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Short communication

Encapsulation of methyl and ethyl salicylates by β-cyclodextrin HPLC, UV–vis and molecular modeling studies

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ABSTRACT

The complexation of methyl salicylate (MS) and ethyl salicylate (ES), non-steroidal analgesic, anti-inflammatory and antirrheumatic drugs with β-cyclodextrin (βCD) has been studied from thermodynamic and structural points of view. The complexation with βCD has been investigated using reversed-phase liquid chromatography. Retention behavior has been analyzed on a reverse-phase column Luna 18(2) 5 μm. The mobile-phase was methanol:water in different ratios (55:45 to 70:30) in which βCD (1–9 mM) was incorporated as a mobile-phase additive. The decrease in retention times with increasing concentrations of βCD enables the determination of the apparent stability constant of the complexes. Values at 30 °C with 55% methanol were $K_{MS:\beta CD}$: 15.84 M^{-1} and $K_{ES:\beta CD}$: 12.73 M^{-1} for MS and ES, respectively. The apparent stability constants decrease as the polarity of the solvent decreases. The low solubility of MS and ES in aqueous solution has been improved by complexation with βCD (1–9 mM). The stability constants of the complexes obtained from the phase-solubility diagrams using a UV–vis spectrophotometric method were $K\dot{i}_{MS:\beta CD}$: 229 M^{-1} and $K\dot{i}_{ES:\beta CD}$: 166 M^{-1}. In addition, semi-empirical quantum mechanics calculations using AM1 and PM3 methods in vacuum were performed. The energetically favorable inclusion structures were identified and the most favorable orientation for the inclusion process was found to be the head-down orientation for both complexes. Enthalpy for encapsulation processes was found to be favorable ($\Delta H^\circ < 0$), while entropy ($\Delta S^\circ < 0$) and Gibbs free energy were unfavorable ($\Delta G^\circ > 0$). By means of HPLC and UV–vis measurements and quantum mechanics calculations, it was found that MS and ES form a 1:1 inclusion complex with βCD. The theoretical results are in agreement with the experimental parameters associated with the encapsulation process.

1. Introduction

Cyclodextrins (CDs, α, β, γ) are torus shaped, naturally occurring, enzymatically synthesized, cyclic oligomers composed of six, seven and eight α-1,4-linked glucose units per molecule (α-,β-, and γ-cyclodextrin, respectively). The exterior of the molecule is hydrophilic and its hydrophobic cavity may selectively include molecules with appropriately sized organic compounds by forming non-covalent inclusion complexes [1,2]. The internal cavity being less polar than the surrounding water molecules, chemical properties of the guest, once included, may be dramatically affected. Cyclodextrins are used as pharmaceutical excipients, principally as solubilizing and stabilizing agents for lipophilic substances in aqueous preparations. The guest molecules are solubilized in cyclodextrins solutions through formation on an inclusion complex. The cyclodextrins are also known to affect the chemical stability of drug molecules. The observed effects have been extensively examined in the literature [3–13].

The role of cyclodextrins in a HLPC system is based on their different extent of complexation depending on the structural features of the guests. This fact leads to differences in the retention behavior which can improve the selectivity of the chromatographic method. There are two approaches for applying cyclodextrins in reversed-phase HPLC either cyclodextrins can be either in the stationary phase, or used as mobile-phase additives. As a result of host–guest interactions the guest retention time will change, it will be shorter when complexation occurs in the mobile-phase and longer in the stationary phase. These changes in retention behavior are closely related to the stability constants of the complexes formed [14].

The numerous therapeutic effects of salicylates, as well as their possible adverse effects, have been known for a long time. They have been used, for example, as diuretics (lithium salicylate), intestinal antiseptics (bismuth salicylate) and also analgesics, anti-inflammatory and antipyretic agents (sodium salicylate, methyl

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salicylate and ethyl salicylate) [15]. As salycilates are practically insoluble in water, complexation with cyclodextrins provides a way to increase their solubility, stability and bioavailability.

Structural and thermodynamic information, such as stoichiometry and geometry of the complex, association constant ($K_{drug:CD}$) and changes in the enthalpy (ΔH°) and entropy (ΔS°) of binding are necessary to draw a complete picture of the driving forces governing the drug:CD interaction. The major driving forces for the complex formation have been proposed to include the release of entropy-rich water molecules from the cavity, van der Waals interactions, hydrophobic interactions, hydrogen bonding and release of ring strain in the CD molecule. However, the relative contributions and even the nature of the different forces are not well known. It is generally accepted that the overall stability of the complex [16,17] is dependent on a balance between van der Waals contacts and solvent effects.

The purpose of this work is to study the HPLC retention behavior of methyl salicylate and ethyl salicylate in the presence of βCD and to evaluate the stability constants of the respective inclusion complexes from the influence of the cyclodextrin concentration on their capacity factors. Besides, taking into consideration the low aqueous solubility of methyl and ethyl salicylates, an attempt has been made to increase it by means of complexation with βCD. These assays were carried out using a spectrophotometric method.

Molecular modeling methods by molecular mechanics (MM), quantum mechanics (QM) and/or molecular dynamics (MD) are frequently used for deriving information on the geometry and energy interaction of the inclusion compounds.

No complex stability constants values between MS and/or ES with βCD can be found in literature. The aims of this work were the use of two different experimental methods to estimate the stability constants of the respective inclusion compounds and their comparison with theoretical results.

2. Materials and methods

2.1. Materials

β-Cyclodextrin (βCD) was purchased from MP Biomedicals, methyl salicylate (MS) and ethyl salicylate (ES) were purchased from Aldrich. HPLC grade methanol was obtained from Merck. Bidistilled water was purified by using a Super Q Millipore System, with conductivity lower than 1.8 μS cm^{-1}. Methanol (MeOH) and water were also degassed and filtered with Minisart RC filters (0.5 μm).

2.2. HPLC measurements

Chromatographic experiments were performed using a Gilson 322 series pump with HPLC temperature controller ThermaSphere TS-130, a Rheodyne 7725i sample injector and a Gilson 152 UV–vis detector (302.5 nm for methyl and ethyl salicylates). The system was controlled by UniPoint v2.10 (Gilson) software. The analysis was carried out with a reverse-phase column Luna 18(2), 5 μm, 250 mm × 4.6 mm. Column temperature was maintained constant at 30 °C. Chromatographic studies were performed with a mobile-phase of MeOH:water at different ratios with a relative permittivity range of 46–54 (55:45 MeOH:H_2O to 70:30 MeOH:H_2O, v/v). Several amounts of βCD were dissolved in the mobile-phase (1–9 mM). The concentration of the solutes in the injected solution was 0.4 mM and the volume of injection was 20 μL. The mobile-phase was pumped at a flow rate of 1.1 mL/min. The capacity factor (k) was measured from chromatographic data, $k = (t_R - t_o)/t_o$. The mobile-phase holds up times were measured by injecting a solution of $K_2Cr_2O_7$ (1 mM). The HPLC data were obtained as an average of three measurements for each determination.

2.3. UV–vis measurements

Excess volumes of MS and ES were added to 20 mL glass tubes containing 5 mL of aqueous solutions of different βCD concentrations. The tubes were placed in a water bath at constant temperature (27 °C) and shaken until equilibrium was reached (24 h). The solutions were then filtered and diluted. The concentrations of MS and ES were spectrophotometrically determined at 302.5 nm using a Shimadzu UV-160A spectrophotometer with temperature control. All studies were carried out in duplicate.

2.4. Computational details

The theoretical calculations were performed using the GAUSSIAN 03 software package [18]. The initial molecular geometries of βCD, guest molecules and inclusion complexes were fully optimized using the AM1 [19] (Austin Model vs.1) and PM3 [20] (Parametric Model 3) methods. The corresponding frequencies were calculated to ensure that the obtained stationary points were true minima. These semiempirical methods are very convenient for the modeling of large molecular systems, such as cyclodextrin inclusion complexes [21–23], and they are much less time-consuming compared to *ab initio* methods.

Although PM3 seems to perform better than AM1 in biochemical systems due to its enhanced description of the interactions of non-bonded atoms [24], both methods were applied here since the study of cyclodextrin complexes with the AM1 method is widely reported in the bibliography [25–28].

The initial structures of methyl and ethyl salicylates were constructed using the CS Chem3D (Version 5.0) program [29]. Initial βCD coordinates were obtained from crystallographic data [30]. In order to simulate the inclusion process, the glycosidic oxygens of βCD were placed onto the XY plane and their center was defined as the center of the coordination system. The βCD was then kept in this position while the guest molecule was introduced along the *Z*-axis into the βCD cavity.

The relative position between the host and the guest molecules was measured by the distance along the *Z*-axis of the salicylates C1 atom to the coordination center. Inclusion was emulated by manually moving the guest molecule from 6 to −3 Å, with a stepwise of 1 Å. For each step the geometry of the complex was fully optimized using semiempirical AM1 and PM3 methods.

Two possible orientations were considered for the inclusion process of each guest. The "head down" orientation, were the alkyl group of salicylate points toward the primary hydroxyls of the βCD cavity, and the "head up" orientation where the alkyl group points toward the secondary hydroxyls of the βCD cavity. The stabilization energy (ΔE) of the salicylates with βCD was calculated as the difference between the energy of the inclusion complex ($E_{S:\beta CD}$), and the sum of the energies of the free salicylate (E_S) and free βCD ($E_{\beta CD}$):

$$\Delta E = E_{S:\beta CD} - (E_s + E_{\beta CD}) \quad (1)$$

3. Results

3.1. HPLC results

The retention behavior of the solutes in RP-HPLC is governed by their partition coefficients between the mobile and stationary phases. In presence of cyclodextrins there is an additional contribution which is the complexation process. The capacity factors for MS

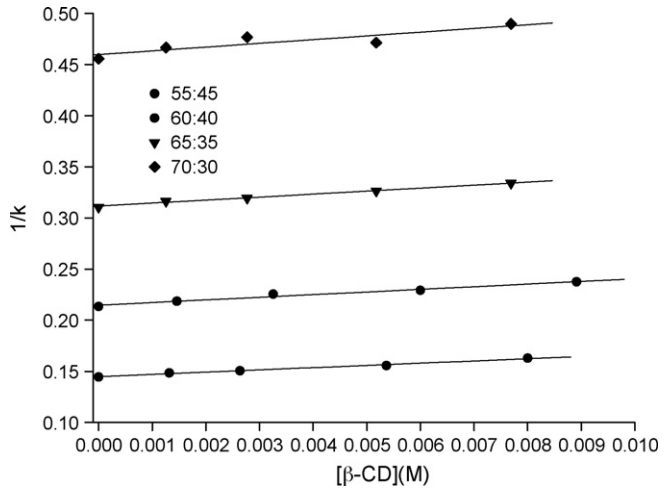

Fig. 1. Plot of $1/k$ vs. βCD concentration for MS at various percentages MeOH:H_2O at 30 °C.

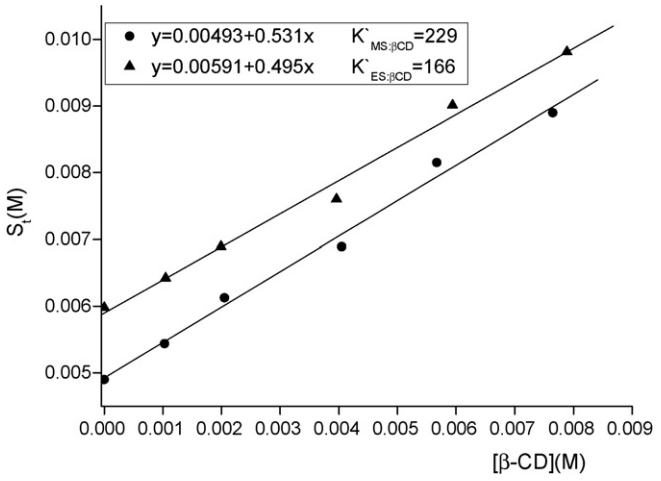

Fig. 2. Solubility diagrams of MS and ES with βCD in aqueous solution at 27 °C.

and ES were monitored in the presence of increasing concentrations of βCD.

Assuming that MS and ES form a 1:1 inclusion complex with βCD, the following equilibrium can be written:

$$S + \beta CD \leftrightarrow S - \beta CD \quad (2)$$

and the formation constant ($K_{S:\beta CD}$) of the complex is

$$K_{S:\beta CD} = \frac{[S - \beta CD]}{[S][\beta CD]} \quad (3)$$

The relationship between the capacity factor and βCD concentration in mobile-phase is given by

$$\frac{1}{k} = \frac{1}{k_0} + K_{S:\beta CD}\frac{[\beta CD]}{k_0} \quad (4)$$

where k is the capacity factor of the solute at the concentration of βCD in mobile-phase, k_0 the capacity factor in the absence of βCD, and $K_{S:\beta CD}$ the apparent formation constant or apparent stability constant [31].

When $1/k$ is plotted versus [βCD], a linear relationship was observed, reflecting a 1:1 drug/CD complex. The slope/intercept ratio gives the value of $K_{S:\beta CD}$.

The formation constants of the complexes between the studied salicylates and βCD have been calculated. The linear relationship between $1/k$ and [βCD] is shown for MS in Fig. 1. It indicates that the behavior of these compounds is well described by the model assuming 1:1 stoichiometry between the guest and βCD. Table 1 reports the apparent constants obtained for MS and ES.

3.2. Solubility studies

The stoichiometry of the drug/cyclodextrin complexes and the numerical values of their stability constants are frequently obtained from phase-solubility diagrams which plot drug solubility versus βCD concentration. Linear phase-solubility diagrams (A_L-type systems) indicate that the complex is first order with respect to cyclodextrin and first order with respect to the drug. The experimental data obtained from UV–vis measurements fit the following equation [32,33]:

$$S_t = S_0 + \frac{K'_{S:\beta CD} S_0 [\beta CD]}{1 + K'_{S:\beta CD} S_0} \quad (5)$$

where S_0 is the molar solubility of MS and ES, S_t is the molar solubility of drugs in presence of βCD, and $K'_{S:\beta CD}$ is the stability constant for the complexes. In this case, the slope is always less than unity [34]. The apparent stability constant of the complex formed, $K'_{S:\beta CD}$ can be obtained from the slope and the intercept of the straight line.

Fig. 2 shows the apparent stability constants, and the diagrams obtained present a linear increase of solubility with the concentration of βCD so they can be classified as A_L type.

3.3. Molecular modeling

In order to find the stabilization energies (Eq. (1)), the resulting complexes with the lowest energy were used. Fig. 3 illustrates the structures of MS-βCD complexes with minimum energies for both orientations. As listed in Table 2, the negative ΔE changes upon complexation demonstrate that ES forms a more stable complex with βCD than MS.

It can also be seen that the head-down orientation was more favorable than head up for both complexes. AM1 and PM3 calculations show the same results. However, Table 2 shows that the PM3 stabilization energies are lower than the corresponding AM1 energies.

Taking into consideration that both PM3 and AM1 are parameterized methods, the first one presents an improved description and

Table 1
Apparent formation constants, $K_{S:\beta CD}$ (M^{-1}) for drug/βCD complexes at 30 °C determined by HPLC

MeOH (%)	ε	MS				ES			
		Slope	Intercept	R	$K_{MS:\beta CD}$	Slope	Intercept	$K_{ES:\beta CD}$	R
55	53.61	2.293	0.1448	0.996	15.84	0.897	0.0705	12.73	0.956
60	51.28	2.579	0.2147	0.989	12.01	1.228	0.1135	10.82	0.949
65	48.89	2.926	0.3116	0.997	9.39	1.452	0.1606	9.041	0.990
70	46.44	3.165	0.4600	0.900	6.88	1.945	0.2713	7.169	0.999

Table 2
Interaction energies and thermodynamic properties for the inclusion complexes of βCD with MS and ES

	MS:βCD				ES:βCD			
	AM1		PM3		AM1		PM3	
	Head down	Head up	Head down	Head up	Head down	Head up	Head down	Head up
ΔE (kJ/mol)	−34.0	−26.6	−33.9	−31.8	−37.6	−22.4	−44.9	−38.2
ΔH° (kJ/mol)	−27.4	−21.0	−27.4	−26.3	−30.8	−16.3	−36.9	−31.8
ΔG° (kJ/mol)	38.2	35.21	37.8	38.6	45.2	47.0	41.8	32.9
ΔS° (J/mol)	−220.0	−188.5	−218.5	−217.8	−254.9	−212.4	−264.1	−216.9

parameterization between non-bonded atoms, hydrogen bonds and steric effects for this kind of chemical systems [24]. This fact might be the reason for the lower stabilization energies obtained with the PM3 method.

Table 2 also shows the thermodynamic parameters: enthalpy changes (ΔH°), entropy contribution (ΔS°) and Gibbs free energy (ΔG°) for the association of MS and ES with βCD. It can be observed that both MS and ES drugs bind to βCD with a favorable enthalpy term ($\Delta H^\circ < 0$). Furthermore, for both complexes, enthalpy changes for the head-down orientation are more negative than for the head up orientation. The ΔS° for the MS:βCD and ES:βCD inclusion process was found to be negative. The positive ΔG° value can be attributed to the underestimation of the calculated ΔS°. There is a gain in entropy in the assimilation of the solvation water molecules by the medium after inclusion takes place [35], and this effect is neglected in the semi-empirical calculations. Therefore, the Gibbs free energy obtained from semiempirical calculations has no absolute meaning and should be considered only in a comparative way.

4. Discussion

In HPLC analysis, as expected, retention times decrease as the concentration of βCD in the mobile-phase increases, due to the formation of drug–cyclodextrin complex, which enhances the guest solubility in the mobile-phase and reduces its residency time in the column.

Since the mobile-phase is an aqueous methanolic solution, the possible encapsulation of the MeOH molecule by the cyclodextrin, in competition with the drug:βCD complex formation must be considered. However, values reported in the literature for $K_{MeOH:\beta CD}$ can be neglected. As a consequence, all of the effects observed in the presence of alcohols may only be attributed to a change in the solvophobic characteristics of the medium which affect the affinity of an apolar drug in binding cyclodextrin [36].

The values of $K_{MS:\beta CD}$ and $K_{ES:\beta CD}$ reported in Table 1 are plotted as a function of dielectric constant (ε) in Fig. 4. As can be seen, the apparent stability of complexes decreases in a linear relationship as the hydrophobicity of the mobile-phase increases, due to the increase in the percentage of MeOH. It can be observed that $K_{MS:\beta CD} > K_{ES:\beta CD}$, however, for percentages of MeOH higher than 67.5% or dielectric constants (ε) lower than 47.8 this relationship is inverse, $K_{ES:\beta CD} > K_{MS:\beta CD}$. This fact reveals a clear contribution of the hydrophobic effect as a driving force for both complexation processes.

The formation of inclusion complexes of MS and ES with βCD involves solubility enhancements. This fact has been widely employed for improving the bioavailability of drugs. The results in

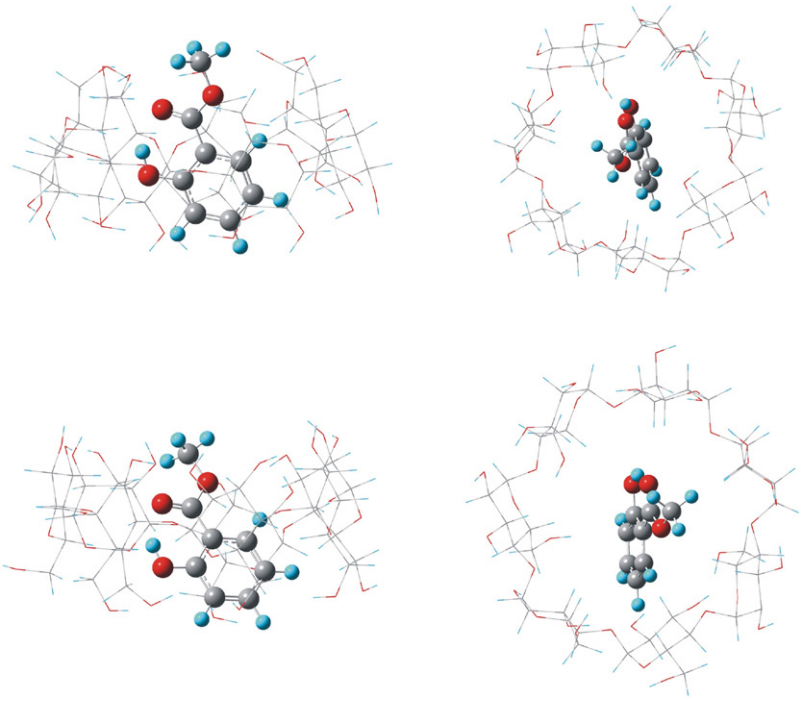

Fig. 3. Structures of the MS:βCD complex with minimum energy obtained by PM3 calculations at different orientations. Top left: head down perpendicular to the cavity axis; top right: head down along the cavity axis; bottom left: head up perpendicular to the cavity axes; bottom right: head up along the cavity axis.

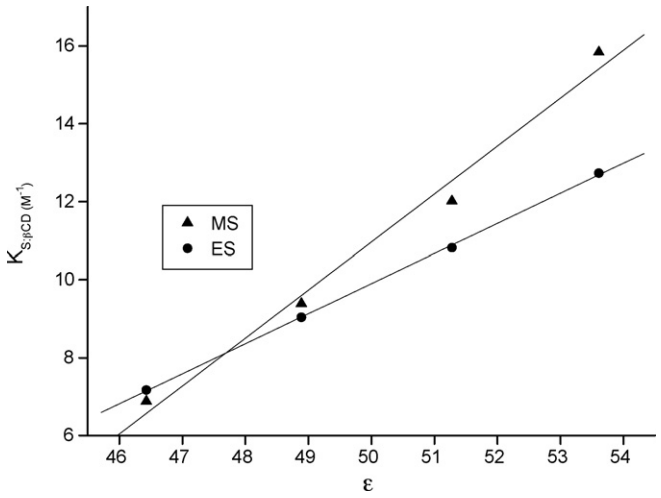

Fig. 4. $K_{S:\beta CD}$ (MS and ES) values vs. dielectric constant (ε) determined by HPLC.

Table 1 demonstrate that the apparent stability constant for MS is clearly higher than that for ES. It can be seen that the slopes of the solubility phases diagrams are less than 1.

Semiempirical quantum chemical calculations (using the AM1 and PM3 methods) were applied to the study of the complexation of βCD with MS and ES. The preferred orientation for the inclusion complexes obtained for both methods was head down for MS and ES because it is the most energetically favorable structure. Values of ΔH° and ΔS° were obtained for the inclusion processes and while these results indicate that the inclusion of MS and ES into the βCD cavity is favored by the enthalpy ($\Delta H^\circ_{MS:\beta CD} \approx -27$ kJ/mol; $\Delta H^\circ_{ES:\beta CD} \approx -37$ kJ/mol), the processes are entropically unfavorable ($\Delta S^\circ_{MS:\beta CD} \approx -219$ J/mol; $\Delta S^\circ_{ES:\beta CD} \approx -264$ J/mol). Therefore, the balance between the hydrophobic effect ($\Delta H^\circ \approx 0$; $\Delta S^\circ > 0$) and the van der Waals forces ($\Delta H^\circ < 0$; $\Delta S^\circ < 0$) seem to be inclined towards the latter [22].

5. Conclusion

Using the HPLC and UV–vis measurements, we found that both MS and ES form inclusion complexes with βCD with 1:1 stoichiometries. Values of complex apparent formation constants were determined and $K_{MS:\beta CD} > K_{ES:\beta CD}$. We also determined that $K_{S:\beta CD}$ decreases as the dielectric constant decreases. However, the energy differences for the complexes obtained in the AM1 and PM3 calculations in vacuum indicate that βCD forms more stable complexes with ES than it does with MS. For both MS and ES, $K_{S:\beta CD}$ obtained for solubility phases diagrams in aqueous medium ($\varepsilon \approx 80$) > $K_{S:\beta CD}$ obtained for HPLC ($\varepsilon = 56$–47) and $K_{MS:\beta CD} > K_{ES:\beta CD}$. For $\varepsilon \leq 47.8$, $K_{ES:\beta CD} > K_{MS:\beta CD}$. This fact can be clearly seen in Fig. 4. By theoretical calculations ($\varepsilon = 1$) $K_{ES:\beta CD} > K_{MS:\beta CD}$. This fact demonstrates that the hydrophobic effect plays a key role in the inclusion process.

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