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Investigation of curcumin-cyclodextrin inclusion complexation in aqueous solutions containing various alcoholic co-solvents and alginates using an UV-VIS titration method

Studies of curcumin and curcuminoides, XXXV

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The effect of pharmaceutical excipients like alcoholic co-solvents and water-soluble polymers on the inclusion complexation of curcumin in hydroxypropyl-β-cyclodextrin and hydroxypropyl-γ-cyclodextrin was investigated with a UV-VIS titration method. The association constants and the stoichiometries of the inclusion complexes in buffered media containing various amounts dl of alcoholic co-solvents and alginates were determined. The results showed a 1 : 1 stoichiometry between curcumin and both the cyclodextrins investigated in buffered media containing 10% (v/v) alcoholic co-solvents, although some 1 : 2 (host : guest) complexation was suspected between curcumin and hydroxypropyl-β-cyclodextrin. The presence of 0.1% (w/v) sodium alginate or propylene glycol alginate did apparently not change the stoichiometry of the complexes formed. Curcumin was found to have a more than 30-fold higher association constant with hydroxypropyl-γ-cyclodextrin compared to hydroxypropyl-β-cyclodextrin in buffer containing 0.5% ethanol. Large variation in the association constants between curcumin and the cyclodextrins as a result of different co-solvents in the aqueous complexing media were found. A decrease in the association constant was seen as the chain lenght of the added co-solvent increased. Further, a decrease in the association constants was observed by addition of alginates in the case of hydroxypropyl-γ-cyclodextrin at 0.5 or 5% (v/v) ethanol. The trend was opposite in the case of hydroxypropyl-β-cyclodextrin, where a 30–90% increase in the association constant was observed in the presence of alginates. The results in the current study showed the large variations in the complexation between curcumin and hydroxypropyl-β-cyclodextrin and hydroxypropyl-γ-cyclodextrin, resepctively, as a result of various alcoholic co-solvents and alginates in the complexing media. The results also illustrated the importance of optimizing the solvent systems when utilizing cyclodextrins as drug carriers.

1. Introduction

Curcumin (Cur) is a natural, highly lipophilic dye with various pharmacological effects like antioxidant, anti-inflammatory and anti-cancer activity (Conney 2003; Duvoix et al. 2005; Kuriakose and Sharan 2006; Kuttan et al. 2007; Li et al. 2002; Menon and Sudheer 2007; Sharma 2005). Cur is considered as safe for human use, even at high doses, but has poor bioavailability (Anand et al. 2007). Cur's solubility in water is a critical parameter when it comes to bioavailability. Extremely low water-solubility at acidic pH ($<3 \times 10^{-8}$ M) and limited hydrolytic stability in water at pH above 7, are factors limiting the pharmaceutical application (Tønnesen and Karlsen 1985a, b; Tønnesen et al. 2002). Approaches to increase the solubility and stability of Cur include incorporation into various micellar systems, the use of polymers as solubility enhancing agents and complexation with cyclodextrins (Ribeiro et al. 2003; Tønnesen 2002, 2006; Tønnesen et al. 2002; Valero et al. 2007). Cyclodextrins (CDs) are natural, cyclic oligosaccharides of six, seven or eight α (1–4)-linked glucopyranose units. They have a hydrophilic outer surface and a hydrophobic inner cavity and can therefore form water-soluble inclusion complexes with hydrophobic compounds like Cur. CDs are commonly utilized in pharmaceutical formulations to increase the apparent solubility of a hydrophobic drug. They can also be used as drug-carriers that affect physiochemical properties of the drug molecule like drug permeability, drug stability, drug dissolution and drug bioavailability without altering the structure of the drug molecule (Challa et al. 2005; Loftsson and Duchene 2007; Loftsson et al. 2006, 2002b;

Masson et al. 1999). Complexation with CDs can lead to 10^4-fold increase in water-solubility of Cur at pH 5 and a more than 500-fold increase in the hydrolytic stability at pH 8 (Baglole et al. 2005; Tomren et al. 2007; Tønnesen et al. 2002). However, a good fit between the drug molecule and the CD cavity is important. This will limit the amount of CDs in the formulation, which is important due to toxicological considerations, production costs, formulation bulk and osmolarity considerations (Loftsson et al. 2005; Stella and He 2008). Thus the selection of the CD is important, but additional formulation excipients can also affect the complexation. The intrinsic solubility of the drug and the presence of various additives like alcoholic co-solvents and polymers will affect inclusion complex formation (Evans et al. 2000; Loftsson et al. 2005; Valero et al. 2003a, 2004). Alcoholic co-solvents may reduce the polarity of the complexing media, and thus alter the drug's affinity for the CD-cavity and also influence on the structures of the complex formed (Connors 1997; Garcia-Rio et al. 2006; Giangiacomo 2006; Li et al. 1999; Loftsson and Brewster 2008; Mrozek et al. 2002; Parsons et al. 2001; Pitha and Hoshino 1992). Water-soluble polymers can increase the complexation efficiency and thus improve the solubility and bioavailbility (Loftsson et al. 2005; Savolainen et al. 1998; Valero et al. 2004, 2007). Water-soluble polymers are commonly used in pharmaceutical preparations as emulsifying and suspending agents, as coating materials or in controlled release formulations. Alginate is a water-soluble polymer that can be used to solubilise Cur (Tønnesen 2006). We have recently been investigating the release of Cur from vehicles containing sodium alginate or propylene glycol alginate in combination with HPβCD and HPγCD (Hegge et al. 2008). Formulations based on these alginates and CDs are currently under investigation in our laboratory.

The aim of the current study was to investigate the effect of pharmaceutical excipients like alcoholic co-solvents and water-soluble polymers on the inclusion of Cur with two CD derivates, hydroxypropyl-β-cyclodextrin (HPβCD) and hydroxypropyl-γ-cyclodextrin (HPγCD). The association constants and the stoichiometries of the inclusion complexes in buffered media containing various amounts (v/v) of alcoholic co-solvents have been determined by a UV-VIS titration method (Singh et al. in progress). The co-solvents investigated in the current study (methanol, ethanol, 1-propanol, glycerol, polyethylene glycol 200 and polyethylene glycol 400) were chosen due to their relevance as pharmaceutical excipients or to study the effect of alcohols of various chain lengths. The association constants and the stoichiometries of Cur with HPβCD and HPγCD at various concentrations of ethanol in the presence and absence of alginates were determined to evaluate the effect of water-soluble polymers on the complexation. The two alginates chosen for this investigation were sodium alginate and propylene glycol alginate.

2. Investigations, results and discussion

The association constants and the stoichiometries of the inclusion complexes were determined in aqueous complexing media containing a constant concentration of Cur (5×10^{-6} M). The concentration of CDs varied from 0% to 15% (w/v) and various amounts (v/v) of the selected co-solvents were added. Alginate was added at 0.1% (w/v) in selected experiments. Representative absorption spectra of Cur with increasing concentrations of HPβCD are shown in Fig. 1. The enhanced Cur absorption observed as the concentration of HPβCD in solution was increased, together with a simultaneous small spectral shift towards higher wavelengths and the appearance of an isobestic point around 385 nm were indications of Cur inclusion into the CD-cavity. The high electron density prevailing inside the CD-cavity mobilizes the electrons of the incorporated molecule which results in changes in the absorption spectra of Cur (Frömming and Szejtli 1994). Similar enhancement in the Cur absorption caused by the inclusion of Cur into β-CD has previously been reported (Frömming and Szejtli 1994; Tang et al. 2002). Inclusion complexation between Cur and HPβCD and HPγCD in aqueous complexing media are also described previously (Hegge et al. 2008; Tønnesen et al. 2002).

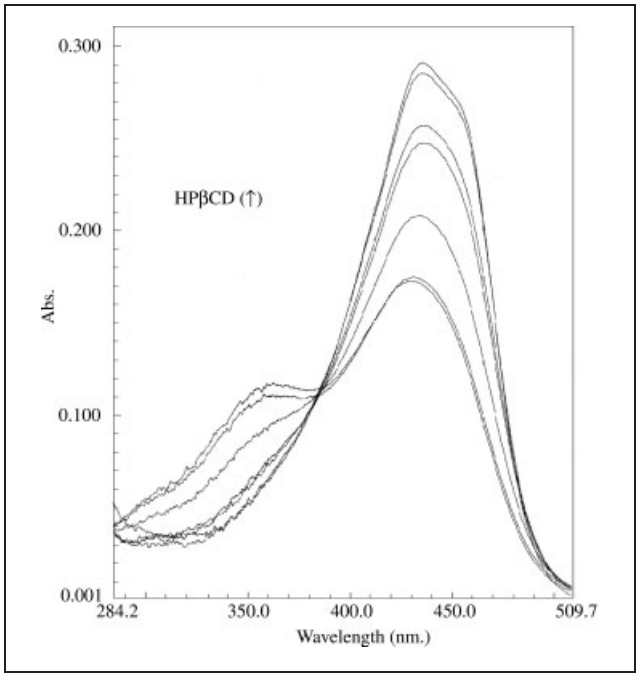

Fig. 1: Absorption spectra of Cur (5×10^{-6} M) in phosphate buffer containing 5% (v/v) ethanol at increasing concentrations of HPβCD (0.0005%, 0.005%, 0.05%, 0.5%, 1%, 10% and 15% (w/v), respectively)

2.1. Stoichiometry

The association constants were calculated from the 1 : 1 binding isotherm (see 3.4.). The experimental data could be fitted to the binding isotherm ($R^2 = 0.976–0.998$) by non-linear curve fitting. Figures 2 a) and 2 b) show representative examples of data sets obtained in case of HPβCD and HPγCD, respectively, in the current study. A good correlation between the experimental data and the 1 : 1 binding isotherm was found (see 2.2). However, a systemic deviation in the obtained data in case of HPβCD from the 1 : 1 binding isotherm was observed at higher CD concentrations (>0.0001 M) in some cases, which might indicate the formation of complexes with other stochiometries than 1 : 1. Still, the good correlation between the experimental data and the 1 : 1 binding isotherm confirms that Cur-CD complexes with 1 : 1 stoichiometry were formed and that the complexation led to significant changes in the spectra. The Cur absorption spectra showed a shoulder at the red edge of the peak at 10% and 15% (w/v) HPβCD (Fig. 1). Addition of increasing amounts of ethanol to the aqueous complexing media counteracted these characteristics of the absorption spectra and higher

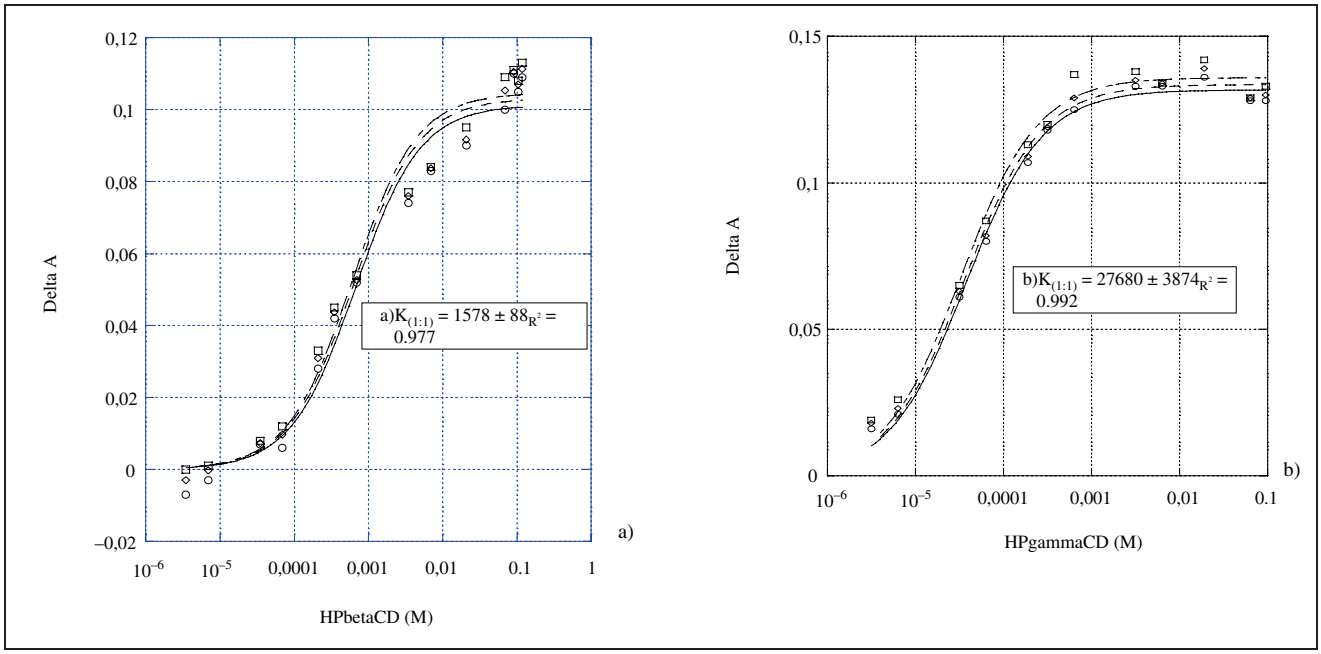

Fig. 2: a) The increase in Cur (5×10^{-6} M) absorbance, expressed as ΔA at 434 nm, at increasing amounts of HPβCD. ΔA ($A - A_0$) is plotted against increased HPβCD-concentrations (12 concentrations) and fitted to the 1 : 1 binding isotherm in Kaleidagraf. 10% ethanol is used as co-solvent. B, C and D are 3 replicates of the same samples, b) The increase in Cur (5×10^{-6} M) absorbance, expressed as ΔA at 429 nm, at increasing amounts of HPγCD. ΔA ($A - A_0$) is plotted against increased HPγCD-concentrations (12 concentrations) and fitted to the 1 : 1 binding isotherm in Kaleidagraf. 5% ethanol is used as co-solvent. B, C and D are 3 replicates of the same samples —○— B, —□— C, —◇— D

concentrations of HPβCD were required to obtain the shoulder. In case of 10% (v/v) 1-propanol as co-solvent, the shoulder was absent event at a 10% and 15% (w/v) HPβCD concentration (Fig. 3) and the fit to the 1 : 1 binding isotherm appeard to be improved ($R^2 = 0.996$). This suggests a different type of complexation in this case. Cur is a symmetrical molecule with two 3-methoxy 4-hydroxy substituted phenyl moieties, which can fit into the cyclodextrin cavity on each end. Thus the formation of inclusion complexes with 1 : 2 (guest : host) stoichiometry is possible as well as the proposed 1 : 1 complexes. The observed shoulder in the absorption spectra could indicate

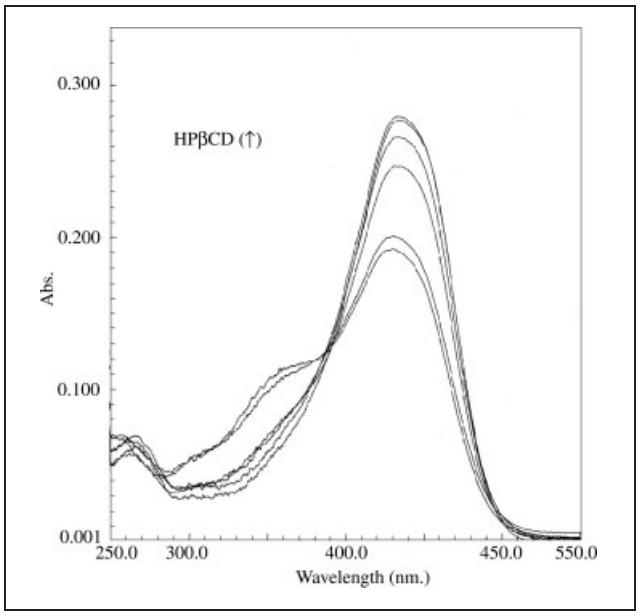

Fig. 3: Absorption spectra of Cur (5×10^{-6} M) in phosphate buffer containing 10% (v/v) 1-propanol at increasing concentrations of HPβCD (0.0005%, 0.05%, 0.5%, 1%, 10% and 15% (w/v), respectively)

that 1 : 2 complexes are also present. Spectrophotometric investigations indicated 1 : 2 (guest : host) complex with βCD (Tang et al. 2002) and a fluorescence spectroscopy study in 1% methanol/water indicated that 1 : 1 and 1 : 2 complexes were formed with HPβCD and HPγCD (Baglole et al. 2005). The current data fit well with a 1 : 1 complex formation. However the data do not contradict some 1 : 2 complex formation, especially if the 1 : 2 complexation results in much smaller spectral changes than the 1 : 1 complex formation. The variations in the reported Cur-CD stoichiometries may be due to solvent effects (e.g. buffer type) and Cur quality, as commercial Cur normally also contains other curcuminoids in addition to Cur (Tønnesen et al. 1995). Stoichiometry studies performed in solutions saturated with the guest molecule (i.e. phase-solubility studies) (Tønnesen et al. 2002) cannot easily be compared to the studies performed with lower concentration of the host (e.g. NMR, fluorescence, UV-VIS titration method). The ability to separate between 1 : 1 and 1 : 2 complex formation can also depend on the method. Previously performed phase-solubility studies, e.g. saturated solutions of Cur in HPβCD and HPγCD using a buffer system identical to the present buffer system, indicated complex stoichiometries of higher order with respect to the CD, at least in case of HPγCD. (Hegge et al. 2008). The presence of 0.1% (w/v) sodium alginate or propylene glycol alginate, or the presence of various co-solvents, did apparently not change the stoichiometry of the complexes formed. The method is therefore considered suitable for determining the 1 : 1 association constants and these can therefore be compared in the following discussion.

2.2. Calculated association constants K(1 : 1)

The 1 : 1 association constants ($K_{(1:1)}$) and regression factors (R^2) obtained for different solvent systems are presented in Tables 1 and 2. The UV-VIS titration method requires a reference value of Cur in the abscence of CD. The extremely

Table 1: Association constants ($K_{(1:1)}$) of Cur and HPβCD or HPγCD in the presence of 10% (v/v) alcoholic co-solvents in phosphate buffer pH 5. $K_{(1:1)}$ in 0.5% ethanol are 980 and 33708 M^{-1} for Cur and HPβCD and HPγCD, resepectively

Co-solvent	% Co-solvent (v/v)	CD	$K_{(1:1)} \pm$ SD M^{-1}	R^2
Methanol	10%	HPβCD	1613 ± 21	0.980
Methanol	10%	HPγCD	17675 ± 1270	0.990
Ethanol	10%	HPβCD	1578 ± 88	0.977
Ethanol	10%	HPγCD	7530 ± 326	0.995
1-Propanol	10%	HPβCD	535 ± 61	0.996
1-Propanol	10%	HPγCD	1992 ± 131	0.992
Glycerol	10%	HPβCD	38 ± <1	0.990
Glycerol	10%	HPγCD	7748 ± 387	0.994
PEG 200	10%	HPβCD	304 ± 25	0.985
PEG 200	10%	HPγCD	1842 ± 173	0.998
PEG 400	10%	HPβCD	189 ± 25	0.992
PEG 400	10%	HPγCD	775 ± 98	0.985

low solubility of Cur in water ($<3 \times 10^{-8}$ M) prevents complexing studies in pure aqueous solvents (Tønnesen et al. 2002). Samples containing 0.5% (v/v) ethanol in buffer were therefore used as reference to estimate the complexation in plain buffer solutions. Table 2 shows that HPγCD has a more than 30-fold higher association constant with Cur than HPβCD in buffer containing 0.5% ethanol, $33708 \pm 3874\ M^{-1}$ and $980 \pm 22\ M^{-1}$ respectively. Observations from previous complexation studies on curcuminoides suggest that the bulky moieties of the two phenyl groups of Cur fit the γ-cavity better than the β-cavity which is in accordance with the results in the present study (Tomren et al. 2007). Tønnesen et al. (2002) reported the estimated stability constants of Cur in HPβCD and HPγCD to be $> 5 \times 10^4\ M^{-1}$ and $> 16 \times 10^4\ M^{-1}$ respectively (Tønnesen et al. 2002), while Baglole et al. reported the association constants for the 1 : 1 complex to be $3400 \pm 1800\ M^{-1}$ (HPβCD) and $21000 \pm 1200\ M^{-1}$ (HPγCD) (Baglole et al. 2005). Phase-solubility and solubilization studies performed with the same CD derivates further emphasize the higher affinity of Cur for the γ-cavity (Baglole et al. 2005; Hegge et al. 2008; Tomren et al. 2007; Tønnesen et al. 2002). The difference between the association constant of Cur in HPβCD compared to HPγCD obtained in buffer containing 0.5% ethanol was found to be higher than what was previously reported. This can possibly be explained by differences in the method of investigation. The possibility of uncertainties in the results caused by tentative supersaturated Cur solutions at the low concentrations of ethanol cannot be disregarded. However, the differences in association constants of Cur obtained in HPβCD compared to HPγCD were also clear in case of 10% (v/v) co-solvent added to the complexing media, which should reduce the effect of tentative supersaturation. The Cur association constants were 3–11 times higher for HPγCD compared to HPβCD in the presence of 10% methanol, ethanol, 1-propanol, PEG 200 and PEG 400 buffered solutions. This is consistant with previous reports (Baglole et al. 2005; Tønnesen et al. 2002).

Table 2: Association constants ($K_{(1:1)}$) of Cur and × % HPβCD or HPγCD in the presence of 0.1% (w/v) alginate at various ethanol concentrations (0.5%, 5% and 10%, v/v) in phosphate buffer pH 5

Co-solvent	% Co-solvent	CD (v/v)	$K_{(1:1)} \pm$ SD M^{-1}	R^2
Ethanol	0.5%	HPβCD	980 ± 22	0.980
+ SA			1330 ± 168	0.982
+ PGA			1661 ±157	0.984
Ethanol	5%	HPβCD	1113 ± 182	0.976
+ SA			1459 ± 159	0.988
+ PGA			2122 ± 256	0.981
Ethanol	10%	HPβCD	1578 ± 88	0.977
+ SA			1525 ± 57	0.988
+ PGA			1197 ± 82	0.984
Ethanol	0.5%	HPγCD	33708 ± 3874	0.995
+ SA			16382 ± 2740	0.998
+ PGA			22877 ± 2848	0.993
Ethanol	5%	HPγCD	27680 ± 3874	0.992
+ SA			8666 ± 454	0.997
+ PGA			11716 ± 475	0.997
Ethanol	10%	HPγCD	7530 ± 326	0.995
+ SA			5609 ± 136	0.996
+ PGA			5986 ± 316	0.998

SA = Sodium alginate; PGA = Propylene glycol alginate

2.3. Effect of alcohols and PEGs

There were obvious changes in the inclusion complexation of Cur as a result of the different co-solvents in the complexing media. Table 1 shows that there was a decrease in the association constant with both CDs as the chain lenght of the added co-solvent increased, both in case of the monoalcohols and the PEGs. Alcohols are able to form complexes with CDs (Evans et al. 2000; Garcia-Rio et al. 2006; Matsui and Mochida 1979; Mrozek et al. 2002; Munoz de la Pena et al. 1991). A size-dependent complexation between the CDs and the alcohols (including the PEGs) is possible. Especially, alcohols with longer alkyl chains can compete with Cur for the CD-cavities, and such a competition corresponds to the results obtained (Table 1). A reduced complexation was observed with 1-propanol as co-solvent compared to the shorter co-solvents. The trend is especially clear in the case of HPγCD. The presence of a co-solvent also affects the bulk solution polarity. Addition of a water-miscible alcohol to the aqueous bulk is expected to decrease the polarity of the complexing medium and consequently increase the intrinsic solubility of Cur (Bastos et al. 1997; Dashnau et al. 2006; Moreira and Bastos 2000; Parsons et al. 2001; Pitha and Hoshino 1992). The Cur concentration was constant in the current study. A decreased polarity of the bulk solution will therefore reduce the affinity for the lipophilic CD-cavity and the association constant between Cur and CD was therefore expected to decrease (Huang et al. 1992). Methanol, ethanol, 1-propanol, glycerol and PEG 400 have dielectric constants of 33, 24.3, 20.1, 42.5 and 13.6, respectively (Loftsson and Brewster 2008; Loftsson et al. 2007; Rubino and Yalkowsky 1987). The effect of the alcohols on the polarity of the aqueous bulk solution should to some degree correspond to their dielectric constants (ε). However, the dielectric constant of the aqueous bulk solutions is dependent on the molar concentration of the alcohols and the phosphate buffer. Studies to investigate the relationship between the complexation and the ε of the bulk solutions will be performed in our laboratory.

Ethanol as a co-solvent clearly affected the complexation between Cur and HPγCD. The effect was not as pronouced in the case of HPβCD. Addition of 10% (v/v) ethanol to the solvent decreased the association constant for HPγCD by a factor of ∼4.5 (i.e. from 33708 M^{-1} to 7530 M^{-1}) compared to the buffer solution containing 0.5% ethanol. A competition between Cur and small alco-

hol molecules like methanol and ethanol for the relatively large γ-cavity seems unlikely (Pitha and Hoshino 1992). The co-solvent effect on the intrinsic solubility of Cur resulting in a decrease in Cur-HPγCD complexation, is a more likely explanation for the observed effects of the shorter co-solvents like ethanol and methanol. The Cur complexation with HPβCD seems to be quite different from the complexation with HPγCD because of the large difference in the Cur affinity for HPβCD compared to HPγCD. The obtained $K_{(1:1)}$-values for Cur with HPβCD containing 10% (v/v) methanol or 10% and 5% (v/v) ethanol in solution appeard to be higher than the value obtained in buffer solution containing 0.5% ethanol (Tables 1 and 2). Previously performed phase-solubility studies with Cur and HPβCD in 10% ethanolic buffered solution compared to plain buffer showed a decrease in complexation and a reduction in the solublity in presence of ethanol (Hegge et al. 2008). This might be due to ethanol counteracting any 1 : 2 complexation (Cur : CD) as well as the effect on the bulk solubility. The absolute differences in the $K_{(1:1)}$-values between complexation with different amounts of ethanol and 10% methanol were small (Table 2). There is however a possibility that ethanol and methanol affect the formation of 1 : 2 complexes in the case of Cur and HPβCD, which will influence on the calculated $K_{(1:1)}$. The tendency towards increased complexation between Cur and HPβCD as a result of increasing concentrations (v/v) of ethanol or 10% methanol (Table 2) can also be due to other effects of the alcohols. Generally, most guest molecules are too large to be completely engulfed in the CD-cavity (Connors 1997). A preferred moiety of the molecule will enter the CD-cavity, which would be one of the phenyl moieties in case of the symmetric Cur molecule. Tomren et al. suggested that the bulky moieties on the phenyl group of Cur fitted the larger HPγCD-cavity better than the smaller HPβCD-cavity and Baglole et al. speculated in the possibility of a folded Cur molecule residing in the cavity of HPγCD and γCD (Baglole et al. 2005; Tomren et al. 2007). The Cur molecule has been reported to be approximately 19 Å long and 6 Å wide and it is probably too large to be completely embedded in the relatively small HPβCD-cavity (I.D. 6–6.5 Å) (Challa et al. 2005; Tang et al. 2002). The formation of a Cur-HPβCD complex in which a relatively large part of the Cur molecule is residing outside the CD-cavity (compared to the complex with HPγCD) would result in significant Cur-aqueous bulk interactions. These Cur-aqueous bulk interactions might be stabilized in the case of increasing amounts of ethanol and 10% methanol. The possibility of such interactions between one part of the Cur molecule and the bulk solution is supported by observed changes in the Cur absorption maxima at 10% HPβCD (where a high degree of complexation is assumed). The changes are previously reported to correspond with the dielectric constant of the monoalcohols added (Tønnesen et al. 1995). The addition of the least polar co-solvents like 1-propanol ($\varepsilon = 20$) and ethanol ($\varepsilon = 24$) resulted in Cur absorption maxima at 436 nm and 435 nm in 10% HPβCD, respectively. The addition of more polar methanol ($\varepsilon = 33$) to the complexing media resulted in an absorption maximum of 434 nm in 10% HPβCD. The absorption maxima in pure methanol, ethanol and 1-propanol of Cur are 422.0 nm, 429.0 nm and 432.0 nm, respectively. However, no trend in the shift of the absorption maximum was observed for Cur combined with HPγCD in the different solvent systems investigated. Neither was there a difference in the absorption maximum in the case of Cur and HPβCD in 10% PEG 200 buffered solutions compared to 10% PEG 400 buffered solutions (absorption maximum at 434 nm in the presence of HPβCD and 431 nm in pure PEG 400). The low association constant observed for PEG 400 and PEG 200 co-solvents might be explained by a competition between the PEG-chain and Cur for the CD-cavities. The effect was more pronounced in case of PEG 400 (average number of oxyethylene groups = 8.7) compared to PEG 200 (average number of oxyethylene groups = 4.2) (Rowe et al. 2003). Another possible explanation for the observed effects of ethanol and methanol on the complexation between Cur and HPβCD, is the formation of ternary complexes caused by the presence of small alcohols. Previous studies on the effect of methanol and ethanol on the complexation with CDs indicated that both a decrease and an increase in the complexing is possible (Mrozek et al. 2002; Pitha and Hoshino 1992). Li et al. observed a concentration dependent formation of a ternary complex composed of fluasterone, HPβCD and ethanol (Li et al. 1999). Loftsson et al. showed an increase in the apparent intrinsic solubility, but a decrease in the apparent stability constant between two antibacterial agents and HPβCD as a result of addition of ethanol (Loftsson et al. 2005).

The results presented in Table 1 show that the association constant with both CDs to some extent are decreasing as the chain lenght of the added co-solvent increases. This may be explained by the competition between Cur and the alcohols for the CD cavities. Due to the small absolute differences in $K_{(1:1)}$-values obtained in buffer containing 0.5% ethanol compared to 10% (v/v) methanol or increasing amounts of ethanol, it was not possible to determine whether addition of ethanol and methanol in fact did increase the association constant of Cur with HPβCD. It is, however, clear that ethanol and methanol as co-solvents had different effects on the two Cur-CD complexes studied. This might be due to the different extent of inclusion of Cur into the two different CD-cavities. While Cur is believed to be included deep in the cavity of the HPγ-derivate, a shallow and "incomplete" inclusion might be the case with HPβCD. The results obtained might indicate that the small alcohols like ethanol and methanol had a stabilizing effect on the Cur-HPβCD complex, possibly through effects on the Cur part of the inclusion complex residing outside the CD-cavity or through formation of ternary complexes where the alcohols caused a better match between Cur and the HPβ-cavity. However, the trend observed for the experiments with HPβCD can contain uncertainties due to formation of a 1 : 2 complexation which will influence on the association constants calculated. PEGs as co-solvents resulted in lower association constants between Cur and both of the CDs investigated compared to the monoalcohols as co-solvents, possibly due to a competition between the PEG-chain and Cur for the CD-cavities. The results clearly illustrated the large variation in the complexation observed for the same host and guest in various solvent systems, and showed the importance of optimizing the solvent systems when utilizing CDs as drug carriers.

2.4. Glycerol as co-solvent

Cur revealed an unexpected low association constant in a 10% glycerol (85%)/buffer solution, especially in the presence of HPβCD. An increase in Cur absorption in the wavelenght range 350 nm to 500 nm was observed in a sample containing Cur in 10% (w/v) HPβCD and 10% (v/v)

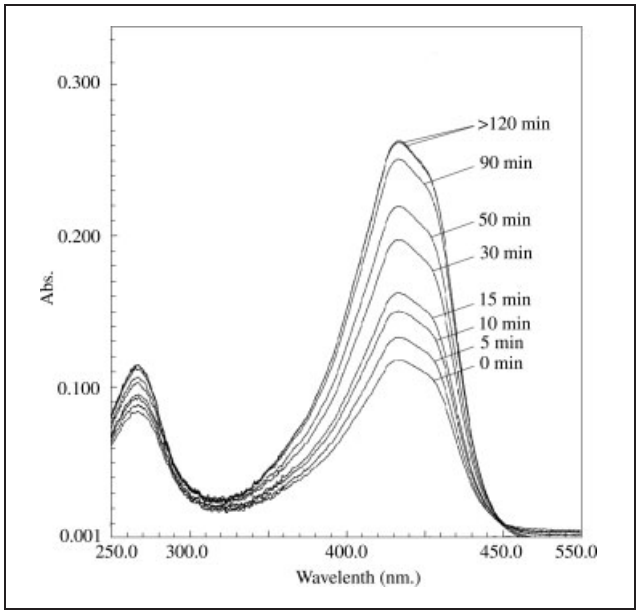

Fig. 4: Increase in Cur absorbance during 140 minutes in a sample containing Cur (5×10^{-6} M), 10% (w/v) HPβCD and 10% (v/v) glycerol (85%) in phosphate buffer pH 5

glycerol (85%) that was stored in the dark for two hours (Fig. 4). The increase in absorption is probably caused by a time-dependent solubilization of Cur in a supersaturated system. Such an increase in absorption as a function of time could not be detected in the samples containing HPγCD or in samples containing any of the other co-solvents. All the samples were prepared from stock solutions, where Cur was dissolved in the appropriate co-solvent e.g. glycerol. Cur seemed to form a supersaturated solution in this particular co-solvent. The association constants obtained for the samples containing 10% glycerol should therefore be treated with caution. However, the obtained data indicated a very low association constant between Cur and the two CDs investigated, in the presence of 10% glycerol. Glycerol ($\varepsilon = 42.5$) is probably too hydrophilic to enter the hydrophobic CD-cavity, thus a competition between glycerol and Cur for the CD-cavities is unlikely (Bastos et al. 1997; Moreira and Bastos 2000; Tang et al. 2002). However, glycerol is postulated to form an equatorial complex with α-CD through hydrogen bonding at the wider end of the CD-molecule. This would prevent simultaneous inclusion complexation of other molecules with the CD (Bastos et al. 1997). The larger diameter of the HPγCD-cavity might result in a lower tendency to form such an equatorial complex with glycerol compared to the smaller HPβCD-cavity. This may result in a less pronounced competitive effect between glycerol and Cur for the HPγCD compared to HPβCD. In addition, glycerol affects the bulk solution and behaves as a chaotrope co-solvent (order-breaking) at low concentrations and a cosmotrope co-solvent (order-making) at higher concentrations (Giangiacomo 2006; Loftsson and Brewster 2008). The effect on the hydrogen bond network in the aqueous bulk might affect the driving force of the complexation. The effect of glycerol on the complexation between Cur and HPβCD and HPγCD needs to be further investigated.

2.5. Effect of alginate

The association constants obtained in samples containing Cur, various amounts of ethanol (0.5, 5 and 10% respectively) and 0.1% (w/v) of alginates, are presented in Table 2. Neither the presence nor the type of alginate did affect the complexation between Cur and the two CDs with 10% (v/v) ethanol in the complexing medium. However, a decrease in the association constants was observed by addition of alginates in the case of HPγCD at 0.5 or 5% (v/v) ethanol. The decrease was most pronounced for sodium alginate (SA). Interestingly, the trend was opposite in the case of HPβCD, where a 30–90% increase in the association constant was observed in the presence of alginates. The most pronounced increase in the complexation was seen with propylene glycol alginate (PGA) for 0.5 or 5% (v/v) ethanol. Both an increase and a decrease in complexation between guest molecule and CDs have previously been reported in the presence of water-soluble polymers (Savolainen et al. 1998; Valero et al. 2003a, b, 2004, 2007). SA and PGA act as solubilizers of Cur, probably through formation of intermolecular hydrogen bonds and/or electrostatic interactions between the polymer molecules and the Cur molecules (Tønnesen 2006). A competition between the polymer molecules and the CDs for the constant amount of Cur molecules present in the solution could lead to a reduction in Cur-CD complexation. Such a competition could explain the observed effect of alginates on samples containing HPγCD, but not those containing HPβCD. With a reference to the discussion above on the Cur-HPβCD-complex (see 2.3), a significant portion of Cur-bulk solution interactions are possible. SA and PGA might have the ability to stabilize the Cur molecule residing outside the HPβCD-cavity, or possibly the whole supramolecule, with a resultant increase in $K_{(1:1)}$. The exception is at high ethanol concentration (10%, v/v), where the interactions between ethanol and Cur might dominate over the weaker Cur-alginate interactions. A preliminary study using buffered complexing medium containing SA 0.4% (w/v) and 0.5 % (v/v) ethanol, resulted in association constants comparable with the constants obtained in a 10% ethanolic buffered solution (without polymer) in case of both CDs (data not shown). A decrease in the apparent stability constant as the polymer concentration reached a certain optimal concentration is previously reported (Loftsson 1998). An alginate concentration of 0.4% (w/v) seemed to decrease the complexation in case of both CD-derivates studied. However, SA and PGA in small amounts affected the association constant between Cur and HPβCD or HPγCD in different ways depending on the type of CDs. Both SA and PGA can be considered useful polymers in pharmaceutical preparations containing Cur and HPβCD due to the small positive effect seen on $K_{(1:1)}$ and the previously reported solubilization effect on Cur (Tønnesen 2006).

3. Experimental

3.1. Materials

Cur was synthesized according to the method of Pabon (1964). Two types of alginate; sodium alginate (Protanal LF10/60 LS, lot number s17261) and propylene glycol alginate (Protanal ester SD-LB, lot number SLP3908) were generously provided by FMC Biopolymers, Sandvika, Norway. The cyclodextrins were purchased from Wacker Chemie AG, München, Germany and were as follows: 2-hydroxypropyl-β-cyclodextrin (Cavasol® W7 HP, Mw ∼ 1380–1500) and 2-hydroxypropyl-γ-cyclodextrin (Cavasol® W8 HP, Mw ∼ 1576). The water content of the CDs (∼5% w/w) was determined by heating the CDs to 130 °C and calculating the water loss whenever a new item was opened and further every third month. The water content was taken into account in the further calculations. The phosphate buffer pH 5 (0.05 M) was prepared in distilled water from sodium dihydrogen phosphate and disodium hydrogen phosphate. Sodium chloride was used to adjust the ionic strength ($\mu = 0.085$). Polyethylene glycol 200 (for synthesis) and polyethylene glycol 400 (Ph. Eur.) were delivered by Merck. Ethanol (96%) was delivered by Arcus (Nor-

way). Methanol and propanol were of analytical grade and glycerol (85%) (Ph. Eur) was delivered by Norsk Medisinaldepot AS, Norway.

3.2. Sample preparation

Stock solutions of CD (HPβCD or HPγCD) were prepared in phosphate buffer or in 0.1% (w/v) alginate in phosphate buffer. Cur stock solutions were prepared in each of the alcohols to be investigated (without buffer). The appropriate volume of aqueous CD stock solution was then added to a volumetric flask (10 ml) before adding the selected volume of alcoholic Cur stock followed by dilution with phosphate buffer to the final volume. Thirteen concentrations in the concentration range from 0% (reference solution) to 15% (w/v) CD were chosen to calculate each association constant. The Cur concentration was kept constant at 5×10^{-6} M in all the samples regardless of the composition of the solvent. All the samples were prepared in triplicates. They were manually shaken and the absorption spectra were recorded within approximately 5 min after the preparation. The temperature was kept at 21 ± 1 °C. A minimum amount of co-solvent in the aqueous medium is necessary to be able to measure Cur absorption in samples without CDs due to the extremely low water-solubility of Cur. However, some of the samples in this study, especially the samples containing low amounts of co-solvent and/or CDs are probably supersaturated with respect to Cur and therefore unstable. All the samples were because of this measured within approximately 5 minutes after preparation to avoid problems related to the instability of the tentatively supersaturated samples.

3.3. UV-Vis spectroscopy method

Absorption measurements were performed by use of a Shimadzu UV-2101 PC UV-VIS scanning spectrophotometer equipped with a 1 cm quartz cell to determine the inclusion complex stoichiometries and association constants. The Cur absorbance in the reference samples (without any CDs) and in the samples containing CD at various concentrations was recorded at 250–550 nm against sample blanks prepared from the same reagents as the sample without Cur. A detection wavelength was chosen to give the most accurate and largest possible ΔA-values (i.e. the difference in absorbance of sample containing CDs (A) and sample without CDs (A_0)) for each of the different solvent systems investigated. The absorption maximum in the samples containing 10% (w/v) CDs in the presence of Cur and co-solvent was regarded as the absorption maximum of the Cur-CD inclusion complexes formed in the respective solvent system and therefore selected as the detection wavelength in that medium. The absorbance was measured at 21 ± 1 °C. The calculated ΔA-values were plotted against the logarithm of the CD-concentrations (M) in Kaleidagraf 4.0. Non-linear curve fitting to the 1 : 1 isotherm was used to determine the stoichiometry and the association constant ($K_{(1:1)}$) from the experimental data.

3.4. The 1 : 1 binding isotherm

The formation of an inclusion complex is usually described as a 1 : 1 complexes Eq. (1) between one CD molecule and one drug molecule e.g. Cur, although higher order complexes and CD-aggregates do exist (Challa et al. 2005; Gabelica et al. 2002; Loftsson et al. 2002a; Polyakov et al. 2004).

$$[\mathrm{Cur_{free}}] + [\mathrm{CD_{free}}] \leftrightarrow [\mathrm{Cur/CD}]$$
$$\mathrm{K_{(1:1)}} = [\mathrm{Cur/CD}]/[\mathrm{Cur_{free}}] \times [\mathrm{CD_{free}}] \quad (1)$$

The measured absorbance of Cur in solutions containing CDs can be regarded as the sum of the absorption by free Cur molecules, free CD molecules and Cur-CD complexes Eq. (2):

$$\mathrm{A_{total}} = \varepsilon_{\mathrm{Cur}} \times \mathrm{b} \times [\mathrm{Cur}]_{\mathrm{free}} + \varepsilon_{\mathrm{CD}} \times \mathrm{b} \times [\mathrm{CD}]_{\mathrm{free}} + \varepsilon_{\mathrm{complex}} \times \mathrm{b} \times [\mathrm{CD/Cur}] \quad (2)$$

$\mathrm{A_{total}}$ is the total absorbance of Cur in the selected solvent, $\varepsilon_{\mathrm{Cur}}$, $\varepsilon_{\mathrm{CD}}$ and $\varepsilon_{\mathrm{complex}}$ are the molar absorbtivity of free Cur molecules, free CD molecules and Cur-CD complexes respectively, b is cell path length and $[\mathrm{Cur}]_{\mathrm{free}}$, $[\mathrm{CD}]_{\mathrm{free}}$ and [CD/Cur] are the concentrations of uncomplexed Cur, uncomplexed CDs and Cur-CD complexes, respectively. When the cell path length is 1 cm, the absorbance of uncomplexed CDs at wavelengths around 420–434 nm is very low ($\varepsilon_{\mathrm{CD}} \approx 0$) and the equation can be simplified:

$$\mathrm{A_{total}} = \varepsilon_{\mathrm{Cur}} \times [\mathrm{Cur}]_{\mathrm{free}} + \varepsilon_{\mathrm{complex}} \times [\mathrm{CD/Cur}] \quad (3)$$

The total amount of Cur is expressed as:

$$[\mathrm{Cur}]_{\mathrm{total}} = [\mathrm{Cur_{free}}] + [\mathrm{Cur/CD}] \quad (4)$$

Combining Eq. (3) and Eq. (4) gives:

$$\mathrm{A_{total}} = \varepsilon_{\mathrm{Cur}} \times [\mathrm{Cur}]_{\mathrm{total}} + \Delta\varepsilon_{\mathrm{complex}} \times [\mathrm{CD/Cur}] \quad (5)$$

where

$$\Delta\varepsilon_{\mathrm{complex}} = \varepsilon_{\mathrm{complex}} - \varepsilon_{\mathrm{Cur}} \quad (6)$$

In case of a 1 : 1 complex, where A_0 is the Cur absorbance without CD and A is the Cur absorbance at x% CD:

$$\Delta\mathrm{A} = \mathrm{A} - \mathrm{A_0} = \Delta\varepsilon \times [\mathrm{CD/Cur}] = \Delta\varepsilon \times \mathrm{K_{(1:1)}} \times [\mathrm{Cur_{free}}] \times [\mathrm{CD_{free}}] \quad (7)$$

$$[\mathrm{Cur}]_{\mathrm{total}} = [\mathrm{Cur}]_{\mathrm{free}} + [\mathrm{Cur/CD}] = [\mathrm{Cur}]_{\mathrm{free}} + \mathrm{K_{1:1}} \times [\mathrm{Cur_{free}}] \times [\mathrm{CD_{free}}] \quad (8)$$

Which can be rearranged to:

$$[\mathrm{Cur}]_{\mathrm{free}} = [\mathrm{Cur}]_{\mathrm{total}} - \mathrm{K_{1:1}} \times [\mathrm{Cur_{free}}] \times [\mathrm{CD_{free}}] = [\mathrm{Cur}]_{\mathrm{total}}/(1 + \mathrm{K}(1:1) \times [\mathrm{CD_{free}}])$$

$$\Delta\mathrm{A} = \Delta\varepsilon \times \mathrm{K_{(1:1)}} \times [\mathrm{Cur_{free}}] \times [\mathrm{CD_{free}}] = \Delta\varepsilon \times \mathrm{K_{1:1}} \times [\mathrm{CD_{free}}] \times [\mathrm{Cur}]_{\mathrm{total}}/(1 + \mathrm{K_{(1:1)}} \times [\mathrm{CD_{free}}]) = \Delta\varepsilon \times [\mathrm{Cur}]_{\mathrm{total}} \times (\mathrm{K_{1:1}} \times [\mathrm{CD_{free}}])/(1 + \mathrm{K_{(1:1)}} \times [\mathrm{CD_{free}}]) \quad (9)$$

The experimental data are fitted into Eq. (9) using the Kaleidagraph (4.0) software and values for the association constants were determined through iterations.

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