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^{1}H-Nuclear Magnetic Resonance and Phase Solubility Studies of the Stoichiometries in 2,4-D: α- and β-Cyclodextrins Inclusion Complexes

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Abstract. The interaction in solution between 2,4-dichlorophenoxyacetic acid with α- and β-cyclodextrins was evaluated by phase solubility studies. Association constants were calculated by this technique. The stoichiometries were 1 : 2 and 1 : 1 for the α- and β-cyclodextrin complexes, respectively. In order to corroborate the complexation and the knowledge of structural aspects of the host : guest interaction, proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy was employed. The application of the continuous variation technique corroborated the calculated complex stoichiometries by solubility assays. Complementary NOE studies were applied in order to corroborate the proposed complex structures.

Key words: solubility, ^{1}H-NMR, stoichiometry, cyclodextrins

1. Introduction

2,4-Dichlorophenoxyacetic acid (2,4-D) is a systemic herbicide widely used for weed control in cereals and other crops. The 2,4-D molecule is characterised by the presence of two groups: the aromatic ring and the aliphatic chain and the use of cyclodextrin (CD) complexation improved its solubility in water [1].

Nuclear magnetic resonance spectroscopy (^{1}H-NMR) allows a clear distinction between true inclusion and any other possible external interaction between CDs and guests [2]. It is interesting to determine the extent of molecular interaction between host and guest molecules in a quantitative fashion. Knowledge of the stoichiometry and equilibrium constants in solution for inclusion complexes will allow a prediction of the further dissolution behaviour. The aim of this paper is to investigate the stoichiometries, the nature of the interactions and a structural

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approach for the 2,4-D complexes with α- and β-CDs in solution, by using phase solubility techniques [3] and ^{1}H-NMR spectroscopy.

2. Experimental

2,4-D was supplied by Sigma (St. Louis Missouri, USA) and α- and β-CD by Ringdex (Paris, France). D_2O was purchased from SDS (Barcelona, Spain). All other materials were of analytical reagent grade.

2.1. PHASE SOLUBILITY STUDIES

The solubility studies were carried out according to the method reported by Higuchi and Connors [3]. Excess of 2,4-D (50 and 20 mg for the α- and β-CD assays, respectively) were accurately weighed into 50 mL Erlenmeyer flasks, adding 10 mL of unbuffered water containing increasing concentrations of α-CD (0.01–0.1 M range) and β-CD (0.002–0.030 M range). In the β-CD system, with the purpose of making the curve asymptotic, it was necessary to add an excess of β-CD much greater than the solubility limit (0.016 M). These flasks were sealed and shaken at 25 °C for one week. After equilibrium, the samples were filtered with a syringe through a 0.22 μm Millipore cellulose nitrate membrane filter, properly diluted and analysed spectrophotometrically at 284 nm (Hitachi U-2000).

2.2. ^{1}H-NMR SPECTROSCOPY

^{1}H-NMR experiments were run at 25 °C using a Bruker AC 200 spectrometer operating at 200 MHz. The concentrations employed were: 0.5 mg/mL of 2,4-D; 4.88 mg/mL of α-CD; 3.0 mg/mL of β-CD; 5.38 mg/mL of 2,4-D-α-CD binary system (1 : 2) and 3.5 mg/mL of 2,4-D-β-CD binary system (1 : 1). The conditions were: acquisition time 2.818 μs; pulse width 5 μs; time domain 1.6 K; spectral width 2906.97 Hz.

A continuous variation method [4] was performed in order to confirm the results that the former studies revealed about the stoichiometry of the complexes. The sum of the concentrations of both components was kept constant ([2,4-D] + [CD] = 4.52 mM) and the molar fraction of each component (r = [2,4-D]/([2,4-D] + [CD]) ranged between 0 and 1. In order to calculate the stoichiometry, the chemical shift variations (ppm) of the H3 and H5 CD signal protons ($\Delta\delta$) were plotted versus the molar fraction (r).

NOE difference measurements were carried out during *steady-state* experiments, by irradiation of the H3 signal of CDs, at a temperature of 25 °C. The solutions employed were the same as for the NMR experiments.

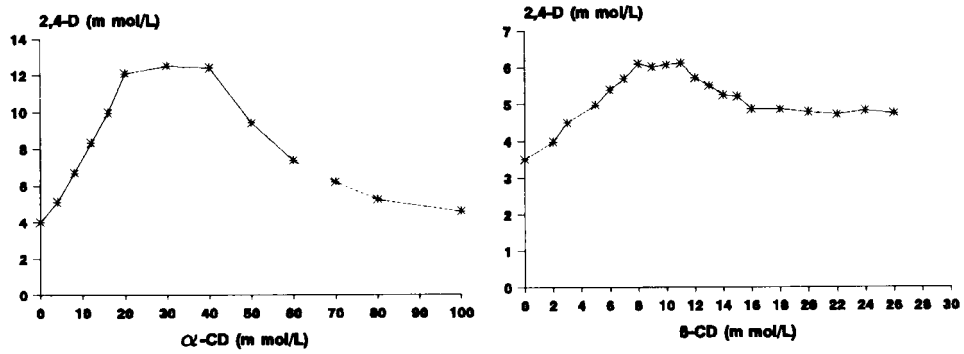


Figure 1. Phase solubility diagrams of the 2,4-D-α-CD and 2,4-D-β-CD systems in water at 25°C.

Table I. Precipitated complex stoichiometry (R) and different parameters of the solubility assay

	S_T (mM)	S_M (mM)	S_C (mM)	L_C (mM)	S_C/L_C	R
α-CD	22.64	12.41	10.23	20	0.51	1 : 2
β-CD	9.06	6.1	2.96	3	0.98	1 : 1

3. Results and Discussion

3.1. PHASE SOLUBILITY STUDIES

The phase solubility diagrams obtained for 2,4-D with α- and β-CD are shown in Figure 1. According to Higuchi and Connors, both diagrams can be classified as B_S type. An insoluble microcrystalline complex was formed from the solution at 0.020 M for the α-CD system and 0.009 M for the β-CD one.

Therefore, the 2,4-D content of the precipitated complex (S_c) is equal to the difference between the total amount of 2,4-D added to the system (S_T) and the pesticide in solution when the plateau region is reached (S_M). The CD content in the complex (L_c), corresponds to the CD concentration during the plateau region. So, the precipitated complex stoichiometry could be expressed as follows:

$$R = S_c/L_c. \tag{1}$$

Table I reports the different calculated values from the solubility assay for both systems.

The precipitates have the stoichiometries SL_2 for the α-CD complex (two α-CD molecules are involved for the inclusion of one 2,4-D molecule) and SL for the β-CD complex.

The solubility of the α-CD complex (8 mM), estimated from the initial straight line portion, is different from that at high α-CD concentrations (4 mM). The fact

that the ascending part of the solubility curve has a variable positive tendency (it can be ascribed to an A_P model), indicates that more than one complex is being formed simultaneously in solution, i.e., SL and SL_2. In this zone, however, the prevalent complex is SL, while the SL_2 complex formation is responsible for the descending zone on the diagram. Similar examples were reported in the literature [5, 6].

A more accurate description of the initial rise in the solubility diagram for α-CD, assuming the formation of the two complexes SL and SL_2, characterised by $K_{1:1}$ and $K_{1:2}$ stability constants, may be achieved according to the following equilibrium:

$$\begin{array}{ccccc} & & K_{1:1} & & K_{1:2} \\ S+ & L & \leftrightarrow SL & + & L \leftrightarrow SL_2 \end{array} \tag{2}$$

where S, L, SL, and SL_2 are the concentrations of the 2,4-D, α-CD, 1 : 1 complex and 1 : 2 complex, respectively, with $K_{1:1} = (SL)/(S)\ (L)$ (Equation (1)) and $K_{1:2} = (SL_2)/(SL)\ (L)$ (Equation (2)).

The material balance equations are:

$$S_T = (S) + (SL) + (SL_2) \tag{3}$$

with $S = (S_0)$, and

$$L_T = (L) + (SL) + 2(SL_2). \tag{4}$$

S_T and L_T being the total concentrations of the substrate and ligand, respectively. If Equations (1)–(3) are combined and, if it is assumed that the extent of complexation is fairly small it may be permissible to set $(L) \approx (L_T)$ (see Equation (4)), and we obtain the following Equation (5):

$$S_T - S_0/L_T = K_{1:1}S_0 + K_{1:1}K_{1:2}S_0L_T. \tag{5}$$

Plotting $(S_T - S_0)/L_T$ against L_T, a straight line was obtained. The calculated values for $K_{1:1}$ and $K_{1:2}$ from the slope and intercept point were $K_{1:1} \approx 94.5\ \mathrm{M}^{-1}$ and $K_{1:2} \approx 6.5\ \mathrm{M}^{-2}$.

On the other hand, in the β-CD system, the precipitated complex shows the same stoichiometry (1 : 1) and similar solubility (4.5 mM) to the complex formed in solution. Thus, only one complex (SL) is present along the curve. In this case, the apparent 1 : 1 stability constant K_C can be calculated from the initial straight line portion of the phase solubility diagram, following the well-known equation proposed by Higuchi and Connors [3]. From this study, the K_C of the complex was found to be equal to $336\ \mathrm{M}^{-1}$.

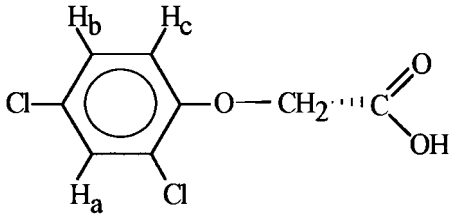


Figure 2. Structure and proton assignments for the 2,4-D molecule.

Table II. ^{1}H Chemical shifts corresponding to free and complexed (1 : 2) 2,4-D and α-CD

2,4-D	δ_{free}	$\delta_{complex}$	$\Delta\delta$ (ppm)
Ha	7.361	7.381	0.020
Hb	7.147	7.186	0.039
Hc	6.799	6.768	−0.031
CH_2	4.544	4.494	−0.050
α-CD			
H2	3.508	3.506	−0.002
H3	3.808	3.766	−0.042
H4	3.407	3.399	−0.008
H5	3.635	3.673	0.038
H6	3.708	3.717	0.009

3.2. ^{1}H-NMR SPECTROSCOPY

Figure 2 summarises the peak assignments of 2,4-D. The molecular structure is characterised by the presence of two complexing moieties that are potentially able to interact with the CD cavity, i.e., the aromatic ring and the aliphatic chain. Tables II and III report the chemical shift values of 2,4-D and α-CD and β-CD binary systems.

The use of the continuous variation technique helps us to confirm the complex stoichiometry (Figure 3). For the β-CD complex the maximal shift variations are observed at $r = 0.5$, showing a highly symmetrical shaped diagram. It verifies that the maximal interaction occurs at 1 : 1 (mol : mol) ratio. For the α-CD complex, however, the maximum displacement value appears at $r = 0.333$, establishing the maximal interaction at 1 : 2 (mol : mol) ratio.

From the results reported in Table II, the upfield (negative difference) shift of the signal corresponding to H3 of the α-CD can be observed. This displacement is due to the anisotropic magnetic effect induced by the presence of the aromatic group of the guest molecule. Quite similar results were found by Fronza et al. [7]

Table III. ^{1}H Chemical shifts corresponding to free and complexed (1 : 1) 2,4-D and β-CD

2,4-D	δ_{free}	$\delta_{complex}$	$\Delta\delta$ (ppm)
Ha	7.360	7.360	0.000
Hb	7.158	7.160	0.002
Hc	6.790	6.775	−0.015
CH_2	4.521	4.500	−0.021
β-CD			
H2	3.489	3.496	0.007
H3	3.807	3.771	−0.036
H4	3.399	3.381	−0.018
H5	3.673	3.641	0.032
H6	3.724	3.695	0.029

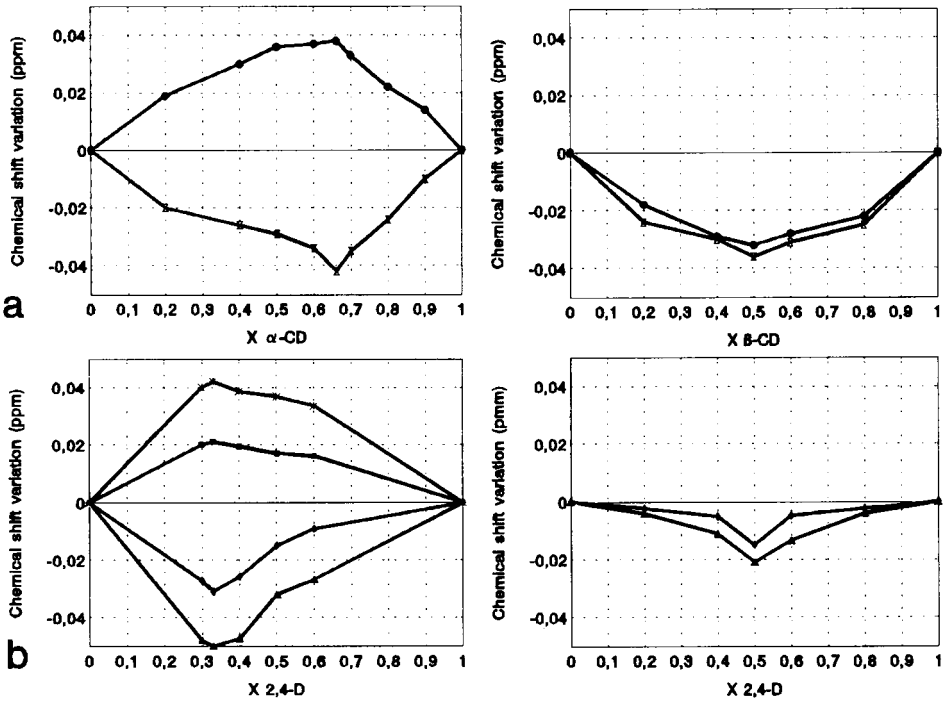
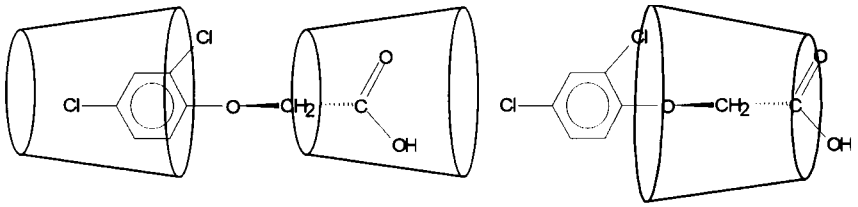


Figure 3. Continuous variation plots for: (a) H3 (▼) and H5 (●) CD protons; (b) Ha (■), Hb (★), Hc (◊) and CH_2(△) 2,4-D protons.



a) **b)**

Figure 4. Proposed structures for the 2,4-D complexes with α-CD (a) and β-CD (b).

for the piroxicam-β-CD system. Besides, it shows a downfield shift for the H5 protons of the α-CD. This behaviour can be ascribed to the presence of the halogen chlorine atom in position 4, experiencing van der Waals deshielding. Other authors observed a similar phenomenon with the iodine atom [8]. Also, H5 protons are in a zone where a deshielding by the ring current of the dichlorophenoxy moiety is expected. Thus, the sum of both effects is responsible for this downfield (0.038 ppm) shift.

In consequence, these results indicate that penetration of the aromatic moiety of 2,4-D is achieved through the wider side of the α-CD cavity. It also seems reasonable since the presence of the chlorine atom in position 2 makes unlikely a penetration through the narrow side of the CD, due to its steric hindrance. Moreover, the presence of the chlorine substituent at position 2 makes incomplete the penetration of the aromatic ring into the CD cavity. For 2,4-D, the chemical shifts corresponding to Ha and Hb go downfield, which is in accordance with their position, situated into the CD cavity. The Hc and aliphatic chain signals go upfield due to the local polarity of the 2,4-D molecule. For the aliphatic chain it is not clear if the cavity side is involved in the interaction with the second CD. For steric reasons, it seems more probable that interaction through the narrow side of this one, where a small distance between the host and guest atoms is more favourable to the establishment of non-covalent interactions. Figure 4 reports the proposed structure for this complex.

In the case of the β-CD system (see Table III), upfield shifts were observed for the H3, H4, H5 and H6 signals, the changes registered for the H3, H5 and H6 ones being more relevant. For the 2,4-D molecule, the changes correspond to the signals of the aliphatic moiety and the Hc proton of the aromatic one, the signals of the Ha and Hb protons remain unmodified. This fact, together with the modification of the H6 proton signal, can be ascribed to the entrance of the 2,4-D molecule by the narrow side of the CD cavity, with an incomplete penetration of the aromatic ring. The proposed orientation is reported in Figure 4, where one molecule of 2,4-D interacts with only one molecule of β-CD.

With the aim of confirming these hypotheses, NOE difference studies were carried out. In the case of the 2,4-D-α-CD complex, the most relevant NOE effects upon H3 irradiation corresponded to the Ha and Hb aromatic protons (1.1

and 1.0%, respectively), being lower for the Hc signal (only 0.6%). These results indicate that complexation takes place through the wider side of the α-CD ring.

On the other hand, the H3 irradiation for the 2,4-D-β-CD system does not produces significant NOE effects on the aromatic proton signals of the guest, demonstrating the low participation of the ring in the inclusion process.

These NOE results are consistent with the chemical shift variations observed in the NMR section, thus confirming the above-mentioned structures of the complexes. Only the interaction of the second CD molecule in the case of the 2,4-D-α-CD complex is not completely clear, where parallel or antiparallel orientation of the α-CD which interacts with the aliphatic moiety of 2,4-D is possible.

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