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Cyclodextrins: Efficient biocompatible solubilizing excipients for bromhexine liquid and semi-solid drug delivery systems

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ABSTRACT

Bromhexine hydrochloride (bromhexine) is a mucolytic agent with very low aqueous solubility. However, with addition of cyclodextrins (CD) to the formulation, this disadvantage may be limited and therapeutic doses of bromhexine in solution can be achieved.

The interaction of bromhexine with α-, β-, γ- and sulfobutylether (SBE)-β-CD, respectively, was elucidated by means of phase solubility diagrams and calorimetric analysis. The complexes were further characterized by size, and the effect of the CD concentrations used was evaluated in a viability assay.

From phase solubility diagrams with α-, β-, γ- and SBE-β-CD and bromhexine, it was determined that the solubility of bromhexine significantly increased with addition of CDs, showing an A_L type solubility curve for bromhexine/α- and β-CD, and an A_N type for bromhexine/γ- and SBE-β-CD. The highest soluble concentrations of bromhexine were achieved with α- and SBE-β-CD, i.e. when using a 100 mM α- or SBE-β-CD solution, 4 and 5.5 times more bromhexine was solubilized, respectively, compared to pure aqueous solubilization of bromhexine. The apparent association constants determined from the phase solubility studies showed very low values of 34, 17, 8 and 156 M^{-1} for bromhexine/α-, β-, γ- and SBE-β-CD, respectively, as compared to the association constants determined by ITC which exhibited values of 89, 307 and 1680 M^{-1} for bromhexine/α-, β- and SBE-β-CD, respectively. The formation of aggregates aided solubilization of bromhexine in the phase solubility studies explaining the difference in the association constants between the two methods. Due to very low signal to noise ratio, no information was extracted for bromhexine/γ-CD solutions from the ITC measurements. The effect on cellular viability of the CDs ranked β- > α- > SBE-β- > γ-CD. In conclusion, the results altogether demonstrated that SBE-β-CD is the most suitable CD for future drug delivery systems from the aspect of high amounts of solubilized bromhexine and high safety of the SBE-β-CD.

1. Introduction

Bromhexine is a mucolytic agent used in the treatment of respiratory disorders associated with production of viscous and/or excessive mucus (Martindale, The Extra Pharmacopoeia, 31st ed.).

The production of therapeutically efficient liquid or semi-solid pharmaceutical drug delivery systems is challenged by the limited solubility of bromhexine, which also is likely to limit bromhexine release from solid dosage forms. Furthermore, very low bromhexine solubility might be a rate limiting step for achieving efficient therapeutical plasma concentrations in systemic drug delivery. Use of slightly more soluble salts as well as addition of appropriate solubilizing excipients (e.g. polyethylene glycol, polyvinylpyrrolidone, hydroxypropylmethylcellulose, carboxymethylcellulose, and cyclodextrins) to the drug delivery system will be advantageous for obtaining higher solubility of bromhexine in aqueous formulations (Chaubal and Katdare, 2006).

Cyclodextrins (CDs) are well-known excipients used in the pharmaceutical industry to enhance the solubility of poorly soluble APIs (active pharmaceutical ingredients) (Chaubal and Katdare, 2006; Uekama et al., 1998; Hedges, 1998; Brewster and Loftsson, 2007; Del Valle, 2003; Stella and He, 2008; Loftsson and Brewster, 1996). The native CDs are cyclic oligosaccharides composed of 6 (α-CD), 7 (β-CD) and 8 (γ-CD) glucopyranose units, respectively, with the outer surface of the CD being hydrophilic and the inner cavity slightly hydrophobic. The anionic sulfobutylether (SBE) substituted β-CD may be a superior excipient as compared to the native CDs due to its predicted higher biocompatibility (Stella and He, 2008; Irie and Uekama, 1997) while maintaining high aqueous solubility and complexation properties (Stella and He, 2008). The most interesting feature of the CDs is their ability to form inclusion complexes with various guest molecules. By inclusion complex formation, CDs

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most often improve the stability, solubility, bioavailability, as well as decrease side effects and mask any unpleasant taste of the APIs, without altering their activity (Uekama et al., 1998, Hedges, 1998; Del Valle, 2003; Singh et al., 2002; Loftsson and Duchene, 2007). When the aqueous solubility and thus dissolution of the drug after, e.g. oral delivery are rate limiting factors, CDs may be the appropriate excipient to solve the problem. Such a drug is bromhexine, which exhibit a very low intrinsic aqueous solubility (11 mM at 20 ± 1 °C) (Schubert and Müller-Goyman, 2001).

Previously, a study of bromhexine and β-CD complex was reported, in which phase solubility diagrams were employed for determination of the association constant and the stoichiometry of the complex (Higuchi and Connors, 1965). The present study is carried out to gain detailed information on the effect of both α-, β-, γ- and SBE-β-CD on bromhexine solubilization and to attain detailed information about the bromhexine complex forming properties of the appropriate CDs. UV spectroscopy, isothermal titration calorimetry (ITC) and dynamic light scattering (DLS) are employed for determining the association constants, the stoichiometry, the thermodynamic parameters of the complexes, their solubilization properties and their physical appearance in terms of size. Further, the biocompatibility property of the CDs is determined.

This study thus provides essential information important for the direction of any further development of bromhexine pharmaceutical dosage forms containing CDs.

2. Materials and methods

2.1. Materials

Bromhexine hydrochloride used for all experiments (PhEur 5th ed.) was obtained from Unikem A/S, Copenhagen, Denmark. The CDs (α-, β- and γ-CD) were purchased from Wacker Chemie AG, (Burghausen, Germany), and SBE-β-CD (6.6 degree of substitution) was from CyDex Pharmaceuticals, Inc., Lenexa, KS, USA. Hanks balanced salt solution (HBSS) was obtained from Invitrogen A/S (Taastrup, Denmark), N-(2-hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid) sodium salt (HEPES) and phenazine methosulfate (PMS) was from Sigma–Aldrich Denmark A/S (Copenhagen, Denmark). MTS (3-(4,5-dimethylthiazol-2-yl)-5-(carboxymethoxyphenyl)2-(4-sulfenyl)-2H-tetrazolium) was from Promega Corp. (Madison, WI, USA). All other chemicals were obtained from Sigma–Aldrich Denmark A/S (Copenhagen, Denmark) and used as received. For cell culturing, the medium consisted of complete Eagle's minimum essential medium (EMEM) containing 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate (all from Invitrogen A/S, Taastrup, Denmark), and 10% (v/v) fetal bovine serum (FBS) (Fisher Scientific Biotech Line, Slangerup, Denmark).

2.2. UV spectrophotometry

For the UV spectroscopic measurements, a Varian Cary 50 Bio UV–VIS Spectrophotometer (Varian Medical Systems Netherland BV, Houten, The Netherlands) was used with 1 cm quartz cell. From the prepared bromhexine stock solution in demineralised water, standard solutions were prepared by further dilution with demineralised water to a range of 0.005–0.1 mM (0.005, 0.01, 0.02, 0.04, 0.06, 0.08 and 0.1) bromhexine. Both absorption maxima at 245 nm and 310 nm present for bromhexine, demonstrated very good concentration–absorption linearity (0.9995 and 0.9993). The UV experiments were performed at room temperature (21 °C).

2.2.1. Phase solubility

The phase solubility method (Higuchi and Connors, 1965) was used for evaluation of the CDs ability to form complexes with bromhexine for interpretation of solubility, stoichiometry and determination of the association constants. Stock solutions of α-, β-, γ- and SBE-β-CD were prepared in demineralised water and various amounts of the respective CD solution were added to test tubes containing an excess of bromhexine. This resulted in solutions with solubilized amounts of bromhexine way above its intrinsic aqueous solubility using α-, γ- and SBE-β-CD concentrations in the range of 10–140 mM (10, 20, 40, 60, 80, 100, 120, and 140), whereas for β-CD, the concentration ranged between 2 and 20 mM (2, 4, 6, 8, 10, 12, 14, 16, 18 and 20) due to its limited aqueous solubility. The pH value of the solutions was not controlled. The solutions were placed on a shaking table for 6 days at a temperature of 30 °C. The content was filtered using 0.45 μm syringe filters, the samples were subsequently diluted with demineralised water and the amount of solubilized bromhexine was determined using UV spectrophotometry by measuring the absorption at the isosbestic point present at 246 nm for the solution containing SBE-β-CD, 251 nm for β-CD and 250 nm and 249 nm for the solutions containing α- and γ-CD, respectively. The experiments were performed in duplicates, and the average solubility is reported.

2.3. Particles sizes

Dynamic light scattering (DLS) measurements were employed for revealing if other mechanisms than complexation are involved in increase of bromhexine aqueous solubility. The DLS experiments were performed on readymade 5 mM and 10 mM of pure CD solutions (α-, β-, γ- and SBE-β-CD), bromhexine/CD (α-, β-, γ- and SBE-β-CD) solutions with concentration of 5 mM bromhexine and 5 mM and 10 mM of CD, respectively, and on 5 mM pure bromhexine solution. The samples were run on 25 °C on Zetasizer nano ZS (Malvern Instruments Ltd., Malvern, UK) DLS instrument. Before each DLS scan, the samples were stabilized for 1 min in the DLC instrument for optimisation of the measurement temperature.

2.4. Calorimetry

Calorimetric titrations were carried out on a VP-ITC microcalorimeter (MicroCal Inc., Northampton, MA, USA) controlled by MicroCal's VP viewer software (ver. 5.0, MicroCal Software, Inc., Northampton, MA, USA). The titration experiment consisted of 30 successive injections of a 10 μL 10 mM solution of the respective CD to a reaction cell loaded with 1.4095 mL of 2 mM bromhexine in demineralised water, with injection intervals of 250 s. The first injection of 1 μL was discarded to eliminate the error that may be present due to diffusion of material from the syringe into the calorimetric cell. All experiments were conducted at 30 °C. The titration of CD solutions into the bromhexine solution resulted in heat signals, which were integrated and the data fitted by non-linear regression analysis (Origin® SR2 v 7.0383 software (OriginLab Corporation, Northampton, USA) with the Microcal LLC ITC Add-On). Applying the "one set of sites model", based on the Wiseman isotherm (Wiseman et al., 1989) as shown in Eq. (1), the association constant (K_a) and the molar enthalpy (ΔH) were determined as varying parameters. The dQ is defined as the heat flow, $[\text{bromhexine}]_{\text{tot}}$ is the total concentration of bromhexine, both in free and complexed form, V_0 is the volume of the calorimetric cell, [CD] is the concentration of CD both in free and complexed form, and K_a is the association constant. Using Origin v 7.0383 software, the number of binding sites (n) was set to 1 for systems of relatively low "Wiseman c" parameter, which is the product of the receptor concentration and the binding constant (for $c < 5$), as

recommended by Turnbull and Daranas (2003):

$$\frac{dQ}{d[\text{bromhexine}]_{\text{tot}}} = \Delta H^{\circ} V_0 \left[\frac{1}{2} + \frac{1 - [\text{bromhexine}]_{\text{tot}} - (1/[\text{CD}])K_a}{2\sqrt{(1 + [\text{bromhexine}]_{\text{tot}} - (1/[\text{CD}])K_a)^2 - 4[\text{bromhexine}]_{\text{tot}}}}\right] \quad (1)$$

The dilution heats of both bromhexine and CDs were negligible, which was confirmed by addition of demineralised water into a solution of bromhexine, as well as by injection of solutions of the respective CD to pure water.

2.5. Viability

To evaluate the effect of the CDs on the cellular viability, an MTS/PMS assay was performed essentially as described previously (Nielsen et al., 2004) using wild type HeLa cells (ATCC, Manassas, VA, USA) cultured for 24 h in flat-bottomed 96-well plates (9000 cells/well) (Corning, Inc., New York, NY, USA) under standard conditions (5% CO_2–95% O_2 at 37 °C). The CD samples in HBSS were buffered to pH 7.4 by 20 mM HEPES; the concentration ranges were 1–15 mM for β-CD; 5–100 mM for α-CD; 10–100 mM for γ-CD; 50–200 mM for SBE-β-CD. 0.2% (w/v) sodium lauryl sulfate in HBSS served as the positive control. Three replicates were carried out and the results are presented as mean ± standard deviation (SD). The haemolysis of erythrocytes by CDs dissolved in 0.9% (w/v) sodium chloride were determined by incubation of 300 μL horse erythrocyte suspension with 1.5 mL of the test solution rotated end-over-end for 30 min at room temperature. As a positive control, 0.15% (w/v) sodium chloride was used. Upon centrifugation of the samples, the UV absorption of the supernatant was measured at a wavelength of 543 nm. For all CDs tested, the lowest concentration was 20 μM, and ranged to 15 mM for β-CD, 110 mM for α-CD, 120 mM for γ-CD and 140 mM for SBE-β-CD.

The osmolality of CDs in 0.9% (w/v) sodium chloride was determined for various concentrations of the respective CDs using an Osmomat 030-D (Gonotec, Berlin, Germany). The samples were determined in triplicates for α-CD (20–100 mM), β-CD (2–15 mM), γ-CD (40–100 mM) and SBE-β-CD (110–200 mM), and pure water and 0.9% (w/v) sodium chloride were used as controls.

3. Results and discussion

3.1. The solubility pattern of bromhexine is dependent on the type of CD used

Phase solubility diagrams of bromhexine and α-, β-, γ- and SBE-β-CD, respectively, are presented in Fig. 1. The solubility of bromhexine in the absence of CD was found to be 11.5 mM at 30 °C. As it can be seen from Fig. 1a–d, the water solubility of bromhexine linearly increased with gradual addition of the respective CD. In the case of bromhexine/α- and β-CD, respectively, the solubility of bromhexine increased linearly up to the point of solubility saturation of the CD (Fig. 1a and b) (A_L type of phase solubility diagrams). With addition of β-CD portions (in solid form) that are above its saturation point (16 mM), an increase in bromhexine solubilization was observed. This observation provides information that bromhexine increases the β-CD solubility as well. The phase solubility profiles for bromhexine/γ- and SBE-β-CD, respectively, are classified as A_N type with linear increase of the bromhexine solubility followed by a decrease in the slope until a point where the concentration of the solubilized bromhexine begins to decrease. For γ-CD, this point is approximately 100 mM, but for SBE-β-CD

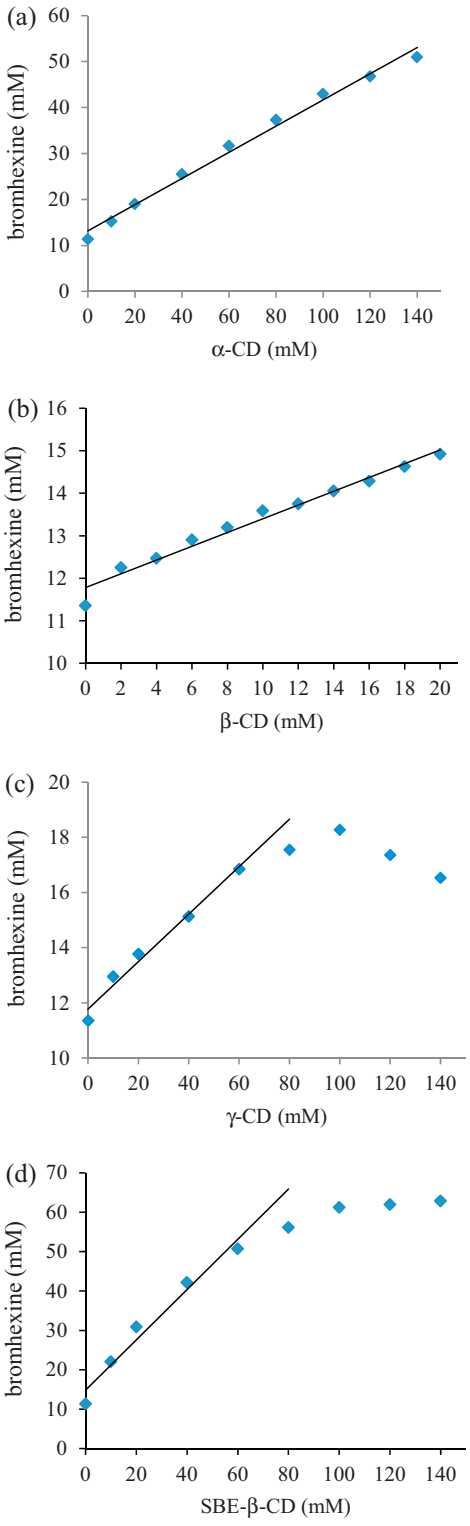

Fig. 1. Phase solubility diagrams for bromhexine: (a) α-CD, (b) β-CD, (c) γ-CD and (d) SBE-β-CD.

the maximum point is not reached within the SBE-β-CD aqueous saturation range. Overall, the highest bromhexine concentrations were obtained with addition of SBE-β-CD as compared to the other CDs investigated. For a 100 mM SBE-β-CD solution, the concentration of solubilized bromhexine was 5.5 times (61 mM) higher than without CD. Beside the inclusion complex formation, it is believed that this effect could partially be attributed to the ionic interaction between the anionic SBE-β-CD and positively charged

Table 1
Average diameter of particles fractions (P1, P2 and P3) and their percentage in readymade solutions containing 5 mM bromhexine and 5 and 10 mM CD (α-, β-, γ- and SBE-β-CD, respectively). The samples were run on 25 °C.

	P1		P2		P3	
	Diameter (nm)	Intensity (%)	Diameter (nm)	Intensity (%)	Diameter (nm)	Intensity (%)
α-CD 5 mM + bromhexine 5 mM	183	75.67	1.34	24.02	–	–
α-CD 10 mM + bromhexine 5 mM	208	74.25	1.36	25.75	–	–
β-CD 5 mM + bromhexine 5 mM	183	73.27	1.48	26.72	–	–
β-CD 10 mM + bromhexine 5 mM	139	68.27	1.23	31.72	–	–
γ-CD 5 mM + bromhexine 5 mM	253	85.55	1.69	9.15	58	5.3
γ-CD 10 mM + bromhexine 5 mM	468	95.32	1.58	1.45	55	3.22
SBE-β-CD 5 mM + bromhexine 5 mM	140	68.9	1.32	31.1	–	–
SBE-β-CD 10 mM + bromhexine 5 mM	127	75.82	1.00	24.17	–	–

bromhexine. Another contribution to the solubilization of bromhexine could be by CD aggregation. It has been reported that α-, β- and γ-CD solutions at concentrations of about 3 mM and above tend to form aggregates (Bonini et al., 2006; Coleman et al., 1992) and that different kinds of interaction between the CD and the API might coexist, such as non-inclusional association (Gabelica et al., 2002) or even aggregation of complexes and formation of micelle-like structures with a core consisting of hydrophobic guest surrounded by CDs (Mele et al., 1998).

Beside increase in the solubilized bromhexine with addition of excess of CD, the phase solubility diagrams display that with gradual addition of CD, the ratio of interaction CD/bromhexine increases. This suggests association of bromhexine/CD complexes with free CD molecules and formation of complex aggregates.

From the slope of the linear fit, the apparent association constant was determined for bromhexine and α-, β-, γ- and SBE-β-CD complexes. From the phase solubility diagrams, at the linear increase of bromhexine solubility, the complex formation is assumed to be 1:1 and the isotherm according to Higuchi and Connors was applied for determining the apparent association constant (Higuchi and Connors, 1965) $K_{1:1}$:

$$K_{1:1} = \frac{\text{Slope}}{[\text{bromhexine}]_0(1 - \text{Slope})} \quad (2)$$

where $[\text{bromhexine}]_0$ was found to be the intersection at $[\text{CD}]_t = 0$.

The apparent association constant for bromhexine/α-, β- and SBE-β-CD was found to be: 34 M^{-1}, 17 M^{-1}, 8 M^{-1} and 156 M^{-1}, respectively.

The complexation efficiency (CE) was calculated as a product or the intrinsic solubility (S_0) and the association constant $K_{1:1}$. The values for the CE from the phase solubility studies for bromhexine/α-, β-, γ- and SBE-β-CD are 0.38, 0.19, 0.09 and 3.09, respectively.

3.2. Self-association and formation of aggregates

The assumption of self-association of CD molecules was confirmed by the DLS experiments, which detected presence of particles with different sizes in both bromhexine/CD (Table 1) and pure CD (α-, β-, γ- and SBE-β-CD) solutions (Table 2). At the experimental conditions used at least two particles fractions with polydispersal distribution of particle sizes were observed. At the pure CD solutions three fractions of particles are present, whereas at the bromhexine/CD solutions only two fractions are present. Exceptions from the latter are bromhexine/γ-CD solutions, where three particles fractions were detected. The particles fraction (P2) with the smallest size corresponds to the diameters of the CD monomers (Atwood et al., 1996) where the larger particles size fractions (P1 and P3) belong to the formed aggregates. At both bromhexine CD/solutions (Table 1) and pure CD (Table 2) the size of the particles increased with increase of the CD concentration, displaying concentration dependency of the formed aggregates. Furthermore, the sizes of particles of bromhexine/CD solutions were higher compared to the pure CD solutions, indicating influence of bromhexine to the aggregation of the CDs.

In the case of bromhexine/γ-CD solutions (Table 1), aggregates formation was more resilient. This unusual behavior explains the A_N shape of the bromhexine/γ-CD phase solubility diagram, in which the high tendency for aggregates formation continues to a degree where the aggregates become so large that they start to precipitate. For bromhexine/SBE-β-CD and pure SBE-β-CD solutions it was observed the lowest size of the particles. The repulsion of the charges of the sulfonate groups is probably the reason for keeping away SBE-β-CD molecules from each other, this way decreasing the aggregates formation (Zia et al., 2001).

The data presented in Tables 1 and 2 for the particles diameter is an average of four measurements (RSD = 20%). Furthermore, at each measurement, the size of the detected particles is an average from particles with large size distribution, which is displayed by the high value (0.599) of the average polydispersity index (PDI). Large value of the PDI complies with the large value of the RSD and it is due to inconsistency of the formed aggregates. It was observed that shear force applied by shaking and residence time in the DLS cuvettes prior to the measurement has a substantial influence on the stability of the aggregates and thus the particle sizes. This shows

Table 2
Average diameter of particles fractions (P1, P2 and P3) and their percentage in readymade 5 and 10 mM pure CD (α-, β-, γ- and SBE-β-CD, respectively) solutions and 5 mM bromhexine solution. The samples were run on 25 °C.

	P1		P2		P3	
	Diameter (nm)	Intensity (%)	Diameter (nm)	Intensity (%)	Diameter (nm)	Intensity (%)
Bromhexine 5 mM	156	98.57	0.83	1.42	–	
α-CD 5 mM	242	50.37	1.45	35.8	66	12.47
α-CD 10 mM	369	75.9	1.42	13.8	79	10.3
β-CD 5 mM	165	81	1.36	17.2	47	1.77
β-CD 10 mM	232	80.5	1.58	11.1	90	8.37
γ-CD 5 mM	195	78.02	1.75	7.2	57	14.77
γ-CD 10 mM	238	86.35	–		58	13.65
SBE-β-CD 5 mM	173	80.95	0.80	15.85	1.1	3.2
SBE-β-CD 10 mM	172	70.65	0.87	28.75	–	

that the aggregates formed are only weakly stabilized, immensely fragile and thus the choice of method and handling (e.g. the application of mechanical forces and/or filtration) for the study of these aggregates will greatly influence the results.

The DLS measurements furthermore revealed that the fractions with the largest particles sizes (P1) are the most abundant (Tables 1 and 2). The fraction of particles corresponding to the CDs diameter (P2), as well as the P3 fraction, constitute the smallest portion (Tables 1 and 2) or were even absent for bromhexine/CD solutions (P3) (Table 1). The proportion of each particles fraction varied with respect to the CD concentration and whether there is bromhexine present in the CD solutions. The percentage of P1 fraction increased with the presence of bromhexine. With exception of pure SBE-β-CD solutions (Table 2), in all other solutions (Tables 1 and 2) the amount of the fraction with the highest particle sizes (P1) was either steady or increased with respect to the concentration increase, indicating proportional CD concentration dependency of the amount of formed CD aggregates.

The DLS experiments of pure bromhexine solution revealed that even bromhexine molecule tend to form aggregates. At a concentration of 5 mM, 98.5% of the bromhexine is aggregated and with average particle size of 156 nm. This is typical for the molecules with low aqueous solubility, such as bromhexine (Mele et al., 1998).

It is assumed that beside the complexation, the increased solubilization of the bromhexine molecule is mediated by formation of complex aggregates composed of inclusion complexes associated with CDs or non-inclusional solubilization where the hydrophobicity of the bromhexine molecule triggers formation of micelle-like aggregates with core composed of bromhexine surrounded by molecules of CD which enables bromhexine aqueous solubilization (Mele et al., 1998). The former is explained by formation of aggregates for the bromhexine itself (Table 2).

3.3. Calorimetric studies

By ITC, not only information about the association constant (K_a) and the two important thermodynamic parameters of the complex formation such as standard formation enthalpy (ΔH°) and the entropic effect ($T\Delta S^\circ$) are provided, but also direct access to the standard changes of the Gibbs free energy (ΔG°) can be determined. The results from the non-linear regression fitting of the ITC data are presented in Table 1.

High negative values for standard formation enthalpies indicate that the formation of complexes between bromhexine and α-, β- or SBE-β-CD is a strong exothermic process. This exothermic process is due to release of high energy water from the CD cavity into the bulk water solution and from the hydrophobic and van der Waals interaction between the guest and the host molecules (Rekharsky and Inoue, 1998). From Table 3 it can be observed that the bromhexine/α-CD complex formation display the highest negative value for ΔH°. It would thus be expected that bromhexine/α-CD complex exhibits the strongest interaction, but this is not the case due to a highly negative value for the entropy effect, which is very unfavorable for complex formation. On the other hand, the entropy effects for bromhexine/β- and SBE-β-CD complex formation are very favorable and contribute largely to the negative values of standard Gibbs energy change. One very important process in the complex formation that additionally contributes to the strong binding abilities of the bromhexine/SBE-β-CD complex is the interaction between the charges of the sulfonate groups of the SBE-β-CD and the positive charges of the bromhexine molecule, thus enabling additional interaction between the host and the guest. Previous study has demonstrated higher binding capacity of charged CDs (SBE-β-CD) when forming complexes with positively charged molecules such as bromhexine, compared to non-charged CDs (Zia et al., 2001). The association constant values determined by ITC for bromhexine and the CDs are 89, 307 and 1680 M^{-1} for bromhexine/α-, β- and SBE-β-CD, respectively. The quality of the ITC signals for bromhexine/γ-CD titration was too low to produce fits of an acceptable quality and thus extract any data from the complexation process (Fig. 2).

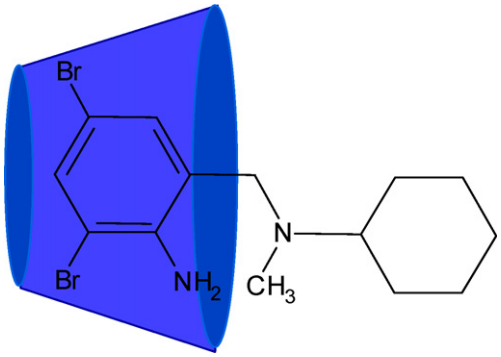

Fig. 2. Schematic description of the complex formed between bromhexine and the CDs.

Theoretically, the association constant determined by different methods should be independent of the method applied. This is the case when the components behave as they are in an ideal solution, i.e. there with no interaction between the individual complexes. The difference in the association constants obtained by the phase solubility and the ITC methods is believed to be due to the different CD concentrations used. When CDs are used in high concentrations like in the phase solubility studies, formation of higher order complex aggregates is likely, leading to non-inclusion solubilization and solubilization by aggregation of the guest molecule, and this might influence the precision of the method (Loftsson et al., 2004). Thus the ITC methodology seems more accurate for studies at a molecular level, due to the low concentrations used. Another influence could be the difference in the pH of the solutions used, since the bromhexine solutions used were not buffered. Different concentrations of bromhexine solutions were used in both methods and this could result in different pH values for the solutions and subsequent difference in the association constant values.

As for the phase solubility studies, the CE calculated based on the ITC K_a results for bromhexine/α-, β- and SBE-β-CD is calculated to be: 1.01, 3.48 and 19.06.

3.4. Effect on cellular viability depends on the type of CD

The results of the MTS/PMS assay shows that the effect on cell viability after exposure to the tested CDs are decreasing in the order of β-> α-> SBE-β-> γ-CD. For α-, β-, γ- and SBE-β-CD, the IC_{50}

Table 3
Association constants and the thermodynamic parameters of bromhexine and α-, β- and SBE-β-CD complexation, obtained by ITC.

Complex	ΔH° (kJ·mol^{-1})	$T\Delta S^\circ$ (kJ·mol^{-1})	ΔG° (kJ·mol^{-1})	K_a (mol^{-1})
Bromhexine				
α-CD	−8554 ± 248	−5851	−2703	89 ± 3
β-CD	−220 ± 10	3244	−3024	307 ± 25
SBE-β-CD	−2907 ± 789	1567	−4474	1680 ± 458

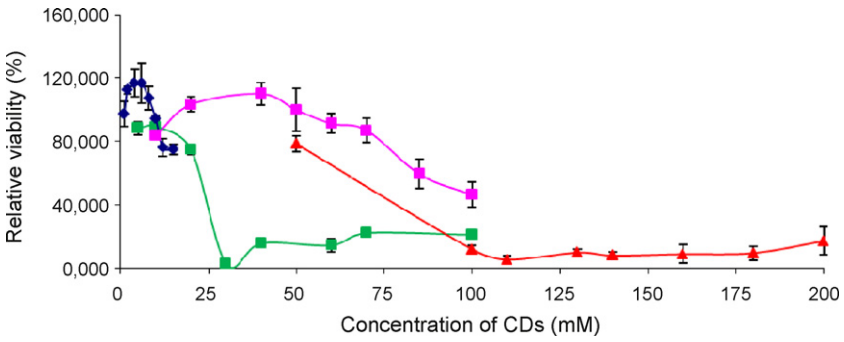

Fig. 3. The relative viability of proliferating HeLa cells after exposure to various concentrations of the CDs (α-CD (■), β-CD (◆), γ-CD (■) and SBE-β-CD (▲)) in buffer. Mean ± SD values are presented ($n = 3$).

values for the present experimental setup is estimated from the curves, and found to be <15 mM, 26 mM, 70 mM and 95 mM for β-, α-, SBE-β- and γ-CD, respectively. Overall, our results support the ranking partly seen in previous studies (Brewster and Loftsson, 2007; Leroy-Lechat et al., 1994; Javrinen et al., 2009). The results from the CDs cytotoxicity are presented in Fig. 3.

The haemolysis data obtained showed that haemolysis started to occur for concentrations above 8 mM for β-CD, 20 mM for α-CD, 50 mM for γ-CD and 110 mM for SBE-β-CD, which to some extend is consistent with no or little effect on the cellular viability, except for the SBE-β-CD, which seems to be cytotoxic to the proliferating cells even at a concentration of 100 mM. However, it was observed that the osmolality of the SBE-β-CD in solution was significantly higher than that of the native CDs at the above mentioned levels; the, i.e. inducing hypertonicity to the delivery vehicle may have a larger effect on the cellular viability than on the haemolysis of erythrocytes.

4. Conclusion

Phase solubility studies demonstrated an increase in the bromhexine solubilization with gradual addition of the respective CD. The highest impact on bromhexine solubility was observed with SBE-β-CD as 61 mM of bromhexine was solubilized by 100 mM of SBE-β-CD. This is partially explained by inclusion complex formation, as a result of the highest association constants as compared to the other CDs tested, and partially due to the strong ionic interaction between the charges of the sulfobutylether groups of SBE-β-CD and the charges of the bromhexine molecule. The DLS experiments revealed that the association of bromhexine/CD complexes with CD molecules and formation of aggregates and micelle-like structures might as well contribute to the increase in bromhexine aqueous solubility. Furthermore, it was revealed that the size and the amount of the formed aggregates are proportional to the CD concentration. Though all four investigated CDs may be suitable complexation candidates, SBE-β-CD and α-CD are the most appropriate for increasing the solubilization properties of bromhexine. However, SBE-β-CD may be much more biocompatible as it can be applied to cells at much higher concentrations without affecting the cell viability. Further, α-CD induced hemolysis at 5-fold lower concentration than SBE-β-CD, and SBE-β-CD would thus be preferred over α-CD. Overall, these results provide essential information for future development of different bromhexine pharmaceutical dosage forms aiming at enhanced solubilization and/or dissolution of bromhexine.

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