

Article

2-Hydroxypropyl- β -Cyclodextrin Aggregates: Identification and Development of Analytical Techniques

André Rodrigues Sá Couto, Alexey Ryzhakov and Thorsteinn Loftsson * 

Faculty of Pharmaceutical Sciences, University of Iceland, Hofsvallagata 53, IS-107 Reykjavik, Iceland; ars70@hi.is (A.R.S.C.); alexey@hi.is (A.R.)

* Correspondence: thorstlo@hi.is; Tel.: +354-525-4464

Received: 13 September 2018; Accepted: 11 October 2018; Published: 13 October 2018



Abstract: It is extremely important for pharmaceutical formulators to have analytical methodology that provides efficient detection and quantification of HP β CD aggregates. Five different methods were then evaluated for their potential to detect these aggregates and to determine critical aggregation concentration (cac): osmometry, viscometry, tensiometry, dynamic light scattering (DLS), and permeability studies. Overall, tensiometry was an inadequate method with which to study HP β CD aggregation, since the addition of HP β CD to water resulted in only minor changes in surface tension. Osmolality and viscosity studies have shown that for HP β CD, solute–solvent interactions are the main contributors for the observed deviation from ideality. These deviations might be related to the presence of aggregates. The DLS method proved to be an effective method with which to detect HP β CD aggregates and estimate their hydrodynamic diameter, although it presented some limitations concerning their quantification. In terms of the assessed methods, permeation studies were shown to be the best to study HP β CD aggregation phenomena, since they were the only method where the detection of aggregates and the determination of apparent cac values was possible. Also, it was the least invasive for the HP β CD samples and the method that provided more conclusive data. Results suggested that HP β CD, as expected, has less tendency to form aggregates than β CD.

Keywords: 2-hydroxypropyl- β -cyclodextrin; aggregation; critical aggregation concentration; permeation; nanoparticles

1. Introduction

It has been well documented that cyclodextrins (CDs), especially the natural ones, can self-assemble in aqueous solutions to form CD aggregates that are kept together by weak intermolecular forces [1–4].

Although the natural β -cyclodextrin (β CD) is the most available and most affordable CD [5], it has very low solubility in water (i.e., only 18.5 mg/mL at 25 °C) [6] and a high tendency to self-aggregate. Formation of β CD aggregates and various methods for their detection is described in several publications. For instance, Bonini et al. used dynamic/static light scattering and cryo-TEM techniques to detect β CD aggregates in aqueous solutions at β CD concentrations as low as 2–3 mM [2]. De Sousa et al., using particle size measuring techniques, reported similar but somewhat lower critical aggregation concentration (cac) values for β CD or approximately 1–2 mM [7]. Mixcoha and co-workers used computational molecular dynamic simulations to show the presence of β CD aggregates in aqueous solutions [8], and Coleman et al. showed that the aggregate size decreased and solubility of β CD increased with increasing pH of the aqueous media or when chaotropic solutes were added to the media [9]. Due to the low solubility and aggregation propensity of β CD and the other natural CDs (i.e., α CD and γ CD), several water soluble derivatives of these CDs were synthesized [10]. Random

substitution of the hydroxy-moieties of the natural CDs decreased their tendency to crystallize and disrupted the formation of intramolecular hydrogen bonds. The substitution with consequent changes in the physiochemical properties led to considerable solubility improvements [11]. One of the most relevant derivatives for the pharmaceutical field is 2-hydroxypropyl- β -cyclodextrin (HP β CD) [12]. Despite the fact that in theory HP β CD should be less prone to aggregate than β CD, some unexpected phenomena were observed with this β CD derivative when included in aqueous pharmaceutical formulations. Haüsler et al. described the presence of aggregates in aqueous HP β CD solutions and observed changes in CD aggregation profile when urea was added to the media [13]. Messner et al. verified by permeation studies and HPLC drug quantification formation HP β CD aggregates that consisted of drug/HP β CD inclusion complexes [4,14]. However, due to the physiochemical properties of CDs (they do not possess chromophore), they were unable to detect aggregates in pure aqueous HP β CD solutions. In order to apply the permeation method to detect HP β CD aggregates, a new type of HPLC detector had to be used. Here, a charged aerosol HPLC detector is used to detect HP β CD in aqueous media.

The objective of the present work is to develop a suitable/reliable analytical method to study the aggregation phenomena in pure aqueous HP β CD solutions and to determine the *cac* value.

2. Results and Discussion

2.1. Validation of UHPLC Method

Due to the lack of chromophores or fluorophores groups, detection of CDs by HPLC using conventional detectors (UV/Visible detectors or fluorometric detectors) was challenging. Recent advances in HPLC detector technologies have overcome this limitation and, nowadays, detectors such as charged aerosol detector (CAD), evaporative light scattering detector (ELSD), and refractive index (RI), among others, are used to detect CDs in different kind of media [15]. A CAD detector was selected for the HP β CD detection, as it is more sensitive than RI and ELSDs detectors, even though CAD has a similar detection mechanism to ELSD.

The linearity between the peak area and the individual HP β CD concentrations on log-log scale was assessed. The tested method proved to be linear for HP β CD over the studied concentration range (0.0008–1% *w/v*). The obtained results also showed a good correlation factor of 0.9993. Using Equations (5) and (6) LOD and LOQ were calculated and found to be 0.00014% and 0.00042% (*w/v*), respectively. To determine these two values, three different concentrations of HP β CD were prepared, namely, 0.5%, 2.5%, and 5% *w/v*. All assessed CD concentration had to be diluted with water prior to injection. The percentages of recovery (accuracy) and intraday precision (repeatability) of the selected CD concentrations are shown in Table 1.

Table 1. Intraday Precision and Percentage Recovery for HP β CD standard concentrations.

HP β CD Concentration (% <i>w/v</i>)	Intraday Precision		Accuracy
	(Average Peak Area \pm SD)	%RSD	(Percentage Recovery \pm SD)
0.5	17.41 \pm 0.04	0.23	101.11 \pm 0.45
2.5	18.17 \pm 0.04	0.20	101.37 \pm 0.20
5	17.41 \pm 0.07	0.28	98.71 \pm 0.27

2.2. Osmolality Measurements and Activity Coefficient Determination

Intermolecular interactions caused by London-van der Waals forces and H-bonding result in significant deviations in system behavior from ideality. In order to evaluate such deviations, the method of activity coefficient can be applied. Numerous publications devoted to the non-ideal behavior of saccharides solutions were based on this method. The analysis of colligative properties of galactose [16], glucose [16–18], maltose [17], maltotriose [17], mannose [16], and sucrose [18–20] have shown the same trend: activity coefficient increased with increasing saccharides concentration, corresponding to

a positive deviation from ideality. In majority of the cases, authors explained such behavior as strong interactions between sugar and water molecules.

Recently [21], we have analyzed osmometry results of native CDs (α CD and β CD) and obtained data pointed to their propensity to self-association (negative deviation from ideality), which is not common for saccharides. In current studies, we would like to know which type of non-ideality behavior HP β CD follows. For that purpose, calculation of activity coefficient was carried out. The concentration dependency of osmolality for HP β CD solutions is presented in Figure 1.

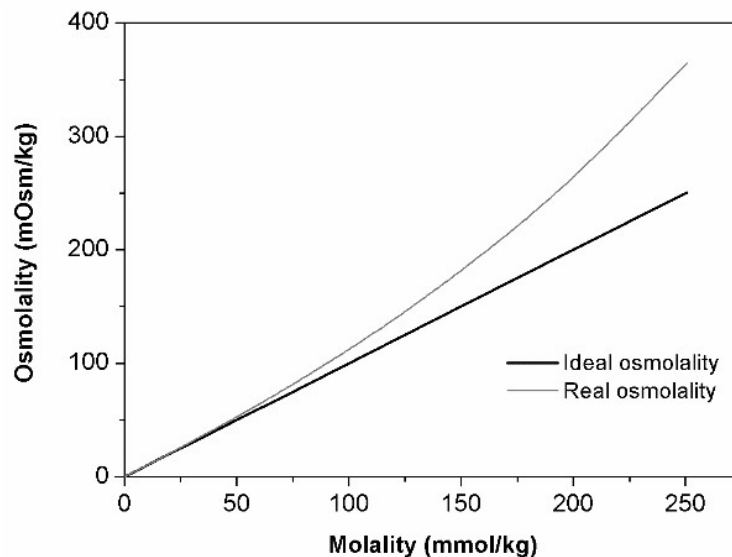


Figure 1. Concentration dependencies of osmolality for HP β CD.

Activity coefficients were derived from the following Equation:

$$\ln \gamma_2 = (\varphi - 1) + \int_0^m \frac{\varphi - 1}{m} dm \quad (1)$$

in which γ_2 is HP β CD activity coefficient; m is molality of HP β CD solution; and φ is osmotic coefficient, which was obtained as proportional coefficient between osmolality (\bar{m}) and molality (assuming that HP β CD does not dissociate in water, $n = 1$):

$$\bar{m} = n\varphi m \quad (2)$$

In order to solve integral part in Equation (1), osmotic coefficient was presented as polynomial function of the type

$$\varphi = 1 + \sum_{i=1}^n A_i m^i \quad (3)$$

Concentration dependencies of osmotic and activity coefficients are illustrated in Figure 2. Calculated activity coefficients obey following empirical Equation:

$$\gamma_2 = 56.81 m^3 - 5.00 m^2 + 2.40 m + 1 \quad (4)$$

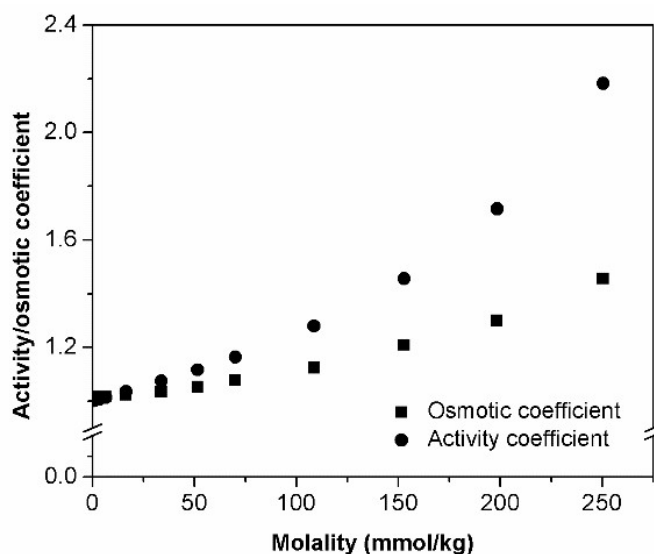


Figure 2. Variation of osmotic/activity coefficients with HP β CD concentration.

As it can be seen on Figure 2, HP β CD aqueous solutions represent the example of positive deviation from ideality—the higher the concentration of solute, the higher the osmotic/activity coefficient. Such behavior can be attributed to two factors that may be concurrent or not. On the one hand, the presence of HP β CD isomers might overly enhance the molecular randomness of solution opposing the tendency to freezing. Therefore, addition of HP β CD causes significant lowering of the freezing point and, consequently, positive deviation of osmolality. Such assumption can be supported by the conclusion that was made by Miyajima and co-workers [16] based on analysis of partial molar entropy of water at increase monosaccharides' concentration. On the other hand, the decrease of water molecules' mobility can be caused by strong solute–solvent interactions that might take place in HP β CD solutions. Similar observations/conclusions are also characteristic for saccharides [17,18,20]. The same presented arguments can possibly explain the high viscosity values registered for HP β CD solutions.

Contrary to what was reported for native CDs and following the same tendency of saccharides, the results of HP β CD pointed to the existence of stronger solute–solvent interactions when compared to solute–solute interactions.

Similar conclusions can be found in previous publications where authors also described a positive deviation from ideality for HP β CD [12,22], as well as other CD derivatives: DM β CD [23], SBE β CD [12,22], HP γ CD [12,22], and SBE γ CD [12].

Overall, obtained results cannot completely exclude the presence of self-aggregated particles (solute–solute interactions); however, in this case, solute–solvent interactions were the main contributors to the observed non-ideal behavior.

2.3. Viscosity Measurements

Figure 3 illustrates of HP β CD viscosity behavior with increasing concentration. A linear increase of viscosity is observed at HP β CD concentration below 11% (w/v), after which a positive deviation from linearity is observed. This upward curvature at higher HP β CD concentrations is not a novel observation, and similar results have also been described before [13,24–27].

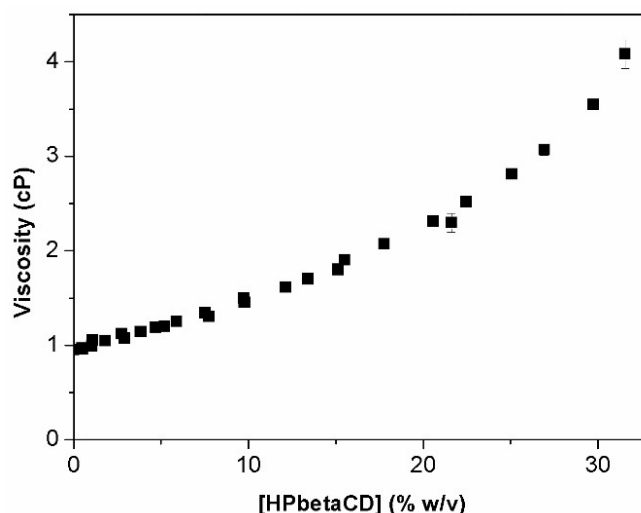


Figure 3. Increase in viscosity of pure aqueous HP β CD solutions with increasing HP β CD concentrations at 25 °C.

Viscosity is a measure of the free movement of water molecules. The linear relationship described above is explained by a linear increase of water networking with increase of HP β CD concentration. The decrease in water molecules mobility due to their binding by HP β CD leads to an increase of media viscosity [28]. The exponential rapid increase of viscosity above 11% (*w/v*) HP β CD is probably due to the ability of HP β CD to behave as a structure maker [25] and the consequent HP β CD self-aggregation and overlapping of hydration shells [25,28]. Also, the entrapped water molecules inside of the formed aggregates can increase their effective hydrodynamic volume, which will also contribute to the increase of apparent viscosity [29]. The inflexion point was calculated from Figure 3 and determined to be approximately 11% (*w/v*) HP β CD. This value is quite similar to the *cac* value for aqueous HP β CD solutions (i.e., 11.8%) obtained from permeation studies with the smallest pore size used (MWCO 3.5–5 kDa). The similarity of the two values indicates that the exponential increase in viscosity with increase of HP β CD concentration can be related to the self-assembly process.

Häusler et al. made similar observations and reported that HP β CD molecules had tendency to associate. Furthermore, they added different amounts of urea, which is a chaotropic agent, and observed a viscosity decrease due to disruption of aggregates and consequent increase in water mobility [13]. Although the attempt to determine *cac* values applying this method was not successful, this parameter was applied during particle size measurements in aqueous CD solutions.

2.4. Surface Tension Measurements

Figure 4 shows that the addition of HP β CD to water results in a small decrease in the surface tension. This small decrease in surface tension is in agreement with earlier observations by various investigators [24,26,30,31].

A decrease of 14 mN/m was observed at 0.5% (*w/v*) HP β CD concentration (corresponding to CMC), after which constant values are obtained even at HP β CD concentration as high as 35% (*w/v*). Yoshida et al. suggested that this decrease in surface tension is due to the surface activity of HP β CD [24,30]. Other substituted β CDs also possess surface activity greater than that of the natural β CD [13,26,31]. The stronger interaction of hydroxypropyl groups with water molecules is probably responsible for the disruption of the water network at the surface. HP β CD results in positive deviation from linearity of the activity coefficient, indicating that solute–solvent interactions are preferred over solute–solute interactions. This can explain why HP β CD promotes a slight change in surface tension. However, here the focus is on the detection of CD aggregates and how these structures could influence or modify the surface tension of a solution when HP β CD is present.

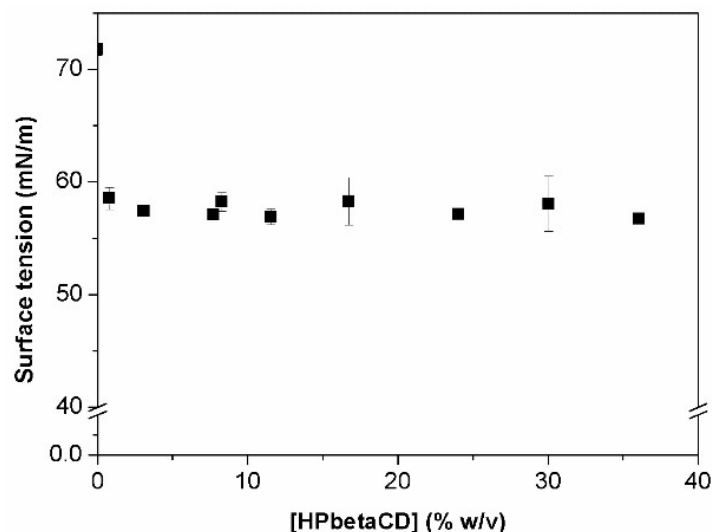


Figure 4. Surface tension variation with concentration of HP β CD aqueous solutions at 25 °C.

The results show that tensiometry does not appear to be a usable method for the detection of aggregates. The cac value cannot be related to the CMC value, as this is really low ($\sim 0.5\%$), and this concentration as aggregation starting point for a substituted CD does not seem to be realistic.

2.5. Size Distribution of HP β CD Particles

In pure aqueous HP β CD solutions, the scattering intensity at concentrations below 1% (w/v) was very low, meaning that, if present, particles cannot be detected accurately. At higher HP β CD concentrations, particles and, especially, aggregates could readily be observed. Figure 5a shows aggregate size distribution in freshly prepared solutions (measured right after their preparation without filtration). This figure clearly shows that particle size increased with increasing HP β CD concentration. In all tested HP β CD solutions, bimodal populations were observed. The smaller populations with hydrodynamic size of about 10 nm might present HP β CD monomers or very small aggregates. The relative contribution of this population size decreases with increasing HP β CD concentration. The hydrodynamic diameter of the larger aggregate populations and their intensity increase with concentrations from about 80 nm at 5% (w/v) HP β CD to about 800 nm at 40% (w/v) HP β CD. Initially, small aggregates are being formed, and then as the HP β CD concentration increases, both their size and abundance increases.

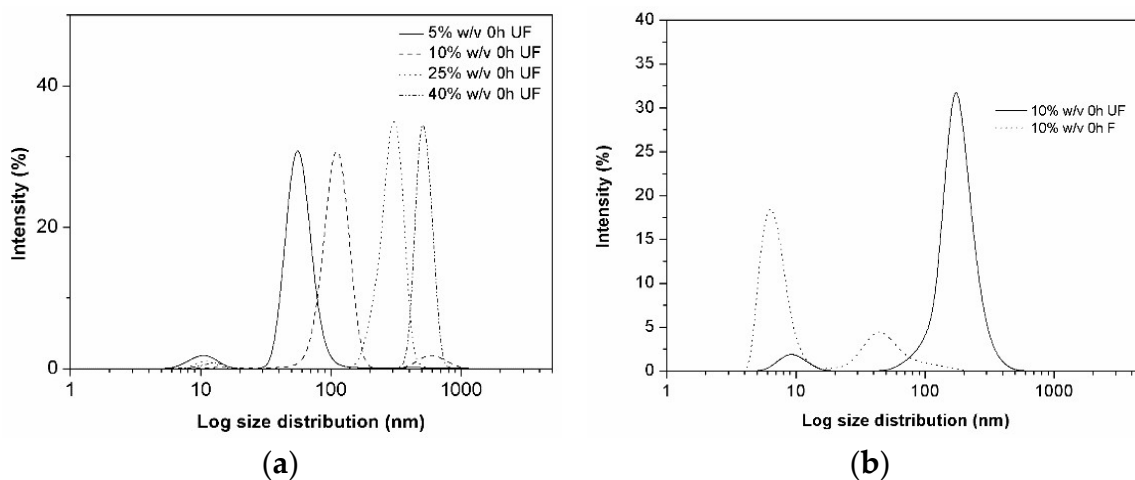


Figure 5. Cont.

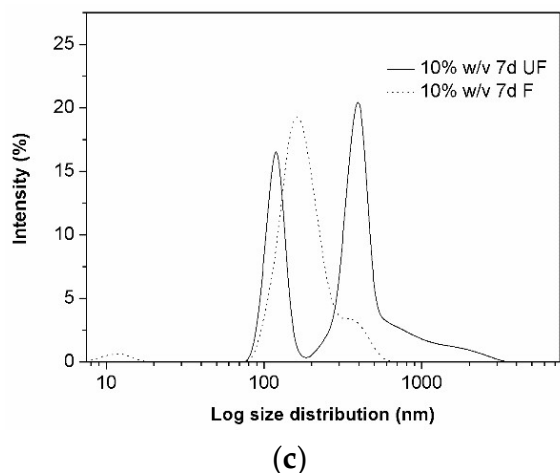


Figure 5. (a) Intensity fraction size distributions of the apparent hydrodynamic radius of HP β CD aggregates in pure aqueous HP β CD solutions, as measured by DLS at 23 °C. (b) Particle size distribution of HP β CD aggregates in freshly prepared aqueous solutions and after equilibrium of the sample solutions for seven days at room temperature (about 23 °C) (c).

The effect of filtration and storage time on the aggregate size distribution was also studied (Figure 5b,c). For this purpose, the 10% (*w/v*) HP β CD solution in water was filtrated through 0.45 μ m membrane filter and then measured immediately after preparation (at time zero) and again after seven days' equilibration without agitation at room temperature. The reference solution was the unfiltered 10% (*w/v*) HP β CD solution.

Figure 5b shows that immediately after filtration (i.e., at time zero), the particle size was considerably smaller in the filtered (F) than in the unfiltered (UF) solution. Both the F and UF solutions had similar bimodal aggregate size population but were comprised of different sizes. Just after preparation of samples and before filtration, a small population of particles (monomers, dimers) around 10 nm is observed, along with a second population of larger aggregates with hydrodynamic diameter of about 200–300 nm. After filtration through 0.45 μ m pore size membrane filter, the same bimodal profile is registered but with a different size distribution. The sample filtration disassembled the larger aggregates with a consequent formation of smaller aggregates with hydrodynamic diameter of about 80 nm. Furthermore, the filtration resulted in a large increase of monomer population, and this population has higher intensity than the aggregate population.

Figure 5c presents the size distribution after equilibrium of the sample solutions for seven days. A shift to the right is observed showing formation of larger aggregates due to further aggregation in the unstirred HP β CD solution with time. The size of particles increased significantly for the UF samples that, however, maintained their bimodal size distribution with size populations of about 100 nm and 800 nm. The size distribution of the F solutions also changed, showing one major size population of about 200 nm. These results show that filtration reduced the hydrodynamic diameter of the HP β CD aggregates, and that storage increased the aggregate diameter. Previously, other investigators have reported similar observations for the natural CDs [2,3]. Furthermore, the study shows that the HP β CD aggregation is a reversible process.

The light scattering analytical method can be a reliable method to describe and study self-aggregation of CDs, but the results depend on several variables. For example, the angle used for determining the hydrodynamic diameter will affect the results [32]. Also, guest compounds will affect the aggregation and the size of the aggregates, and they will depend on if a single CD system is being studied or if an effect of some other molecules on the CD aggregation is being investigated. Consequently, the previously reported *cac* values for pure HP β CD [32] and HP β CD complexes [33–35] can be different from the values obtained in this study. In this present study, the DLS equipment used did not allow determination of the *cac* value.

2.6. Permeation Studies

2.6.1. Optimization of Permeation Studies

Before determination of the HP β CD aggregation profile by Franz diffusion cells experiments, it is important and crucial to know and optimize some of the experimental parameters, namely, determination of the time that is needed to obtain steady state HP β CD permeation through the cellulose semi-permeable membrane (i.e., the lag-time) and duration of the steady state permeation.

HP β CD steady state permeation was obtained at 0.20 h (12 min). After 0.25 h (15 min), a straight line is obtained with a slope representing the flux (J) in Equation (7). With this information, the permeation studies for HP β CD could be planned, starting the first collection point at 15 min and the last one at 75 min.

2.6.2. Determination of Flux and Apparent cac Values for HP β CD

For each HP β CD concentration samples were collected every 15 min after the lag time (15 min) had been reached with time 0 when the donor phase (i.e., the HP β CD solution) was applied to the membrane surface (i.e., to the donor chamber). Samples were analyzed by UHPLC, and graphs describing mass of CD permeated (mg) versus time (s) were drawn. From these graphs, slopes (dq/dt) for each sample concentration were registered and used to determine CD flux (J) using Equation (7) described on materials and methods section.

Figure 6a shows the flux of HP β CD versus its concentration through three semi-permeable membranes of different MWCO (i.e., 3.5–5, 8–10, and 20 kDa). Following Fick's first law (see Equation (3)), one should expect a linear increase of flux with increasing HP β CD concentration, but this is not always the case. Initially, the graphs in Figure 6a display a linear section corresponding to free permeation of HP β CD monomers and possibly small aggregates. When concentration increases, both the amount and size of aggregates increase, affecting the ability of HP β CD to permeate the semi-permeable membrane. Furthermore, small aggregates (e.g., monomers, dimers, and trimers) will permeate the larger MWCO membranes. For this reason, an increase in apparent cac value is observed with increasing of MWCO or 11.8%, 14.3%, and 19.1% (w/v) for the 3.5–5, 8–10, and 20 kDa membranes, respectively. Also smaller particles are able to pass membranes with the larger MWCO, but aggregates larger than the pore size are the ones responsible for the observed negative deviation from Fick's First law. This negative deviation has been observed also by different authors [1,15,27,36–39] which, like in our case, suggested formation of CD self-aggregates.

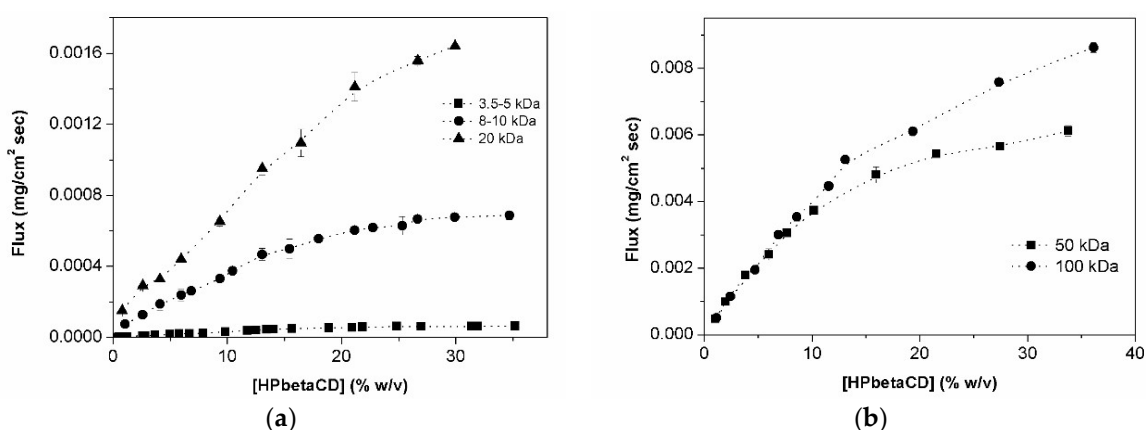


Figure 6. Flux profile of HP β CD through semipermeable membranes: 3.5–5, 8–10, and 20 kDa (a) and 50, 100 kDa (b) at room temperature (about 23 °C).

There are two simultaneous processes ongoing during the permeation studies through semi-permeable membranes: (1) a solute transfer from donor to receptor phase (simple diffusion) and

(2) a solvent transfer in the opposite direction (osmosis). These two processes are not controlled by the same physicochemical properties. The osmotic effect depends on the MWCO of membrane (i.e., larger pore size results in faster diffusion for water molecules) and osmolality difference of the two phases; the larger this difference is, the larger volume of water that will be transferred from the receptor to the donor chamber. The HP β CD diffusion from the donor to the receptor chamber is influenced by the MWCO of membrane but also by other parameters such as aggregate size, concentration, and viscosity of the tested sample (i.e., the donor phase). All these properties can affect the observed HP β CD flux by diluting the HP β CD donor phase concentration or by perturbing the normal HP β CD permeation. For example, extremely high viscosity can prevent or delay the release of permeating molecules from one compartment to the other, and high substrate concentrations can increase the difference in osmolality between the two chambers and by this way increase osmotic pressure that can work as a counter flow force delaying permeation of molecules through the membrane.

The analysis of HP β CD graphic for 50 and 100 kDa membrane (Figure 6b) shows similar profile behavior as displayed in Figure 6a. However, a more careful interpretation and analysis has to be performed. Despite the slight negative deviation obtained in the case of the 20 kDa membrane (Figure 6a), the profile is almost linear (indicates that diameter of the HP β CD aggregates is close to globular molecule of molecular weight of 20 kDa). Looking to the accentuated inflexion of both 50 and 100 kDa MWCO membrane curve, one might suppose that it is also due to aggregates size. However, the difference in osmolality between HP β CD (donor phase) and water (receptor phase) allied to the larger pore size of membranes makes one speculate that under such conditions probably the osmosis process is so fast that it acts like a counter flow force that prevents normal particle permeation with consequent donor phase dilution during the experiment. Also, viscosity of samples can contribute to this phenomenon. The donor phase dilution might be responsible for the negative deviation from linearity. To get better understanding of these phenomena, contribution of osmosis and diffusion for permeation process through the 50 and 100 kDa membranes were calculated. As observed in Figure 7, the point where osmosis starts to be higher than diffusion is coincident with the inflexion point on the flux curves of these membranes. This phenomenon does not affect the three other membranes with much lower MWCO.

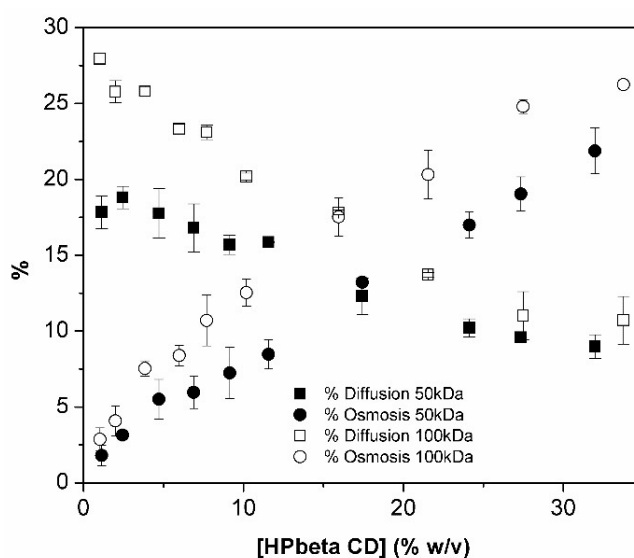


Figure 7. % osmosis (circles) and % diffusion (squares) determined for aqueous HP β CD solutions when studies used different MWCO semipermeable membranes: 50 kDa (black) and 100 kDa (white) (n = 3).

These observations make us believe that under such conditions, we were not longer working under sink conditions [40], and so these two larger pore size membranes cannot be used to study HP β CD aggregation and determine apparent cac values.

From the intersection of the linear portions of the plots at concentrations above and below the inflection points, it was possible to determine the apparent cac (concentration after which one start to detect formation of aggregates) for HP β CD. The estimated apparent cac values for HP β CD in pure water are presented in Table 2.

Permeation showed advantages over the other methods tested with less variability and more precision and seems to be the most valuable method to determine HP β CD aggregates in water.

Table 2. Calculated apparent cac values for HP β CDs in pure water and different semi-permeable membranes.

MWCO (kDa)	HP β CD
	cac% (<i>w/v</i>)
3.5–5	11.8
8–10	14.3
20	19.1
50	ND *
100	ND *

* not determined.

3. Conclusions

Several available analytical approaches were explored in an effort to develop a method that could be used to efficiently detect and quantify HP β CD self-aggregates in aqueous solutions. However, not all of them proved to be useful. Results from tensiometry studies showed that HP β CD had only a minor effect on the surface tension, and the conclusion was that this is not a good method with which to study the aggregation phenomena of HP β CD.

A positive deviation after the linear increase of media viscosity with HP β CD concentration was registered (at approximately 11% *w/v* HP β CD). This exacerbated increase is due to the binding of water molecules by HP β CD with consequent reduced mobility of water molecules that might also be related to formation of HP β CD aggregates. However, there are no scientific bases that enable correlation of this value with an apparent cac value.

Similarly to what has been described for saccharides, osmometry results show a positive deviation from ideality when plotting osmolality against HP β CD molality. An increase of osmotic and activity coefficient with increasing HP β CD concentration was also noticed, suggesting the existence of strong interaction between CD and water molecules. Although these interactions (solvent–solute) are probably favored over solute–solute interactions and are the main contributors to the observed osmolality behaviour, we cannot exclude the presence of HP β CD aggregates. This method did not prove to be suitable for aggregates' quantification.

Contrary to previously published work [32], we could not determine the apparent cac value for HP β CD by a DLS method. Self-assembly process is a fast and dynamic equilibrium (formation/dissemble) and probably too fast to be detectable by the DLS machine used in this study. However, other types of important findings were made applying this method, such as the size range of HP β CD aggregates, the tendency for aggregates' size to increase with increasing HP β CD concentration, and the important effect of filtration (affected the size distribution by disassemble of larger aggregates form smaller ones) and equilibration time (increasing size with time after filtration).

Permeability showed that aggregation and aggregate size increases with increasing HP β CD concentration with determined apparent cac value of 11.8% (*w/v*) for the 3.5–5 kDa MWCO membrane. The analysis of permeation results for HP β CD clearly indicates that the aggregates have hydrodynamic diameter larger than 20 kDa, as they do not permeate freely through the 20 kDa MWCO membrane. For the 50 and 100 kDa MWCO membranes, the flux still deviates from linearity, but one can speculate

that is not only due to the aggregate particle size but also due to other factors such as high osmotic pressure and viscosity. HP β CD is less prone to self-aggregate than its precursor β CD, since the presented apparent cac values for HP β CD (starting from 11.8% *w/v*) were significantly higher than for β CD (starting from 0.8% *w/v*).

As CD aggregates are quite unstable and can be disrupted by forces applied in specific analytical techniques, to have an unstirred and quite still donor phase during permeation experiment is really important. Overall, the permeation method was the best method to evaluate HP β CD aggregation, as it provided more conclusive results and is the least “invasive” for the self-assembled samples.

The main results, advantages, and disadvantages of each analyzed technique are summarized in Table 3.

Table 3. Schematic table of main results, advantages, and disadvantages of assessed analytical methods.

Analytical Method	General Outcome	Advantages	Disadvantages
Osmometry	Positive deviation from linearity (solute–solvent interactions favored). Presence of HP β CD aggregate particles cannot be excluded.	Simple to perform. Sample dilution not required. Fast.	Low accuracy at low CD concentrations. Inadequate method for HP β CD aggregate quantification.
Viscometry	Exacerbated increase of viscosity deviating from linearity at CD conc. of 11% <i>w/v</i> (similar to sugars). Plausible method for detection/quantification of HP β CD aggregates in aqueous solutions.	Sample dilution not required. Fast.	This technique (plate method) can disassemble the aggregates by the mechanical forces involved.
Tensiometry	Addition of HP β CD to water has only minor effect on surface tension.	None.	Inadequate method for detection and quantification of HP β CD aggregates.
Dynamic Light Scattering	Uncertain results with available apparatus. Adequate method for detection of HP β CD aggregates.	Good approximation of aggregate size range.	Not suitable for aggregate quantification (i.e., to calculate the apparent cac value).
Permeation studies	Most useful and reliable method to detect and quantify (i.e., determine their apparent cac values) in aqueous HP β CD solutions.	Most accurate method with most precise results. Least “invasive” method for the aggregates (donor phase is always unstirred and quite still during experiment).	Time-consuming. Extremely laboring. Indirect results.

4. Materials and Methods

4.1. Materials

2-Hydroxypropyl- β -cyclodextrin (HP β CD) DS 0.62 (MW 1380 Da) was kindly provided by Janssen Pharmaceutica (Beerse, Belgium). Milli-Q water (Millipore, Billerica, MA, USA) was used to prepare CD solutions and mobile phases. Acetonitrile of HPLC grade was purchased from Sigma–Aldrich (St. Louis, MO, USA). A set of 5 different pore size Spectra/Por[®] semipermeable cellophane membranes (Spectrum Europe, Breda, The Netherlands) with molecular weight cutoff (MWCO) of 3.5–5, 8–10, 20, 50, and 100 kDa were used for permeation studies.

4.2. Quantitative Determination of HP β CD

Quantitative determination of HP β CD was performed on a reverse-phase, ultra-high-performance liquid chromatography (UHPLC) Ultimate 3000 series system from Dionex Softron GmbH (Germering, Germany) consisting of a LPG-3400SD pump with a built-in degasser, a WPS-3000 autosampler (Dionex, Germering, Germany), a TCC-3100 column compartment (Dionex, Germering, Germany), and a CoronaR ultra RS detector (Dionex, Germering, Germany). During the stationary phase, a Phenomenex Kinetex C18 150 \times 4.60 mm 5 μ m column with a matching HPLC Security Guard (Phenomenex, Cheshire, UK) were used. The mobile phase consisted of equal volumes of acetonitrile and water; the flow rate was 1.0 mL/min, and the injection volume was 5 μ L. Retention time and peak area of all chromatograms were determined using the software ChromeleonR version 7.2 SR4 (ThermoScientific, Waltham, MA, USA).

4.3. Validation of the UHPLC-CAD Method

The validation of the UHPLC analytical method for detection of HP β CD in aqueous solutions was performed according to the Q2(R1) guidelines from the International Conference on Harmonization Guidelines [41]. The parameters that were validated are as follows: linearity and range, repeatability, detection limit (LOD), quantification limit (LOQ), and accuracy. Aqueous HP β CD solutions of various concentrations were prepared through sonication at 60 $^{\circ}$ C for 60 min and then allowed to cool to room temperature. The water content of HP β CD powder was determined using an A&D MX-50 moisture analyser (A&D company, Limited, Tokyo, Japan) and accounted for.

4.3.1. Linearity and Range

Aqueous solutions containing 0.001 to 1% (*w/v*) HP β CD were prepared in order to evaluate the linearity and range. Calibration curves were prepared in triplicate and each sample injected three times. Linearity of the curves obtained was analyzed by plotting logarithm of the peak area vs. logarithm of the CD concentration.

4.3.2. Repeatability

Repeatability is the parameter that evaluates the variability of the assay being expressed in relative standard deviation (%RSD). To validate this parameter, three concentrations of HP β CD were randomly selected and injected six times. Acceptance criteria for this test were established as $RSD \leq 1\%$.

4.3.3. Accuracy (% Recovery)

Accuracy was measured by injecting, three times, three different concentrations of the studied CD solution. Mean recovery (difference between theoretical and real concentration) was then calculated. In order to be accepted and validated, this parameter needed to be $100 \pm 2\%$ at each concentration [15].

4.3.4. Limit of Detection (LOD) and Limit of Quantification Limit (LOQ)

These were obtained from the linearity test as follows [41]:

$$LOQ = \frac{\text{Standard Deviation}}{\text{Slope}} \times 10 \quad (5)$$

$$LOD = \frac{\text{Standard Deviation}}{\text{Slope}} \times 3.3 \quad (6)$$

4.4. Determination of the Water Content

Water of the HP β CD samples (approx. 5 g) was determined using an A&D MX-50 moisture analyzer (A&D company, Limited, Tokyo, Japan). The samples were evenly spread over the aluminium

pans, in order to avoid uneven drying of the samples. The moisture content of HP β CD was measured in triplicate and results presented in average (%) \pm standard deviation (SD). The water content of HP β CD was determined to be $6.21 \pm 0.12\%$.

4.5. Osmolality Measurements (Cryoscopic Osmometry)

The osmolality studies were performed using Osmomat 030 (Gonotec GmbH, Berlin, Germany). Each point was measured at least three times, and the mean values and SD were calculated. MilliQ water was used to determine background, and standard NaCl solutions of 300 and 400 mOsm (Knauer GmbH, Berlin, Germany) were used for initially osmometer calibration. Recalibrations were performed between measurements.

4.6. Viscosity Measurements

The viscosity measurements of aqueous HP β CD solutions were performed with a Brookfield model DV-I + (USA) viscometer equipped with a Polystat circulating thermostatic water bath (Cole Palmer, Vernon Hills, IL, USA) set to 25 °C. All samples were analyzed in triplicate in order to calculate average values and SD.

4.7. Surface Tension Measurements

HP β CD solutions were measured using a Krüss K100 tensiometer (Krüss GmbH, Hamburg, Germany), based on the Wilhelmy plate method. These surface tension measurements were performed at room temperature, and MilliQ water was used for instrument calibration. Calibration with water was repeated before each measurement. Three measurements of each sample were performed, and the average was calculated \pm SD.

4.8. Dynamic Light Scattering (DLS) Determinations

The particle size analysis of HP β CD samples was performed by DLS using Nanotrak Wave (Microtrac Inc., Philadelphia, PA, USA). Several concentrations of aqueous HP β CD solutions were measured three times at 25 ± 0.5 °C. In order to study the possible influence of filtration and time on the formation/disassembly of aggregates, each CD sample concentration was divided in two samples. One sample was not filtered, but the other one was filtered through 0.45 μ m RC membrane filter (Phenomenex, Cheshire, UK). All samples were immediately measured after preparation ($t = 0$ h), then stored at room temperature and measured again after seven days of storage. The intensity in percentage of each population was plotted versus the logarithm of size distribution.

4.9. Permeation Studies

Permeability studies of different HP β CD aqueous solutions (donor phase) were carried out in unjacketed Franz diffusion cells with diffusion area of 1.77 cm² (SES GmbH—Analyse systeme, Bechenheim, Germany) operated at room temperature. Two ml of these aqueous HP β CD solutions were added to the donor compartment, while the receptor compartment was filled with 12 mL of MilliQ water. The receptor phase needed to be autoclaved before usage in order to remove all dissolved air and avoid the formation of air bubbles during the experiment. A single layer semi-permeable cellulose ester membrane (Biotech CE, Spectrum Europe, Breda, The Netherlands) with different molecular-weight-cutoffs (MWCO) was used to physically separate the compartments (i.e., the donor and receptor phases). The MWCO of membranes used were 3.5–5 kDa, 8–10 kDa, 20 kDa, 50 kDa, and 100 kDa. Depending on the aim of a given experiment, different sample times were used. For optimization of experimental conditions, 28 samples were collected from 5 to 360 min. For determination of aggregates in aqueous HP β CD solutions, samples were collected every 15 min for 75 min. A 150 μ L sample (receptor phase) was collected at each time point and immediately replaced with same volume of receptor phase (i.e., MilliQ water). The receptor phase was agitated with a

magnetic stirrer operated at 300 rpm. The samples collected were then quantified using the UHPLC validated method described previously. The calculation of steady state flux (J) of HP β CD was obtained from the slope (dq/dt) applying the linear regression relationship between time (t) and the amount of HP β CD in receptor chamber (q):

$$J = \frac{dq/dt}{A} = P_{app} \cdot C_d \quad (7)$$

in which dq/dt is the slope (in mg/s of CD permeated through the membrane over time, A is the diffusion area (1.77 cm^2), and C_d is the total HP β CD concentration in the donor phase.

In order to assure sink conditions [40] were followed, both volume change and final concentration of components in donor phases were accessed.

The calculation of the apparent critical aggregation concentration for different tested membranes (i.e., concentration from which aggregates dimensions leads a deviation of ideal flux from CD through the membrane due to their size starts to be larger than the studied pore size) was performed by drawing tangent lines in the flux graphics. This deviation point where flux diversion from linearity started to be noticed corresponds to apparent cac value [21].

Author Contributions: Conceptualization, All authors; Methodology, A.R.S.C. and A.R.; Software, A.R.S.C. and A.R.; Validation, A.R.S.C. and A.R.; Formal Analysis, All authors; Investigation, A.R.S.C. and A.R.; Resources, All authors; Writing—Original Draft Preparation, All authors; Writing—Review & Editing, All authors; Visualization, All authors; Supervision, T.L.; Project Administration, T.L.; Funding Acquisition, T.L.

Funding: This research was funded by the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT), grant number 135040.

Acknowledgments: The financial support received from the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT) (Grant No. 135040) is gratefully acknowledged.

Conflicts of Interest: The authors declare no conflict of interest.

References

- Messner, M.; Kurkov, S.V.; Brewster, M.E.; Jansook, P.; Loftsson, T. Self-assembly of cyclodextrin complexes: Aggregation of hydrocortisone/cyclodextrin complexes. *Int. J. Pharm.* **2011**, *407*, 174–183. [[CrossRef](#)] [[PubMed](#)]
- Bonini, M.; Rossi, S.; Karlsson, G.; Almgren, M.; Lo Nostro, P.; Baglioni, P. Self-Assembly of β -Cyclodextrin in Water. Part 1: Cryo-TEM and Dynamic and Static Light Scattering. *Langmuir* **2006**, *22*, 1478–1484. [[CrossRef](#)] [[PubMed](#)]
- González-Gaitano, G.; Rodríguez, P.; Isasi, J.R.; Fuentes, M.; Tardajos, G.; Sánchez, M. The Aggregation of Cyclodextrins as Studied by Photon Correlation Spectroscopy. *J. Incl. Phenom. Macrocycl. Chem.* **2002**, *44*, 101–105. [[CrossRef](#)]
- Messner, M.; Kurkov, S.V.; Palazón, M.M.; Fernández, B.Á.; Brewster, M.E.; Loftsson, T. Self-assembly of cyclodextrin complexes: Effect of temperature, agitation and media composition on aggregation. *Int. J. Pharm.* **2011**, *419*, 322–328. [[CrossRef](#)] [[PubMed](#)]
- Del Valle, E.M.M. Cyclodextrins and their uses: A review. *Process Biochem.* **2004**, *39*, 1033–1046. [[CrossRef](#)]
- Dodziuk, H. Molecules with Holes—Cyclodextrins. In *Cyclodextrins and Their Complexes*; Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, Germany, 2006; pp. 1–30.
- De Sousa, F.B.; Lima, A.C.; Denadai, A.M.; Anconi, C.P.; De Almeida, W.B.; Novato, W.T.; Dos Santos, H.F.; Drum, C.L.; Langer, R.; Sinisterra, R.D. Superstructure based on beta-CD self-assembly induced by a small guest molecule. *Phys. Chem. Chem. Phys.* **2012**, *14*, 1934–1944. [[CrossRef](#)] [[PubMed](#)]
- Mixcoha, E.; Campos-Terán, J.; Piñeiro, Á. Surface Adsorption and Bulk Aggregation of Cyclodextrins by Computational Molecular Dynamics Simulations as a Function of Temperature: α -CD vs β -CD. *J. Phys. Chem. B* **2014**, *118*, 6999–7011. [[CrossRef](#)] [[PubMed](#)]
- Coleman, A.W.; Nicolis, I.; Keller, N.; Dalbiez, J.P. Aggregation of cyclodextrins: An explanation of the abnormal solubility of β -cyclodextrin. *J. Incl. Phenom. Macrocycl. Chem.* **1992**, *13*, 139–143. [[CrossRef](#)]
- Loftsson, T.; Brewster, M.E. Cyclodextrins as functional excipients: methods to enhance complexation efficiency. *J. Pharm. Sci.* **2012**, *101*, 3019–3032. [[CrossRef](#)] [[PubMed](#)]

11. Kurkov, S.V.; Loftsson, T. Cyclodextrins. *Int. J. Pharm.* **2013**, *453*, 167–180. [[CrossRef](#)] [[PubMed](#)]
12. Zannou, E.A.; Streng, W.H.; Stella, V.J. Osmotic Properties of Sulfobutylether and Hydroxypropyl Cyclodextrins. *Pharm. Res.* **2001**, *18*, 1226–1231. [[CrossRef](#)] [[PubMed](#)]
13. Häusler, O.; Müller-Goymann, C.C. Properties and Structure of Aqueous Solutions of Hydroxypropyl-beta-Cyclodextrin. *Starch Stärke* **1993**, *45*, 183–187. [[CrossRef](#)]
14. Messner, M.; Kurkov, S.V.; Flavià-Piera, R.; Brewster, M.E.; Loftsson, T. Self-assembly of cyclodextrins: The effect of the guest molecule. *Int. J. Pharm.* **2011**, *408*, 235–247. [[CrossRef](#)] [[PubMed](#)]
15. Saokham, P.; Loftsson, T. A New Approach for Quantitative Determination of gamma-Cyclodextrin in Aqueous Solutions: Application in Aggregate Determinations and Solubility in Hydrocortisone/gamma-Cyclodextrin Inclusion Complex. *J. Pharm. Sci.* **2015**, *104*, 3925–3933. [[CrossRef](#)] [[PubMed](#)]
16. Miyajima, K.; Sawada, M.; Nakagaki, M. Studies on Aqueous Solutions of Saccharides. I. Activity Coefficients of Monosaccharides in Aqueous Solutions at 25 °C. *Bull. Chem. Soc. Jpn.* **1983**, *56*, 1620–1623. [[CrossRef](#)]
17. Miyajima, K.; Sawada, M.; Nakagaki, M. Studies on Aqueous Solutions of Saccharides. II. Viscosity B-Coefficients, Apparent Molar Volumes, and Activity Coefficients of D-Glucose, Maltose, and Maltotriose in Aqueous Solutions. *Bull. Chem. Soc. Jpn.* **1983**, *56*, 1954–1957. [[CrossRef](#)]
18. Stigter, D. Interactions in aqueous solutions. II. Osmotic pressure and osmotic coefficient of sucrose and glucose solutions. *J. Phys. Chem.* **1960**, *64*, 118–124. [[CrossRef](#)]
19. Miyawaki, O.; Saito, A.; Matsuo, T.; Nakamura, K. Activity and Activity Coefficient of Water in Aqueous Solutions and Their Relationships with Solution Structure Parameters. *Biosci. Biotechnol. Biochem.* **1997**, *61*, 466–469. [[CrossRef](#)]
20. Starzak, M.; Mathlouthi, M. Temperature dependence of water activity in aqueous solutions of sucrose. *Food Chem.* **2006**, *96*, 346–370. [[CrossRef](#)]
21. Sá Couto, A.R.; Ryzhakov, A.; Loftsson, T. Self-Assembly of α -Cyclodextrin and β -Cyclodextrin: Identification and Development of Analytical Techniques. *J. Pharm. Sci.* **2018**, *107*, 2208–2215. [[CrossRef](#)] [[PubMed](#)]
22. Proniuk, S.; Blanchard, J. Influence of degree of substitution of cyclodextrins on their colligative properties in solution. *J. Pharm. Sci.* **2001**, *90*, 1086–1090. [[CrossRef](#)] [[PubMed](#)]
23. Miyajima, K.; Mukai, T.; Nakagaki, M.; Otagiri, M.; Uekama, K. Activity coefficients of Dimethyl-beta-cyclodextrin in aqueous solutions. *Bull. Chem. Soc. Jpn.* **1986**, *59*, 643–644. [[CrossRef](#)]
24. Motoyama, K.; Nagatomo, K.; Abd Elazim, S.O.; Hirayama, F.; Uekama, K.; Arima, H. Potential use of 2-hydroxypropyl-beta-cyclodextrin for preparation of orally disintegrating tablets containing DL-alpha-tocopheryl acetate, an oily drug. *Chem. Pharm. Bull.* **2009**, *57*, 1206–1212. [[CrossRef](#)] [[PubMed](#)]
25. Loftsson, T. Drug permeation through biomembranes: cyclodextrins and the unstirred water layer. *Pharmazie* **2012**, *67*, 363–370. [[PubMed](#)]
26. Evrard, B.; Bertholet, P.; Gueders, M.; Flament, M.P.; Piel, G.; Delattre, L.; Gayot, A.; Leterme, P.; Foidart, J.M.; Cataldo, D. Cyclodextrins as a potential carrier in drug nebulization. *J. Cont. Release* **2004**, *96*, 403–410. [[CrossRef](#)] [[PubMed](#)]
27. Loftsson, T.; Masson, M.; Sigurdsson, H.H. Cyclodextrins and drug permeability through semi-permeable cellophane membranes. *Int. J. Pharm.* **2002**, *232*, 35–43. [[CrossRef](#)]
28. Ryzhakov, A.; Do Thi, T.; Stappaerts, J.; Bertolletti, L.; Kimpe, K.; Sá Couto, A.R.; Saokham, P.; Van den Mooter, G.; Augustijns, P.; Somsen, G.W. Self-Assembly of Cyclodextrins and Their Complexes in Aqueous Solutions. *J. Pharm. Sci.* **2016**, *105*, 2556–2569. [[CrossRef](#)] [[PubMed](#)]
29. Hamberg, L.; Walkenström, P.; Stading, M.; Hermansson, A.-M. Aggregation, viscosity measurements and direct observation of protein coated latex particles under shear. *Food Hydrocoll.* **2001**, *15*, 139–151. [[CrossRef](#)]
30. Yoshida, A.; Yamamoto, M.; Irie, T.; Hirayama, F.; Uekama, K. Some pharmaceutical properties of 3-hydroxypropyl- and 2,3-dihydroxypropyl-beta-cyclodextrins and their solubilizing and stabilizing abilities. *Chem. Pharm. Bull.* **1989**, *37*, 1059–1063. [[CrossRef](#)] [[PubMed](#)]
31. Azarbayjani, A.F.; Lin, H.; Yap, C.W.; Chan, Y.W.; Chan, S.Y. Surface tension and wettability in transdermal delivery: A study on the in-vitro permeation of haloperidol with cyclodextrin across human epidermis. *J. Pharm. Pharmacol.* **2010**, *62*, 770–778. [[CrossRef](#)] [[PubMed](#)]
32. Do, T.T.; Van Hooghten, R.; Van den Mooter, G. A study of the aggregation of cyclodextrins: Determination of the critical aggregation concentration, size of aggregates and thermodynamics using isodesmic and K2-K models. *Int. J. Pharm.* **2017**, *521*, 318–326. [[CrossRef](#)] [[PubMed](#)]

33. Garnero, C.; Longhi, M. Study of ascorbic acid interaction with hydroxypropyl- β -cyclodextrin and triethanolamine, separately and in combination. *J. Coll. Interface Sci.* **2007**, *45*, 536–545. [[CrossRef](#)] [[PubMed](#)]
34. Garnero, C.; Zoppi, A.; Genovese, D.; Longhi, M. Studies on trimethoprim:hydroxypropyl- β -cyclodextrin: aggregate and complex formation. *Carbohydr. Res.* **2010**, *345*, 2550–2556. [[CrossRef](#)] [[PubMed](#)]
35. Duan, M.S.; Zhao, N.; Össurardóttir, Í.B.; Thorsteinsson, T.; Loftsson, T. Cyclodextrin solubilization of the antibacterial agents triclosan and triclocarban: Formation of aggregates and higher-order complexes. *Int. J. Pharm.* **2005**, *297*, 213–222. [[CrossRef](#)] [[PubMed](#)]
36. Loftsson, T.; Masson, M.; Brewster, M.E. Self-association of cyclodextrins and cyclodextrin complexes. *J. Pharm. Sci.* **2004**, *93*, 1091–1099. [[CrossRef](#)] [[PubMed](#)]
37. Jansook, P.; Kurkov, S.V.; Loftsson, T. Cyclodextrins as solubilizers: formation of complex aggregates. *J. Pharm. Sci.* **2010**, *99*, 719–729. [[CrossRef](#)] [[PubMed](#)]
38. Jansook, P.; Moya-Ortega, M.D.; Loftsson, T. Effect of self-aggregation of γ -cyclodextrin on drug solubilization. *J. Incl. Phenom. Macrocycl. Chem.* **2010**, *68*, 229–236. [[CrossRef](#)]
39. Stappaerts, J.; Do Thi, T.; Dominguez-Vega, E.; Somsen, G.W.; Van den Mooter, G.; Augustijns, P. The impact of guest compounds on cyclodextrin aggregation behavior: A series of structurally related parabens. *Int. J. Pharm.* **2017**, *529*, 442–450. [[CrossRef](#)] [[PubMed](#)]
40. Martin, A. Diffusion and Dissolution. In *Physical Pharmacy*, 4th ed.; Martin, A., Ed.; Lea & Febiger: Philadelphia, PA, USA, 1993; pp. 324–361.
41. ICH, H.T.G. Validation of Analytical Procedures: Text and Methodology Q2(R1). Available online: https://www.ema.europa.eu/documents/scientific-guideline/ich-q-2-r1-validation-analytical-procedures-text-methodology-step-5_en.pdf (accessed on 10 October 2018).



© 2018 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).