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Studies on aqueous solubility of 3,3′-diindolylmethane derivatives using cyclodextrin inclusion complexes

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HIGHLIGHTS

- 3,3′-Diindolylmethanes (DIMs)-cyclodextrin (CD) inclusion complexes were prepared.
- Spectroscopic analyses confirmed the supramolecular interaction of complexes.
- 1:1 Stoichiometry of the complexes of DIM-(α, β, γ)-CDs have been observed.
- Aqueous solubility of DIMs in presence of CDs has been enhanced considerably.

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ABSTRACT

The supramolecular interaction of 3,3′-diindolylmethane (DIM) and its derivatives with α-, β-, and γ-cyclodextrins (CDs) has been investigated to improve their aqueous solubility. A series of complexes of α-, β-, and γ-CDs with DIMs were prepared and their inclusion complexation behavior in solution phase assessed by fluorescence, UV–visible spectroscopy and circular dichroism. Circular dichroism spectra revealed that incorporation of DIMs in the chiral environment of the CDs affecting their electric transitions leading to the development of induced circular dichroism bands in the near UV region. A linear increase of aqueous solubility of DIMs in presence of CDs was observed from their phase–solubility diagrams indicating the formation of soluble inclusion complexes. According to the continuous variation method a 1:1 stoichiometry has been proposed for DIM cyclodextrins complexes.

1. Introduction

3,3′-Diindolylmethane (DIM), the most important self-condensation product of indole-3-carbinol (I3C), is found in the acidic environment of the stomach upon ingestion of I3C. The I3C is formed by acid hydrolysis of glucobrassicin present in common vegetables of the genus *Brassicca* such as cabbage, kale, brussels sprouts, and broccoli [1]. DIM has been the subject of much research due to its potential ability in promoting healthier estrogen metabolism in patients with thyroid proliferative disease [2], preventing of human breast cancer [3], and prostate cancer development in the transgenic adenocarcinoma mouse prostate model [4]. Our recent studies have shown that DIM and its derivatives are good plant growth promoters [5] and potent inhibitors of *Leishmania donovani* topoisomerase I [6]. DIM and its derivatives are required in very high doses to provide satisfactory biological activity due to their low aqueous solubility. Therefore, seeking an efficient and nontoxic carrier for these DIM derivatives to overcome the problems of bioavailability has become inevitable.

Among the various compounds generally used as drug carrier, one of the most important ones are the cyclodextrins (CDs) [7], a class of biodegradable cyclic oligosaccharides, mainly with six to eight D-glucose units linked by α-1,4 glycosil units represented as a truncated cone structure with a hydrophobic cavity [8]. Popular feature of CDs is the marked difference of polarity between the internal and external surfaces; the inner part is made nonpolar by the glycosidic oxygen and methylene protons, whereas the external surface is polar by virtue of the presence of secondary and primary hydroxyls on the large and small rims, respectively, thus allowing their solubility in water [9]. Furthermore, this particular structural feature of CDs allows various organic and

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biological guests within their hydrophobic cavities to afford typical host–guest complexes in aqueous solution [10–12]. A number of inclusion complexes of cyclodextrins with drugs like paclitaxel [13] and cinchona alkaloids [14] resulting in increasing their aqueous solubility have been reported.

Herein, we report inclusion complexes of DIM derivatives **1–6** (Fig. 1), previously synthesized and characterized in our laboratory [15], with α-, β- and γ-cyclodextrins, and characterization of the mode of inclusion and stoichiometry of the complexes by means of absorbance [16], fluorescence [17], circular dichroism and phase–solubility studies.

2. Experimental

2.1. General procedures

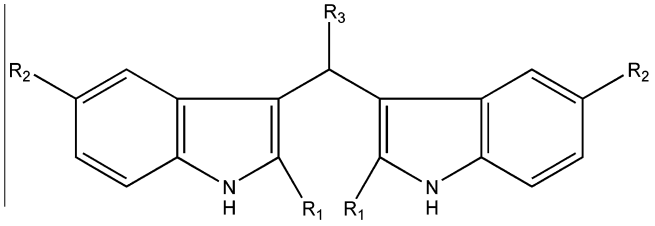
Commercial α-, β- & γ- CDs (Fluka) were dried in a desiccator in vacuo over phosphorus pentoxide at 90 °C for at least 24 h and stored in the same apparatus at 40 °C till use. Guests **5** and **6** were prepared by the reaction of indole (Acros) and corresponding benzaldehyde (Acros) in presence of $InCl_3$ (Aldrich). All other reagents and chemicals were of analytical reagent grade. All solutions were prepared using ultra pure water (MILLIQ). Sodium dihydrogen phosphate and disodium hydrogen phosphate were dissolved in double distilled, deionised water to make a 0.1 (M) buffer solution of pH 7.2, which was used as solvent throughout the measurement. All experiments were performed at 298.15 K.

2.2. Absorption spectral titrations

The inclusion complex formation phenomenon of CDs with guest DIMs in aqueous phosphate buffer solutions was examined at pH 7.2 by means of UV–visible spectral titration in a Shimadzu 1700 Spectrophotometer (Shimadzu Corporation, Kyoto, Japan). A 1.0 mM stock solution of DIMs (5.0 ml) was prepared in methanol, and 6.0 μl of this stock solution was added to the phosphate buffer solution to maintain the final concentration of molecules as 2.5×10^{-7} mol dm^{-3} in the cuvette. Then gradually α-, β-, γ-CD were added in the cuvette so that the concentrations of CDs ranged from 0 to 12.5×10^{-3} mol dm^{-3}. The absorption spectra were measured against an appropriate reagent blank.

2.3. Fluorescence spectroscopy

Fluorescence emission spectra of DIM derivatives (**1–4**) (17×10^{-6} mol dm^{-3}) were measured in presence of α-, β- and γ-CD (0–18.7×10^{-3} mM). 1.0 mM stock solutions of DIMs were prepared in methanol and 3.4 μl of this solution was added to the buffer solution to maintain the final concentration of DIMs as 17.0 μM in 2.0 ml. The α-, β- and γ-CD (1.7 μl) were added gradually to the cell to maintain the concentration of CD's ranged from 0 to 18.7×10^{-3} mM. The solution was kept stirred continuously. Fluorescence emission spectra were acquired on a Shimadzu RF-5301PC fluorimeter (Shimadzu Corporation, Kyoto, Japan) by excitation at the absorption maxima of each compound. Excitation and emission bandwidths were set at 5 nm.



1. R_1, R_2, R_3 = H
2. R_1 = Ph and R_2, R_3 = H
3. R_1 = Me and R_2, R_3 = H
4. R_1, R_3 = H and R_2 = OMe
5. R_1, R_2 = H and R_2 = 2-hydroxy phenyl
6. R_1, R_2 = H and R_2 = 4-(N,N-dimethyl amino) phenyl

Fig. 1. General structure of 3,3′-diindolylmethane derivatives **1–6**.

2.4. Circular dichroism spectroscopy

Circular dichroism spectra of compound **2** (0.5 mM) in presence of α, β, γ cyclodextrin (10.0 mM) were recorded on a JASCO J815 spectropolarimeter (Jasco International Co., Hachioji, Japan) in rectangular quartz cuvettes of 1.0 cm path length at 20 ± 0.5 °C.

2.5. Phase–solubility study

Phase–solubility studies were carried out according to the method reported by Connors [18]. A fivefold molar excess of DIMs were added to the aqueous solutions of α-, β- and γ- cyclodextrins with increasing concentrations of 0.1, 0.2, 0.3, 0.4, and 0.5 mM. The resulting solutions, protected from light by wrapping with black paper, were stirred for 72 h with similar stirring rates. After equilibration, aliquots of the supernatant were filtered through a membrane filter (0.45 μm). The filtrates were then analyzed using UV spectrophotometer at λ_{max} of each compound.

For the determination of stoichiometry of inclusion complexes the total molar concentration of the different DIMs–CD solutions (i.e. the combined concentrations of DIMs and CD) were kept constant (3.0 mM), but the mole fraction of DIMs (i.e. [DIMs]/[DIMs] + [CD]) were varied (0.2, 0.4, 0.6 0.8 mol fraction). The solutions were stirred for 48 h. The absorbance of the resulting solutions was measured at λ_{max} of each compound by using spectrophotometer. The differences in absorbance in the presence of CDs and in the absence of CDs ($\Delta A = A - A_o$) were plotted against molar ratio R; where R = [DIMs]/{[DIMs] + [CD]} for all solutions.

3. Results and discussion

3.1. Absorption spectra

The UV absorption spectrum of **2** in methanolic solution (5×10^{-6} M) exhibited a λ_{max} at 317 nm for $\pi \rightarrow \pi^*$ transition with molar absorptivity of 55.272×10^3 dm^3 mol^{-1} cm^{-1}. Qualitative investigation of the inclusion complexation phenomenon of CDs with guest DIMs was performed at various pH by means of UV–visible spectral titration. The optimum results were obtained at pH 7.2. Table 1 shows the UV spectral wavelength in terms of the ratio of the initial absorbance (A_1) to final absorbance (A_2) and the spectral wave length shift data for all the DIMs and α-, β-, γ- CDs complexes in aqueous phosphate buffer at pH 7.2. Generally, in all cases, maximum absorption bands shifted in presence of CDs, while the absorption intensity gradually decreased with the stepwise addition of CDs. The absorption intensity of compound **2** gradually decreased with the increasing concentration of CDs along with blue shifting of the absorption band. For compound **2** the absorption intensity varied from the initial 0.109 to 0.081 with α-CD and from 0.138 to 0.107 with β- and from 0.137 to 0.099 for γ-CD. This corresponds to 26%, 23% and 28%, respectively, α, β- and γ-CD. This suggests that compound **2** in general experiences harder environmental changes upon inclusion in the narrowest α-CD cavity than in the β-CD and γ-CD cavity, respectively, in agreement with the notion that the effectiveness of non-bonding

Table 1
UV–vis spectral data of DIM–CDs complexes in phosphate buffer solution at pH 7.2 by the excess addition of CDs [conc. 12.5×10^{-3} mol dm^{-3}] to the DIMs [2.5×10^{-7} mol dm^{-3}].

DIMs and DIM–CDs complexes	λ_{max} (nm)	Ratio of change in absorbance (A_1/A_2)[a]
1	292	
1 + α-CD	293	1.39
1 + β-CD	293	1.29
1 + γ-CD	294	1.15
2	317	
2 + α-CD	317	1.35
2 + β-CD	315	1.29
2 + γ-CD	318	1.39
3	276	
3 + α-CD	277	1.56
3 + β-CD	275	1.28
3 + γ-CD	275	1.26
4	293	
4 + α-CD	294	1.59
4 + β-CD	293	1.15
4 + γ-CD	294	1.07
5	276	
5 + α-CD	277	1.36
5 + β-CD	276	1.08
5 + γ-CD	277	1.03
6	295	
6 + α-CD	295	1.32
6 + β-CD	285	2.07
6 + γ-CD	295	1.42

[a] A_1 and A_2 represent the initial and final absorbance values.

interactions in modifying the properties of an included guest strictly depend on the distance from the host inner wall [19].

3.2. Fluorescence spectra

Fluorescence emission spectra of DIM derivatives **1–4** (17×10^{-6} mol dm^{-3}) were measured in presence of α-, β- and γ -CD (0–18.7×10^{-3} mM) in aqueous phosphate buffer at pH 7.2 and 298.15 K. Compounds **5** and **6** were not fluorescent. The maximum absorption wavelengths of the DIMs were chosen for excitation. The excitation and emission maxima of the DIM derivatives **1–6** are presented in Table 2. Accordingly, the maximum excitation and emission wavelengths of compound **2** were 317 and 381 nm, respectively. With the addition of α-, β-, and γ- cyclodextrins to the solution of guest (DIMs, **1–4**), the fluorescence intensity was markedly enhanced (Fig. 2, compound **2**). The increase of fluorescence intensity due to formation of complexes of CDs with DIMs could be explained as the guest molecules were entrapped in the cavity of CDs. The microenvironment around CDs with smaller polarity and stronger rigidity would restrict the freedom of guest molecules inside the cavity and increase the fluorescence quantum yield. Furthermore, the steric hindrance of CDs torus can protect the excited states from non-radiative and quenching process that

Table 2
Excitation wavelength and emission maxima of DIM derivatives **1–6** in phosphate buffer solution at pH 7.2.

Compound	Excitation wavelength (nm)	Emission maxima (nm)
1	292	333
2	317	381
3	276	341
4	293	365
5	276	Non-fluorescent
6	295	Non-fluorescent

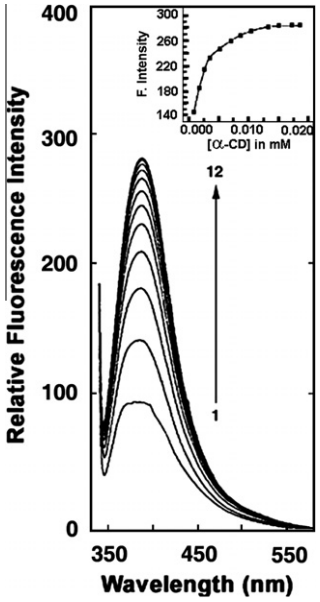


Fig. 2. Fluorescence spectral changes of compound **2** (17×10^{-6} mol dm^{-3}) in presence of α-CD (0–18.7×10^{-3} mM) in aqueous phosphate buffer, pH 7.2 at 298.15 K (the excitation and emission wavelengths were 317 and 381 nm, respectively). The arrows indicate the increase of fluorescence intensity with increasing concentration of α-CD.

normally occur in bulk buffer solution and enhance the fluorescence efficiencies of guest molecules [20]. The fluorescence intensity of **2** gradually increased with increase of concentration of CDs, followed the order, γ > β > α and finally reached a saturation point (Fig. 2, compound **2**).

3.3. Determination of apparent association constant

A very well known way to determine the apparent association constant of host–guest complexes is Benesi–Hildebrand method [21] as per the following equation

$$1/(I-I_o) = 1/[(I_\alpha - I_o)K] + 1/(I_\alpha - I_o) \quad (1)$$

where I is the observed fluorescence intensity of the DIM solutions at each α-, β-, γ-CD concentration tested, I_o and I_α are the fluorescence intensities in the absence of α-, β-, γ-CD and when all the DIMs molecules are complexed. It is presumed that: (i) α-, β-, γ-CDs are in a large excess with respect to DIMs and therefore its free and analytical concentration are the same; (ii) the variation in the fluorescence intensity are proportional to the complex concentration and (iii) at high α-, β- and γ-CD concentration essentially all of the DIMs molecule are complexed.

In case of DIM derivatives, a good linear relationship was obtained when $1/(I–I_o)$ was plotted against 1/[CD] (α-, β-, γ-CD) supported the existence of a 1:1 complex (Fig. 3, compound **2**) [22]. The apparent association constant K of compound **2** was determined to be 4.8×10^5 l mol^{-1} for α-CD and 6.89×10^5 l mol^{-1} for β-CD. In contrary, for γ-CD, the plot is best described by two linear segments each giving association constants of 2.795×10^5 l mol^{-1} and 3.5×10^6 l mol^{-1}. Thus, it can be observed that the DIM compounds form only one type of complex with 1:1 stoichiometry with all three cyclodextrins.

Change in Gibbs energy ($\Delta G°$) associated with various DIM–CD complexations was evaluated from their respective K values. Table 3 reveals that there is a strong host–guest binding ability between cyclodextrins and DIMs quantitatively. It can be concluded

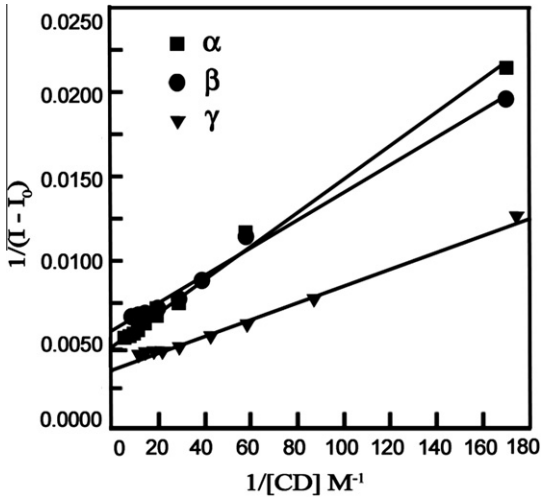


Fig. 3. The change of $1/(I–I_o)$ with 1/[α-CD] (■), 1/[β-CD] (●), 1/[γ-CD] (▲) for DIM–CD complexes for compound **2**.

Table 3
Complex association constant K ($dm^3\ mol^{-1}$) and Gibbs energy change ($\Delta G°$) for inclusion complexation of host CDs with DIMs in aqueous buffer solution (pH 7.2) at 298 K.

Drug	Drug + CD-complexes	Association constant K ($dm^3\ mol^{-1}$)	Gibbs energy change ($\Delta G°$) (kJ mol^{-1})
1	**1** + α-CD	$K_1 = 4.2 \times 10^3$	−20.58
	1 + β-CD	$K_1 = 2.0 \times 10^4$	−24.53
	1 + γ-CD	$K_1 = 1.9 \times 10^4$	−24.40
2	**2** + α-CD	$K_1 = 4.8 \times 10^5$	−32.42
	2 + β-CD	$K_1 = 6.9 \times 10^5$	−33.31
	2 + γ-CD	$K_1 = 2.8 \times 10^5$	−31.07
3	**3** + α-CD	$K_1 = 17.2 \times 10^5$	−35.55
	3 + β-CD	$K_1 = 12.9 \times 10^5$	−34.86
	3 + γ-CD	$K_1 = 8.6 \times 10^5$	−33.86
4	**4** + α-CD	$K_1 = 1.52 \times 10^4$	−23.86
	4 + β-CD	$K_1 = 2.44 \times 10^4$	−25.03
	4 + γ-CD	$K_1 = 7.5 \times 10^4$	−27.82

that these complexation process is highly feasible, which is evident from the negative values of $\Delta G°$ of different DIM–CD complexes.

3.4. Circular dichroism spectroscopy

Induced circular dichroism spectroscopy (ICD) study was performed for compound **2** as a representative case in the presence of CDs. Optical activity was induced at ~317 nm for **2** manifested by the appearance of a positive peak and the shape of the ICD spectra is similar to the corresponding absorption spectra (Fig. 4). The changes in optical activity of compound **2** could be attributed to the perturbation its electronic transition caused its inclusion in the cavity of CDs following complexation. According to Harata's rule, a positive ICD effect is expected for a parallel alignment of the electric transition dipole moment relative to the CD axis, while a negative effect should result from a perpendicular alignment [23]. Thus, the results of circular dichroism spectroscopy revealed that compound **2** is embedded in the asymmetric locus of CDs cavity.

3.5. Solubility studies

Determination of the stability constants from the phase-solubility diagram is a widely accepted method for understanding the effect of CDs complexation on the drug solubility [18]. From

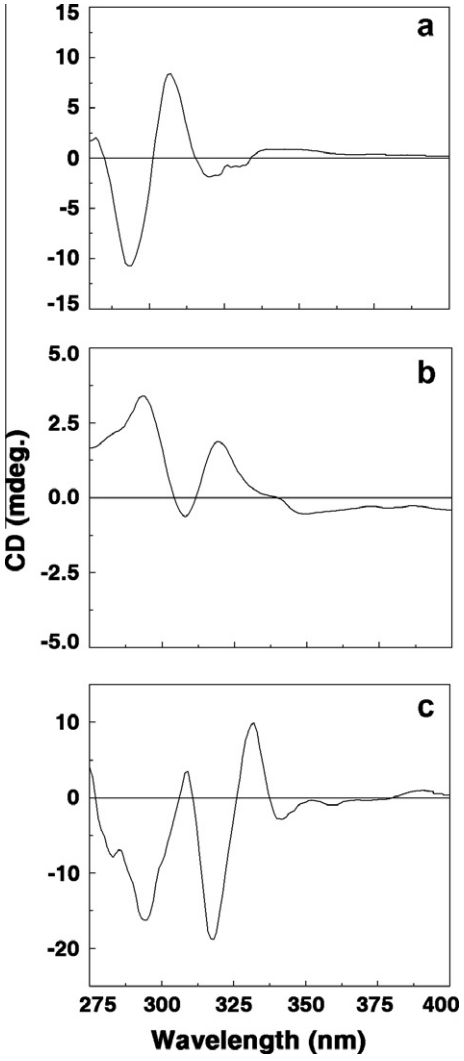


Fig. 4. Induced CD spectra of compound **2** in presence of α-CD (a), β-CD (b) and γ-CD (c).

fluorescence study it has been observed that DIMs and CDs form 1:1 complex which is the most common type of association when a single drug molecule is included in the cavity of a cyclodextrin molecule, with a stability constant K_1:1 for the equilibrium between the free and complexed ones. Fig. 5 presents the phase–solubility diagram for the DIMs studied. There is a linear increase in the solubility of DIMs with increasing concentration of α-, β-, γ-CDs with a slope lower than unity indicating a 1:1 stoichiometry. This is a feature of A-L type of complexes showing the formation of water-soluble complexes between CDs and DIMs [24]. The apparent stability constants (K_1:1) values were calculated from the phase solubility diagram (Fig. 5), using the following equation,

$$K_1 : 1 = \text{Slope}/S_o(1 - \text{Slope}) \quad (2)$$

Here, slope is the value found in the linear regression and S_o is the solubility of the drug previously determined in the absence of CDs [25]. The stability constant values were evaluated to be 5400 M^{-1}, 6130 M^{-1} and 6210 M^{-1} for compound **1**, 4167 M^{-1}, 5769 M^{-1} and 7000 M^{-1} for compound **2**, 5530 M^{-1}, 5710 M^{-1} and 4580 M^{-1} for compound **3**, 7500 M^{-1}, 6370 M^{-1} and 5150 M^{-1} for compound **4**, 7900 M^{-1}, 6320 M^{-1} and 5280 M^{-1} for compound **5**, and 8300 M^{-1}, 6320 M^{-1} and 5280 M^{-1} for compound **6**, respectively, with α-, β-, and γ- CD.

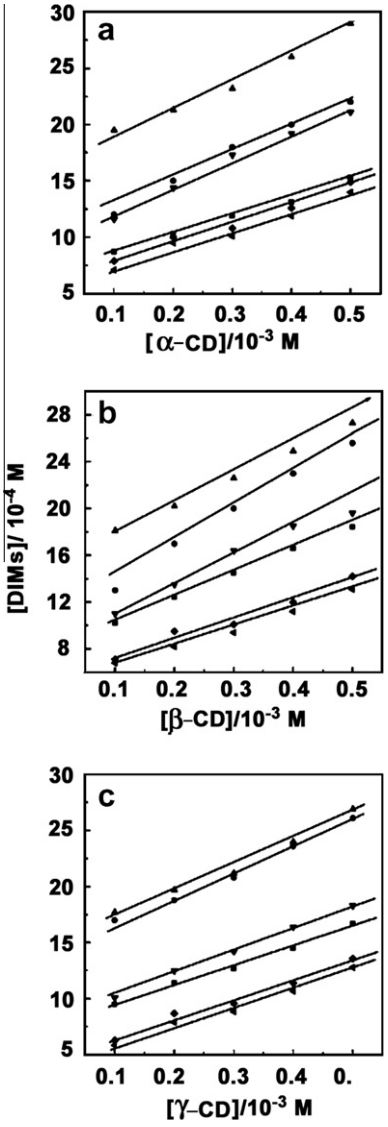


Fig. 5. Phase-solubility diagram of (a) α-CD–DIM, (b) β-CD–DIM and (c) γ-CD–DIM complexes in water at pH 7.2 and 298.15 K. Symbols (-■-), (-●), (-▲-), (-▼-), (-♦-) and ((-◄-)) represent the DIM derivatives **1–6**.

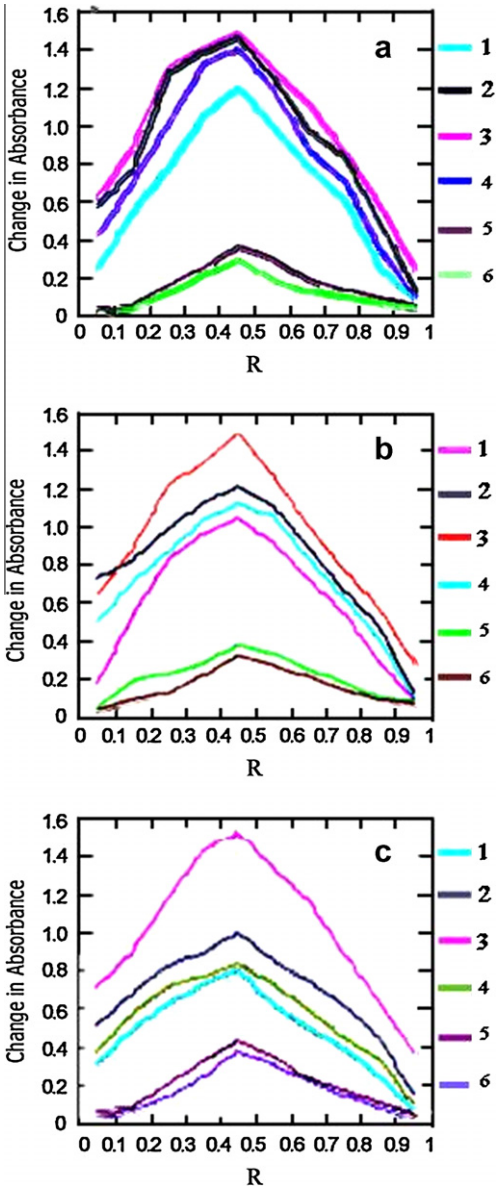


Fig. 6. Job's plot of the (a), α-CD–DIMs (b) β-CD–DIMs and (c) γ-CD–DIMs complexation. Six different colors indicate the plots of six different DIM derivatives **1–6** in presence of α-, β- and γ-CD.

The apparent association constant from fluorescence studies and the stability constants from solubilizations studies differ in their values for many compounds studied here. This may be due to use of the different CD and compound concentrations in the two methods. Such differences are not uncommon and significant variations in solubility and apparent association constants have been recently reported for inclusion complexes of bromhexine with CDs [26].

3.6. Stoichiometry of the complex: job plot

According to the continuous variation method, if a physical parameter can be directly related to the concentration of the complex and that parameter can be measured for a set of samples with continuously variable molar fraction of components, the maximum value of that parameter corresponds to the actual stoichiometry of complex. The molar ratio *R* corresponds to the complexation stoichiometry. The maximum absorbance variation of DIMs in presence of CDs were observed for $R \approx 0.5$ (Fig. 6), which indicates that the inclusion stoichiometry in all cases is 1:1, in agreement with the stoichiometry suggested from the phase solubility study.

3.7. Possible structure of the DIMs–CD inclusion complexes

From the knowledge of theoretical studies of various cyclodextrin–indole inclusion complexes [27] it could be predicted that DIMs penetrate inside the torus of the host molecules through their one of the benzene ring of indole moieties, give rise to a 1:1 stoichiometry with the CDs, although the molecules are perfectly symmetrical in nature.

4. Conclusions

Formation of host–guest inclusion complexes between DIM derivatives (**1–6**) and α-, β-, γ-CDs is revealed by absorption and fluorescence spectroscopy. The ratio of DIM derivatives to α-, β-, γ-CD in the inclusion complexes is 1:1, which is confirmed by their phase solubility studies and Job's plot analysis. Circular dichroism spectroscopy results suggest that the DIMs entered into the

hydrophobic cavity of CDs, which enhance the solubility of DIMs in water for their better bioavailability.

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