. ΔF values in the preparations of (*R*)-**6** were calculated by measuring the fluorescence intensity of (*R*)-**6** in the absence (F_0) and in the presence (F) of the corresponding concentration of HP-β-CD. Also, an equimolar aqueous solution of HP-β-CD was used as a blank, to take into account its refractive index. Subsequently, $\Delta F[(R)\text{-}\mathbf{6}]$ were plotted for the corresponding HP-β-CD against the molar fraction r varying in the range 0–1.

4.3. Mass spectrometry

For mass spectrometry study, experiments were performed using an Applied Biosystems API 3000 Instrument (PE Sciex, Toron-

to, Canada) equipped with an electrospray ion source. ESI-MS measurements were performed in positive mode with an ion spray voltage of 5000 V and a declustering potential of 50 V. Stock solutions of (*R*)-**6** at 2.2 mM in water/methanol/formic acid (74/25/1—v/v/v) and β-CD at 2.2 mM in water were mixed to prepare samples containing 1/10 and 1/100 molar ratios of (*R*)-**6** versus β-CD with different concentrations of (*R*)-**6** (22 μM, 50 μM, and 220 μM). These samples and the appropriate diluted solutions of (*R*)-**6** or β-CD taken alone were introduced into the ion source at a flow rate or 5 μL min^{-1}. Spectra were acquired by scanning *m/z* from 200 to 3000 with a unit resolution.

4.4. Chromatography

For HPLC study, a phosphate buffer (150 mM, pH 3.5 or pH 6) was prepared from a phosphoric acid solution adjusted to convenient pH by addition of TEA. The mobile phases used were A: water/methanol—80/20 (v/v); B: phosphate buffer (pH 3.5)/ethanol—82/18; C: phosphate buffer (pH 6)/ethanol—82/18. Compounds were chromatographied by dissolving them in ethanol to a concentration of about 0.08 mM (concentration 100%) and passed through a 0.45 μm membrane filter prior to loading the column.

Measurements were carried out using a gradient Waters 600E metering pump model equipped with a Waters 996 photodiode array spectrophotometer. Chromatographic data were collected and processed on a computer running with Millennium 2010. The column eluate was monitored at 370 nm for **1** and **4**, at 380 for **2**, **3,** and **6** and at 360 nm for **5**. The sample loop was 20 μL (Rheodyne 7125 injector). For the studies with the immobilized CDs a Cyclobond I 2000 (β-CD) and a Cyclobond I 2000 RSP (HP-β-CD) (Astec, 250 × 4.6 mm, I.D. 5 μm), thermostated at 298 K were used. Mobile phase elution was made isocratically using reversed phase mode, that is, the mobile phase A (eau/methanol—80/20) and a flow of 0.8 mL min^{-1}. The peak of the solvent front was considered to be equal to the dead time (t_o) and was taken from each particular run. It was about 3.3 min for Cyclobond I 2000 and 2.9 min for Cyclobond I 2000 RSP. For the separation with CDs additives, a LiChrospher RP-18 (Merck, 125 × 4 mm, I.D. 5 μm) stationary phase, thermostated at 298 K was used. Mobile phase elution was made isocratically using different mixtures of ethanol and phosphate buffer (150 mM, pH 3.5 or pH 6), with various β-CD and HP-β-CD concentrations (0, 5, 7.5, 10, 12.5 and 15 mM for β-CD and 0, 5, 10, 15, 25, 40 mM for HP-β-CD, respectively). The flow was 0.8 mL min^{-1}. In this separation mode the dead time (t_o) was about 1.50 min. All the mobile phases were filtered through membrane (0.45 μm) and degassed with a Waters in-line degasser apparatus. In all cases retention times were mean values of duplicate determinations.

4.5. Capillary electrophoresis

For CE analysis, a 25 mM phosphate buffer was prepared from a H_3PO_4 solution adjusted to pH 3.5 by addition of TEA. Background electrolyte (BGE; 25 mM phosphate buffer, pH 3.5/ethanol—95/5) containing from 0 to 8.5 mM of β-CD or HP-β-CD was made by appropriate dilutions of 10 mM CD stock solutions and 25 mM phosphate buffer, pH 3.5/ethanol—95/5 solutions. Stock solutions of compounds (*S*)-**3** and (*R*)-**6** prepared in DMSO were dissolved with 2.5 mM phosphate buffer pH 3.5 to obtain 0.1 mM solutions containing 4% of DMSO.

Capillary electrophoresis experiments were performed on a Beckman P/ACE MDQ Capillary Electrophoresis system (Beckman Coulter France, Villepinte, France), including an on-column diode-array UV-detector. The whole system was driven by a PC with the 32 Karat software (Beckman Coulter France) package for system control, data collection, and analysis. It was equipped with a 50.2 cm (effective length: 10 cm) × 75 μm ID untreated fused-silica capillary (Composite Metal Services, Worcestershere, UK). The capillary was mounted in a cartridge and thermostated at 298 ± 0.1 K, unless otherwise specified. An hydrodynamic injection was made with an 8 s injection time at 1 psi (anodic injection) unless otherwise specified. The applied field was 0.50 kV cm^{-1} (normal polarity). Compounds were detected at 220 nm. New capillaries were flushed for 20 min with 0.1 M sodium hydroxide (NaOH) (*P* = 20 psi) and 5 min with water (*P* = 20 psi). For the separation the capillary was each day flushed successively with NaOH (5 min, 20 psi), water (3 min, 20 psi), and then with BGE (3 min, 20 psi). Between each run, it was treated with water (1 min, 20 psi) and BGE (3 min, 20 psi). Electrophoretic parameters presented are averaged values of triplicate determinations.

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