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Effect of hydroxypropyl-β-cyclodextrin-complexation and pH on solubility of camptothecin

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Abstract

The influence of both pH and complexation by hydroxypropyl-β-cyclodextrin (HP-β-CD) on the overall solubility of camptothecin (CPT) was studied, with particular focus on the equilibrium between its lactone- and carboxylate-form. Phase solubility studies at therapeutically relevant pH values (pH 5.5–7.0) and physiologically acceptable HP-β-CD-concentrations (0–25% (w/v)) were performed, and amounts of solubilized CPT quantified by HPLC. The solubility of CPT increased with both increasing pH and HP-β-CD-concentration. The apparent complexation constant (K_C) decreased with increasing pH (245 M^{-1} at pH 5.5; 184 M^{-1} at pH 7.0). The lactone–carboxylate equivalence point shifted from a pH value of 6.8–7.0 and 7.1 with 0, 10 and 25% HP-β-CD, respectively. The lactone–carboxylate-ratios from the equilibrium study were applied to the phase-solubility data, and the lactone- and carboxylate concentrations at 0, 10 and 25% HP-β-CD calculated. Separate complexation constants (K_C) for the carboxylate-CPT and lactone-CPT could thus be derived, and found to be 113 ± 7 and 260 ± 18 M^{-1}, respectively. This allows the prediction of amounts of both lactone- and carboxylate-CPT solubilized at any HP-β-CD concentration and pH-combination.

Keywords: HP-β-CD; Cyclodextrin; Camptothecin; Solubilization; Complexation; pH

1. Introduction

Camptothecin (CPT) is a cytotoxic alkaloid, originally isolated from the stem wood of the oriental tree, *Camptotheca acuminata* (Wall and Wani, 1996; Rothenberg, 1997). Despite of its established in vitro anti-tumour activity, in vivo utilization has not been achieved to date. Reasons for this are the poor water-solubility and the rapid hydrolysis of CPT-lactone to a ring-open carboxylate-form, which is less cell-membrane permeable, has a lower affinity for the target topoisomerase I, and is thus 10-fold less pharmacologically active but more toxic than the lactone

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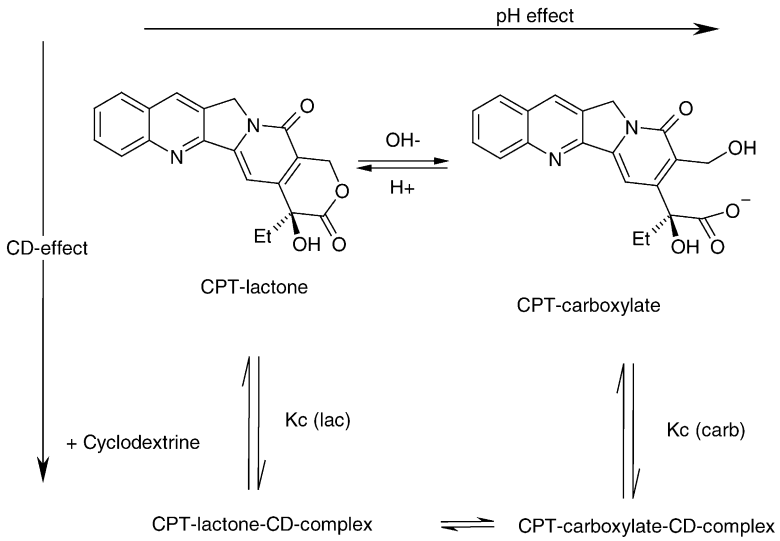

Fig. 1. Camptothecin–cyclodextrin complexation reaction map.

form (Potmesil, 1994; Rivory and Robert, 1995). The lactone–carboxylate equilibrium is reversible and pH-dependent (illustrated in Fig. 1), with the lactone dominating at acidic pH. In plasma, the equilibrium is on the carboxylate side due to its 150-fold stronger affinity to serum albumin (Mi and Burke, 1994).

Two CPT-derivatives that are more soluble and hydrolytically more stable, irinotecan (CPT-11) and topotecan (7-ethyl-10-hydroxycamptothecin) are approved for use in humans. Irinotecan (7-ethyl-10(4(-(1-piperidino)-1-piperidino)carboxycamptothecin) is a water soluble prodrug of the active compound SN38. Another approach to the problem of poor solubility and instability of the mother compound CPT is the variation of the formulation. Micellar, liposomal and lipid nanoparticle-based-formulations have been described (Daoud et al., 1995; Cortesi et al., 1997; Colbern et al., 1998; Lundberg, 1998; Chow et al., 2000). Two studies have reported complexation of CPT by cyclodextrins (CDs) (Kang et al., 2002; Xiang and Anderson, 2002). However, both studies focused on the solubility profile of the CPT-lactone at acidic pH, not suitable for parenteral administration. In the present study the potential of combined control of pH value and complexation of CPT by hydroxypropyl-β-cyclodextrin (HP-β-CD) to achieve formulations for i.v. administration is described. At the same time the equilibrium between CPT and the inactive carboxylate-form during solubilization at physiological pH values is studied.

2. Material and methods

2.1. Material

CPT was purchased from Sigma-Aldrich (Steinheim, Germany). Hydroxypropyl-β-cyclodextrin, Cavasol W7 HP Pharma, was obtained from Wacker-Chemie GmbH, München, Germany. Triethylamine (for analysis), acetic acid (glacial 100%), acetonitrile (gradient grade for LC), di-sodiumhydrogen-phosphate-dihydrate (extra pure), potassium-dihydrogen-phosphate (proanalysi) and ortho-phosphoric acid (proanalysi 85%) were pursed from Merck, Germany. Dimethylsulfoxide (DMSO) of GC quality was obtained from Sigma–Aldrich GmbH, Germany. Solutions were made in freshly distilled water and filtrated through 0.22 μm filters prior to storage and use.

2.2. HPLC-method

An HPLC-method originally described by Warner and Burke (Warner and Burke, 1997) was used with some modifications. The mobile phase had a gradient

from 25 to 35% acetonitrile during 10 min in a 1% (v/v) triethylamine buffer, pH 5.5 and flow rate of 1 ml/min. The Waters HPLC system was equipped with a 474 scanning fluorescence detector, a 2695 separation module, and a Symmetry® C_{18}-column, 3.9 mm × 150 mm (Waters® Milford, Massachusetts). Wavelengths: excitation λ = 360 nm and emission λ = 440. Injection volume was 10 μl if not stated differently. Standard curves for both lactone- and carboxylate-CPT were prepared as follows: Standard samples with CPT concentrations of 25, 50, 100, 150, 200 and 250 ng/ml was made by dilution of a 3.33 mg/ml CPT-stock-solution in DMSO, with a 9 mM phosphate buffer (PB), with pH 10.5 and pH 3.0 for the carboxylate-CPT and the lactone-CPT, respectively. Every standard solution was prepared in triplicate and injected twice into the HPLC. The potential effect of HP-β-CD was investigated by comparing the area under the peak (AUC) of the CPT-lactone and carboxylate from standard solution with 0 and 25% (w/v) HP-β-CD.

2.3. Evaluation of equilibration-time for solubilization

Time for equilibrium of the dissolution and complexation of CPT was investigated by incubation of 1.0 μM CPT-samples with 0 and 5% (w/v) HP-β-CD in 25 mM phosphate buffer (PB), pH 3.0 and 15 °C, and the AUC in the HPLC-chromatograms measured right after preparation (day 0) compared to the AUC of the very same sample at days 3–5, respectively.

2.4. Effect of pH and cyclodextrin on the CPT lactone–carboxylate equilibrium

A 50 μg/ml stock solution of CPT in DMSO was prepared and 25 μl (0.5% (v/v)) samples thereof diluted with 5 ml of the respective solvents; 25 mM PB with pH 3.0, 5.5, 6.0, 6.5, 7.0, 7.5 or 11.0, with 0, 10 or 25% HP-β-CD. The final CPT concentration was 250 ng/ml (0.72 μM). Samples were incubated on a shaking water bath for 5 days at 25 ± 0.5 °C, and thereafter analysed by HPLC (n = 3).

2.5. Phase solubility study

Excess CPT (approximately 5 mg) was incubated with 15 ml 25 mM PB at pH 5.5, 6.0, 6.5 or 7.0 and 0, 5, 10, 15, 20 or 25% (w/v) HP-β-CD. The samples were bath-sonicated for 30 min prior to incubation in a shaking water bath (Type 1086 GFL, GFL Germany) at 25 ± 0.5 °C for 5 days. The 2.5 ml samples were withdrawn and filled into ultra-centrifuge vials (polycarbonate centrifuge tubes, 11 mm × 60 mm, Beckman, USA). An Optime™ from Beckman instrument, USA with a SW60Ti-rotor. Samples were centrifuged at 150,000 × g for 20 min and 25 °C. The supernatant was filtrated through 0.45 μm Millex® filter, (Millipore, Ireland), the first drops were discarded, and the rest collected and upon dilution analysed by HPLC.

3. Results

3.1. Qualification and validation of the HPLC-method

When CPT was quantified, an overall recovery close to 100% was measured irrespective of the amount of HP-β-CD present (data not shown). Apparently the presence of HP-β-CD did not affect the HPLC-method. Neither was the overall CPT-recovery affected by incubation at 25 °C for 5 days (data not shown), which implies chemical stability of CPT.

3.2. Evaluation of equilibration-time for solubilization

The AUC of the very same samples at days 3–5, respectively, are given in Fig. 2. After an initial increase the AUC reached a plateau at day 3 in both cases, which apparently resembles equilibrium. To assure that the solubility equilibrium was reached, an equilibration time of 5 days was used in the phase solubility studies.

3.3. Effect of pH and cyclodextrin on the CPT lactone–carboxylate equilibrium of non-saturated samples

The equilibrium between the lactone- and the carboxylate CPT-forms at pH of 3.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 11.0, containing 0, 10 or 25% HP-β-CD, respectively, were estimated directly from injection of 250 ng/ml (0.72 μM) CPT-samples into the HPLC. Fig. 3 exemplifies the resulting chromatograms. The lactone/carboxylate ratios, calculated from calibration

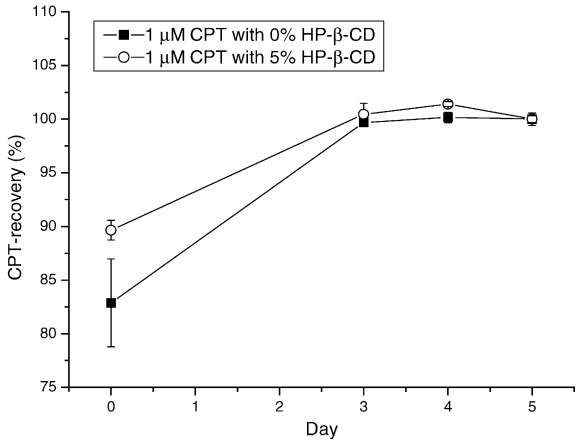

Fig. 2. Relative CPT-concentrations in supersaturated CPT-solutions containing 0 and 5% HP-β-CD vs. storage time (n = 5).

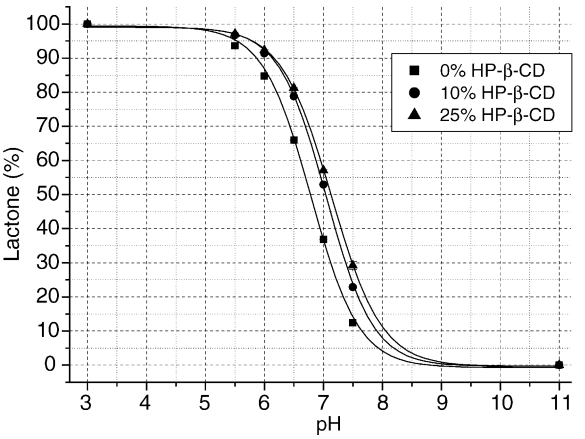

Fig. 4. Lactone-concentrations vs. pH at equilibrium with 0, 10 and 25% HP-β-CD (n = 3).

curves for the carboxylate- and the lactone top in the HPLC-chromatograms, decrease with increasing pH, following a sigmoidal curve (Fig. 4). The equivalence point of the curves increases with increased concentration of HP-β-CD, and were 6.8, 7.0 and 7.1 with 0, 10 and 25% (w/v) HP-β-CD, respectively.

3.4. Phase solubility studies

Solubility diagrams were plotted as described by Higuchi and Connors (Higuchi and Connors, 1965), showing close-to-linear relationships between the overall amount of CPT in solution (a mixture of the carboxylate- and the lactone form) and the HP-β-CD concentration (Fig. 5). The linear relationship indicates the formation of a 1:1 complex. In this case, the apparent stability constant of the complex, K_C, can be calculated from Eq. (1) deduced by Higuchi and Connors (Higuchi and Connors, 1965), which applies for the formation of 1:1 complexes:

$$K_C = \frac{\text{slope}}{S_0\,(1 - \text{slope})} \quad (1)$$

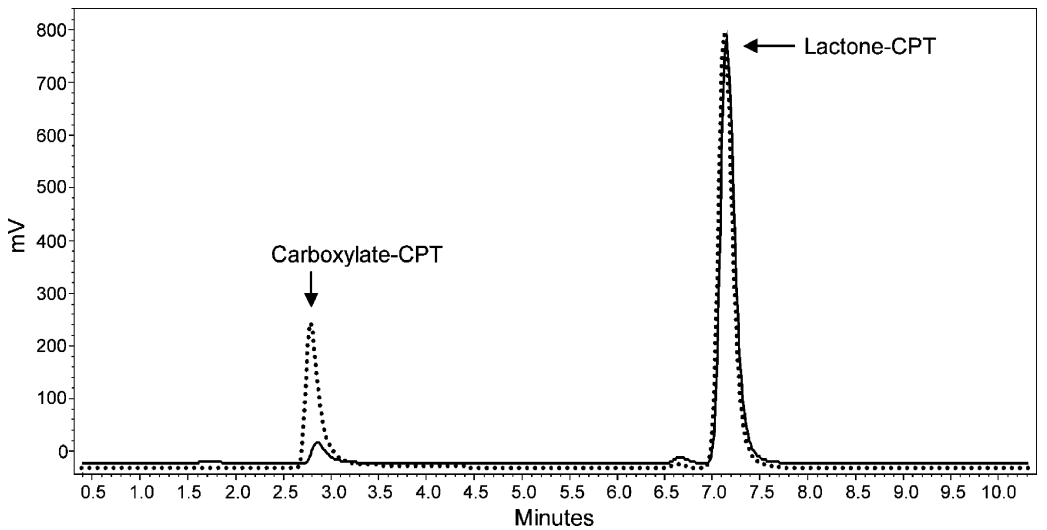

Fig. 3. The HPLC chromatograms of Camptothecin before (· · ·) and after (—) adding 25% (w/v) hydroxypropyl-β-cyclodextrin in PBS pH 6.0.

Table 1
Stability constants (K_C) for camptothecin complexes at different pH calculated from the slope and intercept of the best-fit line of the phase solubility experiments ($n = 3$)

pH	R^2-value	Intercept (μM)	Slope ($\times 10^{-5}$)	K_C (M^{-1})	E_f[a]	[Camptothecin]$_{25\%HP\text{-}\beta\text{-}CD}$ (μM)
5.5	0.996	5.8 ± 0.2	142.0 ± 2.8	245.4	47	270 ± 18
6.0	0.996	6.7 ± 0.1	155.0 ± 1.8	230.8	44	294 ± 14
6.5	0.995	8.9 ± 0.5	180.0 ± 5.0	201.6	41	372 ± 22
7.0	0.999	14.2 ± 2.9	261.0 ± 4.0	184.3	30	484 ± 7

[a] $E_f = [CPT]_{25\%HP\text{-}\beta\text{-}CD}/[CPT]_{0\%HP\text{-}\beta\text{-}CD}$.

The slopes and intercepts of the curves (S_0) were calculated from the linear fits of the curves in Fig. 5. Linear fits with increasing slopes were obtained with increasing pH values. In Table 1, the enhancement factors, E_f, i.e. increase in solubility with 25% HP-β-CD, as compared to solubility without CD, at a given pH value, are listed. E_f decreases with increasing pH. In Fig. 6, K_C is plotted versus pH. There is a sigmoid relationship between K_C and pH, the inflection point of which correlates with the pH equivalence curves shown in Fig. 4. To differentiate between the lactone and the carboxylate, the lactone/carboxylate ratios were taken from the above mentioned equilibrium study, and the concentrations of both the lactone- and carboxylate-forms calculated at 0, 10 and 25% HP-β-CD. This resulted in separate phase solubility plots of the lactone and carboxylate concentrations against HP-β-CD. The slope and S_0 of these curves were calculated using CPT-concentrations at 0 and 25% HP-β-CD; Slope = [CPT-lactone or carboxylate]$_{25\%HP\text{-}\beta\text{-}CD}$ − [CPT-lactone or carboxylate]$_{0\%HP\text{-}\beta\text{-}CD}$ divided by

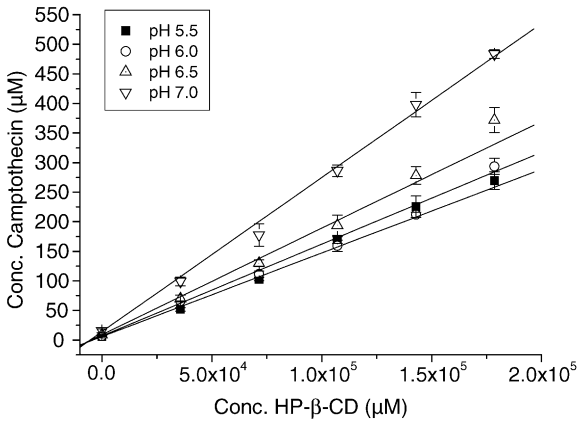

Fig. 5. Phase solubility diagrams obtained for the total camptothecin concentration ($n = 3$).

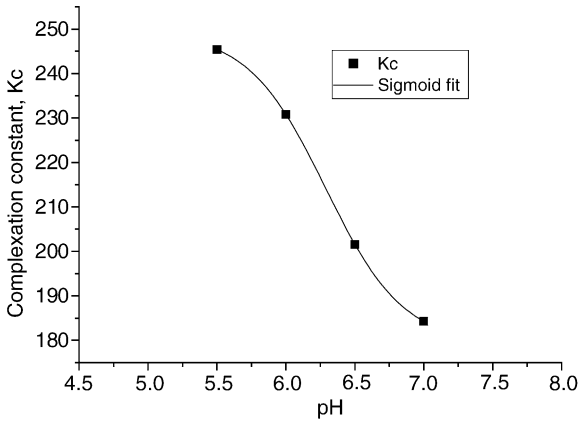

Fig. 6. Correlation between stability constant K_C and pH.

the molar concentration of HP-β-CD corresponding to 25% (178.6 mM) and S_0 = [CPT-lactone] or [CPT-carboxylate] at 0% HP-β-CD. The stability constants, K_C, for the lactone and the carboxylate calculated by this method are given in Tables 2 and 3, respectively. The mean ± S.D. of the K_C values for lactone- and carboxylate-CPT are 260 ± 18 and 113 ± 7 M^{-1}, respectively, with no systematic difference in K_C values with varying pH in the physiologically relevant range of pH 5.5–7.0.

Table 2
Solubility of the lactone-camptothecin (μM)

pH	0% CD	10% CD	25% CD	K_C value (lactone)[a] (M^{-1})
5.5	5.4	103.8	251.8	254
6.0	5.7	107.3	261.6	251
6.5	5.9	108.4	268.4	249
7.0	5.2	106.2	273.9	287

[a] Slope = $[CPT_{lactone}]_{25\%HP\text{-}\beta\text{-}CD}$ (μM) − $[CPT_{lactone}]_{0\%HP\text{-}\beta\text{-}CD}$ (μM)/[HP-β-CD] (μM), $S_0 = [CPT]_{0\%HP\text{-}\beta\text{-}CD}$ (μM), K_C = slope/S_0 (1 − slope).

Table 3
Solubility of the carboxylate-camptothecin (μM)

pH	0% CD	10% CD	25% CD	K_C value (carbox.)[a] (M^{-1})
5.5	0.4	3.5	7.5	109
6.0	1.0	10.1	21.9	114
6.5	3.1	29.1	62.0	108
7.0	9.0	94.5	206.4	123

[a] Slope = $[CPT_{carboxylate}]_{25\%HP\text{-}\beta\text{-}CD}$ (μM) − $[CPT_{carboxylate}]_{0\%HP\text{-}\beta\text{-}CD}$ (μM)/[HP-β-CD] (μM), $S_0 = [CPT]_{0\%HP\text{-}\beta\text{-}CD}$ (μM), K_C = slope/S_0 (1 − slope).

4. Discussion

The hydrolysis of CPT at physiological pH converts the potent anticancer lactone-CPT into the water-soluble, but presumably less active and more toxic carboxylate-CPT. Therefore, it is pivotal to study both isomers during complexation with HP-β-CD at physiological pH values.

The equilibrium time of the complexation/solubilization reaction was found to be less than 5 days, corresponding to the findings of Xiang and Anderson (Xiang and Anderson, 2002) (equilibration time of 3 days) and J. Kang et al. (Kang et al., 2002) (equilibrium time of 5 days).

Close-to-linear correlations of increase in solubility versus HP-β-CD concentration from the phase solubility study were found and indicate the formation of 1:1 complexes (Fig. 5). Both Xiang and Anderson (Xiang and Anderson, 2002) and Kang et al. (Kang et al., 2002) concluded that a 1:1 complex is formed at acidic pH with only the lactone form of the drug. With the present results at pH values where both forms of CPT are expected to be present, it was concluded that CPT forms a 1:1 complex with HP-β-CD irrespective of the isomer present. As dilution of the saturated solutions before HPLC-analysis affect the lactone–carboxylate equilibrium, only the total-CPT could be quantified in the phase solubility study. The apparent overall K_C values were found to decreases with increased pH from a value of $K_C = 245\,M^{-1}$ at pH 5.5 to $K_C = 184\,M^{-1}$ at pH 7.0. It was thus hypothesized that carboxylate-CPT, other than earlier assumed (Kang et al., 2002), also forms a CD-inclusion complex.

In order to distinguish between the lactone- and the carboxylate-form, CPT was incubated at lower CPT-concentrations with 0, 10 and 25% HP-β-CD at different pH values, allowing quantification of CPT on the HPLC without dilution of the samples. The ratios of lactone/carboxylate were supposed to be independent of the CPT-concentration, but only affected by pH. The curves established this way (Fig. 4) revealed a shift of the lactone/carboxylate equilibrium to higher pH values with increasing HP-β-CD concentrations.

Applying the pH-equilibrium data to the above phase solubility data, separate phase solubility plots for both the lactone and carboxylate isomers could be made. The measured lactone–carboxylate equilibrium provided quantitative information on the stabilizing effect of HP-β-CD on the lactone isomer at the respective pHs and HP-β-CD concentrations applied. Consequently, the disturbance of lactone–carboxylate equilibrium during dilution of samples prior to HPLC-analysis was circumvented, and the total CPT-concentration could be separated into the two CPT-forms by calculations, assuming that the lactone–carboxylate equilibrium is the same for saturated, as for diluted CPT-solutions. The resulting separate respective K_C values for the lactone- and the carboxylate-form of the drug were found to be $260 \pm 18\,M^{-1}$ for lactone-CPT and $113 \pm 7\,M^{-1}$ for carboxylate-CPT. The differences of affinity to cyclodextrin between the two CPT-forms is relatively small, supporting the idea that CPT penetrates the cavity of the cyclodextrin molecule with its quinoline ring, which is unaffected by the hydrolysis.

The small complexation constant (K_C) of the CPT-HP-β-CD inclusion complex compared to other K_C values described in literature on drug-cyclodextrin complexation, which normally are between 100 and 20,000 M^{-1} (Stella and Rajewski, 1997) makes the molar ratio between CPT and HP-β-CD in the solution relatively high, i.e. approximately 1/700 and 1/400 at pH 5.5 and 7.0, respectively. This means that at equilibrium <0.25% of the cyclodextrin molecules are occupied by CPT. Furthermore, as no systematic change in K_C values was found neither for the lactone nor the carboxylate-form with changing lactone/carboxylate ratios (Tables 2 and 3), a competitive effect between the two CPT forms for complexation to HP-β-CD was judged not to bias the chosen approach for calculation of the separate K_C values.

At therapeutically relevant pH values, CD stabilizes preferably the lactone form of CPT. This conclusion is drawn from the increase of the pH-equivalence point

Table 4
Comparison of camptothecin-solubility data from the literature with present values

Publication	HP-β-CD substitution[a]	pH	Buffer system	$K_{C(lactone)}$ (M^{-1}) (25 °C)	$K_{C(carboxylate)}$ (M^{-1}) (25 °C)
Xiang and Anderson, 2002	1.07	≤4.6	30 mM citric acid	180	–
Kang et al., 2002	0.85	∼1.58	0.02 N HCl	160.4	–
This study	0.58–0.73	5.5–7.0	25 mM phosphate buffer	260	113

[a] Molar substitution per anhydroglucopyranose unit.

with increased cyclodextrin-concentrations, a decrease in the K_C value for total-CPT with increased pH, and a higher K_C value for lactone-CPT than for carboxylate-CPT. The K_C value of the lactone-form obtained in this study is slightly higher than previously published values (Table 4). This appears reasonable since we have used HP-β-CD with a lower molar substitution than Kang et al. (Kang et al., 2002) and Xiang and Anderson (Xiang and Anderson, 2002). Those cyclodextrin derivatives with lower degree of molar substitution are claimed to be better solubilizers than the same types with a higher degree of substitution (Loftsson, 1998). In the present study, the carboxylate-form of CPT is clearly shown to form an inclusion complex with cyclodextrin at physiological pH. This is in contradiction with the statement of J. Kang et al. (Kang et al., 2002), who postulated that the likelihood of carboxylate-CPT forming a CD-complex was negligible, due to its high water-solubility. At physiological pH, the solubility of both forms is restricted, and the present study demonstrates that the carboxylate-form also has to be taken into account for the complexation equilibrium. The lower observed affinity of the ionized carboxylate compared with the lactone form corresponds well with complexation studies on ionisable drugs (Li et al., 1998, 1999; Sridevi and Diwan, 2002).

The highest total CPT-concentration (168.4 μg/ml/483.5 μM) was reached at pH 7.0 with 25% HP-β-CD. Obviously, the dramatically higher solubility of carboxylate as compared to CPT-lactone overcompensates the two-fold lower affinity of Carboxylate-CPT to HP-β-CD. The CPT-concentration reached in the study by Kang et al., with 25% (w/v) HP-β-CD, was 43.69 ± 0.78 μg/ml (125.4 ± 2.2 μM) in 0.02 N HCl (Kang et al., 2002), which would be at the lower end of the therapeutically useful concentration range. The solubility achieved in the present study is up to almost four times higher dependant on the pH value. But, if only the active lactone-CPT is taken into consideration, concentrations of around 260 μM/90 μg/ml, are obtained with 25% HP-β-CD, for all pH values included in the solubility study (Table 2), whereas the concentration of the carboxylate-form varied between 7.5 and 20.0 μM at pH 5.5 and 6.0, which represents 3 and 7% of the total CPT and is thus regarded acceptable for therapeutical administration.

In summary, complexation of CPT with HP-β-CD at therapeutically relevant pH values gives a linear increase in CPT solubility with increasing HP-β-CD concentration, indicating the formation of a 1:1 complex. The apparent stability constant of the complexes decreases with increasing pH. The pH-equilibrium study demonstrates that both CPT-isomers form HP-β-CD complexes, and that HP-β-CD pushes the carboxylate/lactone-equilibrium towards the more hydrophobic active lactone form of the drug. A combination of phase solubility and pH-equilibrium data allows establishing separate stability constants for CPT-lactone (260 M^{-1}) and CPT-carboxylate (113 M^{-1}).

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