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UV–vis and FTIR-ATR characterization of 9-fluorenon-2-carboxyester/(2-hydroxypropyl)-β-cyclodextrin inclusion complex

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ABSTRACT

In this work, the usefulness of (2-hydroxypropyl)-β-cyclodextrin (HP-β-CyD) as a tool to form an inclusion complex with 9-fluorenonic derivative (AG11) has been investigated, in pure water, by UV absorption. Phase-solubility diagrams allowed the determination of the association constant between AG11 and HP-β-CyD. At the same time, solid binary systems between AG11 and HP-β-CyD have been prepared in 1:1 stoichiometry by co-precipitation method. In order to confirm the complexation, FTIR spectroscopy in ATR geometry measurements have been performed and the results have been compared with the free compounds and the corresponding physical mixture in the same molar ratio. The nature of the interactions between AG11 and HP-β-CyD has been elucidated also by applying mathematical procedures such as deconvolution and curve fitting. Improvement of the aqueous solubility is expected to improve the bioavailability of the drug in oral administration.

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1. Introduction

Cyclodextrins (CyDs) can form a number of inclusion complexes with a wide variety of biologically and pharmaceutically important substances ranging from non-polar organic molecules to rare gases, without the formation of any covalent bonds [1]. Due to this remarkable molecular complexation property, they have been extensively applied in a number of studies as multifunctional pharmaceutical excipients to improve the solubility, stability and bioavailability of many drugs [2–4]. The driving forces of complex formation were thought to be the geometric compatibility or fit and intermolecular interaction between the molecule that encloses (the host) and the molecule entrapped in the cavity (the guest).

Recently, various kinds of chemically derivated CyDs, and especially (2-hydroxypropyl)-β-cyclodextrin (HP-β-CyD), have been largely used in pharmaceutical field in order to extend the aqueous solubility and ability to stabilize poorly water-soluble drug molecules of the parent β-CyD as multifunctional drug carriers [5–8].

Nowadays antiviral therapy, even though of great topicality, gave satisfactory results only for the treatment of few viral diseases. This is because, on one side, the intensive use of antivirals has led to the emergence of resistant strains and, on the other side, these agents have limited oral bioavailability and several side effects [9].

Recently, much attention has been focused to endogenous interferons (IFNs), a large family of multifunctional secreted glycoproteins (cytokines) is involved in antiviral defence, cell growth regulation and immune activation [10,11].

Therefore, the enhancement of the release of endogenous IFN by oral administration of low-molecular weight compounds has been ardently desired [12]. Several synthetic compounds possessing IFN-inducing activity have been reported in the search for novel agents that, due to intercalation, could induce IFN production that, in turn, might stimulate antiviral and/or cytostatic effects in the cell. Among them, Tilorone (2,7-bis[2-(diethylamino)ethoxy]-9H-fluoren-9-one), a well-known DNA-intercalator, was the first low-molecular weight IFN-inducer orally effective *in vivo* against some DNA and RNA viruses [13].

Starting from this molecule, a new structural analogous monosubstituted in the position 2 has been prepared (9-fluorenon-2-carboxyester, AG11, see Fig. 1), by functionalising the 9-fluorenonic moiety with a side ester chain able to improve DNA-intercalation, and hence the antiherpetic and immunostimulating activity. Among the various physico-chemical properties of this drug, poor water solubility is noticed, that is detrimental to its efficiency.

It is our special interest to explore the solubilization of AG11 by complexation with HP-β-CyD. The complex formation and

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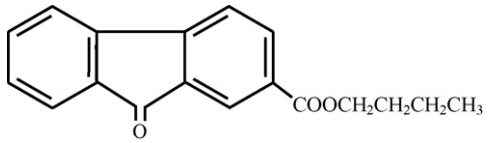

Fig. 1. Chemical structure of 9-fluorenon-2-carboxyester (AG11).

the host–guest interactions have been investigated by UV–vis absorption and FTIR spectroscopy in attenuated total reflectance (ATR) geometry.

2. Experimental

2.1. Chemicals

(2-Hydroxypropyl)-β-cyclodextrin (HP-β-CyD, FW ≈ 1170.0, m.p. ≈ 278 °C dec., degree of substitution ≈0.6) was purchased from Fluka Chemie (Switzerland). The water used was deionized and double-distilled, then filtered through 0.22 μm Millipore® GSWP filters (Bedford, USA). Solutions to be analyzed were prior filtered through 0.45 μm Sartorius Minisart®-SRP 15 PTFE filters (Germany).

2.2. UV–vis absorption measurements

UV–vis absorption spectra were recorded by PerkinElmer (Norwalk, USA) UV–vis double beam spectrophotometer mod. Lambda 45 (resolution, $0.001 \pm 1 \times 10^{-3}$ absorbance units; signal-to-noise ratio, 1×10^{-4}), equipped with a PC for data processing (Softwares: UV–Win Lab®, from PerkinElmer; Peakfit® v. 4.11, from Systat Software Inc.). The measurements were done at room temperature (25.0 ± 0.01 °C) in a 10.00 mm quartz cells (Hellma) in the 220–400 nm spectral range. Baseline was established for each measurement, by placing an aqueous solution of cyclodextrin in the reference compartment at the same concentration of the sample. UV–vis spectroscopy was employed in the study of AG11 alone (10^{-7} M) and in the presence of increasing amounts of macrocycles ($(1.0–8.0) \times 10^{-3}$ M). All data shown represent the average of, at least, three determinations.

2.3. Phase-solubility measurements

The solubility measurements of AG11 with HP-β-CyD were carried out according to Higuchi and Connors [14], by using a Telesystem stirring bath thermostat 15.40 with Telemodul 40 C control unit, which allowed an accuracy of 0.01 °C. Excess amounts of drug were added to 10 ml of an unbuffered aqueous solution containing various concentrations of cyclodextrin ($(0.0–8.0) \times 10^{-3}$ M), and sonicated in a Bandelin RK 514 water bath (Berlin, Germany) for 15 min. Flasks were sealed to avoid changes due to evaporation and shaken in a thermostated bath at 25.0 ± 0.01 °C. At equilibrium, after 3 days, the suspensions were filtered through Sartorius Minisart®-SRP 15 PTFE 0.45 μm filters. An aliquot from each vial was withdrawn by 1 ml glass syringe (Poulten & Graf GmbH, Germany) and assayed spectrophotometrically to evaluate the amount of AG11 dissolved. Each experiment was carried out in triplicate.

2.4. Preparation of solid inclusion complex

Inclusion complex with 1:1 AG11/HP-β-CyD molar ratio was prepared by co-precipitation method, by shaking the appropriate amounts of drug and macrocycle in water. The mixture was stirred for 1 h, under controlled temperature (25.0 ± 0.01 °C). The reaction mixture was cooled in the refrigerator (4 °C) for about 24 h and the water was evaporated under vacuum (∼30 °C), obtaining the solid complexes AG11/HP-β-CyD. Uncomplexed drug was filtered, through Sartorius Minisart®-SRP 15 PTFE 0.45 μm filters, prior to solution evaporation thus, obtaining the solid complex.

2.5. Preparation of physical mixture

The 1:1 molar ratio physical mixture was prepared by homogeneous blending in an agate mortar of previously weighted AG11 and HP-β-CyD.

2.6. FTIR-ATR absorption measurements

The FTIR-ATR spectra of AG11, HP-β-CyD, their physical mixture and solid inclusion complex were recorded in the range 600–4000 cm^{-1} on a Bomem DA8 Fourier transform spectrometer, operating with a Globar source, in combination with a KBr beamsplitter, a DTGS/KBr detector. It was equipped with a Golden Gate diamond ATR system, just based on the ATR technique [15]. An ATR set-up exhibits various advantages with respect to an ordinary absorption set-up. It is non-destructive, it requires only micrograms of sample, and it is at the origin of spectra displaying a very good signal to noise ratio, being in particular easy to avoid saturation of bands. In addition, a chemical analysis can be performed directly on ATR spectra, avoiding implementation of elaborated calculations of optical constants. Measurements have been performed in dry atmosphere to avoid dirty contributions. Each spectrum was recorded at room temperature (25 ± 0.01 °C) by co-addition of 100 repetitive interferograms at a resolution of 2 cm^{-1} and then normalized for taking into account the effective number of absorbers.

No mathematical correction (e.g. smoothing) was done, and spectroscopic manipulation such as baseline adjustment and normalization were performed using the Spectracalc software package GRAMS (Galactic Industries, Salem, NH, USA).

For the O—H/C—H stretching region, second derivative computations have been used for evaluating the wavenumbers of the maxima of the different sub-bands. Multiple curve fitting into Voigt profiles were then applied to the experimental profiles based on these wavenumber values, by using the routine provided in the PeakFit 4.0 software package. The statistical parameters defined in the software manual were used as a guide to "best-fit", and allowed to vary upon iteration until converging solution is reached.

3. Results and discussion

3.1. Phase-solubility studies

The most common and widely used method to evaluate the ability of CyD to complex a molecule is the phase-solubility study.

The solubility diagram shown in Fig. 2 indicated that the solubility of AG11 was increased in a concentration-dependent way by the complexation with macrocycle, indicating favorable interaction between host and guest. The effect of ligand on the UV absorption spectra of AG11 was investigated by holding the concentration of the guest constant and varying the host concentration between 0.0 and 8.0×10^{-3} M. The absorption intensities of the maxima increased as a function of HP-β-CyD concentration, indicating an increased apparent solubility of the guest molecule, up to 4.0×10^{-3} M, above which they were nearly constant. At this point, AG11 must be fully complexed by HP-β-CyD. The concentration of complexed AG11 can be obtained by considering the intrinsic solubility of the drug in pure water ($S_0 = 1.9 \times 10^{-7}$ M), as experimentally determined by distributing the guest molecule between water and butanol, having already calculated the molar

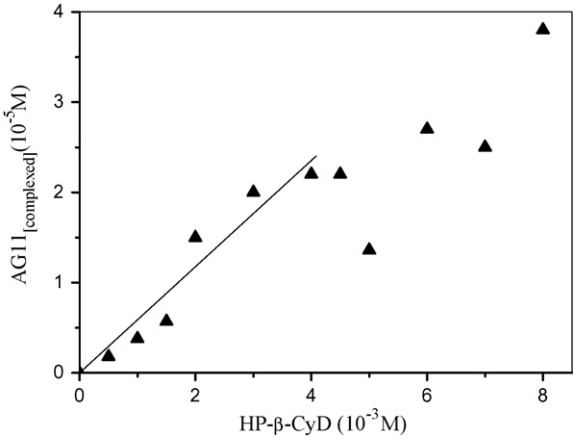

Fig. 2. UV absorption spectra of AG11 in the presence of increasing concentrations of HP-β-CyD (0.0–8.0 mM) in water at 25.0 ± 0.1 °C.

extinction coefficient (ε) of free AG11 in this organic solvent [16]. The binding constant for a 1:1 complex is: $K = [S{\cdot}CyD]/[CyD]\,[S]$, in which [S·CyD], [CyD] and [S] represent the equilibrium concentrations of the complexed substrate, free cyclodextrin and free substrate, respectively. This equation requires careful consideration when, as in the present case, the substrate is not fully soluble and amounts that exceed its solubility are used. In this case the binding constant can be calculated from the phase-solubility technique, which allows for the evaluation of the affinity between cyclodextrin and substrate, according to the method reported by Higuchi and Connors [14]: $K = \text{slope}/S_0(1 - \text{slope})$, where S_0 is the solubility of the substrate and the slope parameter is obtained from the straight-line portion of the plot of complexed substrate concentration [S·CyD], against cyclodextrin concentration. In order to obtain [S·CyD] from the experimental quantities, it has to be considered that the substrate concentration, $C_S - S_0$ (C_S being the analytical AG11 concentration), not dissolved in water, acts as a reservoir for the formation of the complex when the cyclodextrin is added.

The measured molar extinction coefficient of bound AG11 was determined as $\varepsilon_c = A_F - A_0/S_0$ (15643 $mol^{-1}\,cm^{-1}$), A_F being the absorbance value of AG11 when is complexed ($C_S - S_0$) and A_0 the absorbance value of AG11 when $C_{CyD} = 0$. The value of the association constant of the complex was found to be $K_c = 30000 \pm 3000\ M^{-1}$.

3.2. FTIR-ATR absorption measurements

FTIR-ATR spectroscopy was used to confirm, in solid phase, the formation of AG11/HP-β-CyD inclusion complex, as suggested by UV–vis, and to emphasize the functional guest groups affected by the interaction with the host at molecular level.

The 3700–2600 cm^{-1}, 1750–1180 cm^{-1} and 1180–600 cm^{-1} FTIR-ATR spectra of AG11, HP-β-CyD and AG11:HP-β-CyD binary systems are shown in Figs. 3 and 4, respectively. The infrared spectrum of AG11 shows the presence of peaks at ~2920 cm^{-1} (C—H stretching vibration), ~1703 cm^{-1} (C═O stretching) and ~1608 cm^{-1} (twin peak, ring C═C stretching). Going on, the peaks in the 1500–1320 cm^{-1} region are due to the phenyl stretching vibration, probably convoluted with those associated to CH_2 and CH_3 bending vibrations of the alkyl chains, whereas the strong and complex band at 1320–1000 cm^{-1} is connected to the CH rocking vibrations of the rings (~1288 cm^{-1} and ~1255 cm^{-1}), convoluted with those associated to C—O— bonds stretching (in the 1260–1000 cm^{-1} range). Finally, the intense bands that appears in the 900–500 cm^{-1} region correspond to the out-of-plane bending of aromatic C—H bonds. The FTIR-ATR spectrum of HP-β-CyD shows prominent absorption bands at ~3350 cm^{-1} (O—H stretching vibration), ~2930 cm^{-1} (C—H stretching), ~1148 cm^{-1}, ~1077 cm^{-1} and ~1010 cm^{-1} (C—H, C—O stretching vibrations). The 1:1 physical mixture spectrum is a complete superposition of AG11 and HP-β-CyD signals, suggesting no or little interaction of the drug with HP-β-CyD molecule. The spectrum of the 1:1 co-precipitated solid complex revealed changes (shifts, broadenings and/or atten-

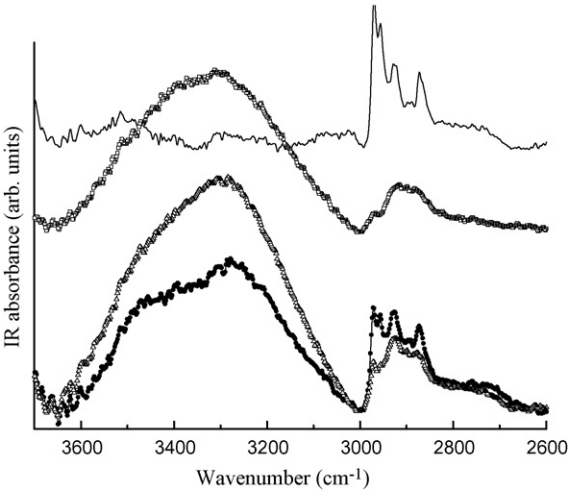

Fig. 3. 3700–2600 cm^{-1} FTIR-ATR spectra of AG11 (solid line), HP-β-CyD (open squares), AG11+HP-β-CyD physical mixture (closed circles), and AG11/HP-β-CyD complex (open triangles).

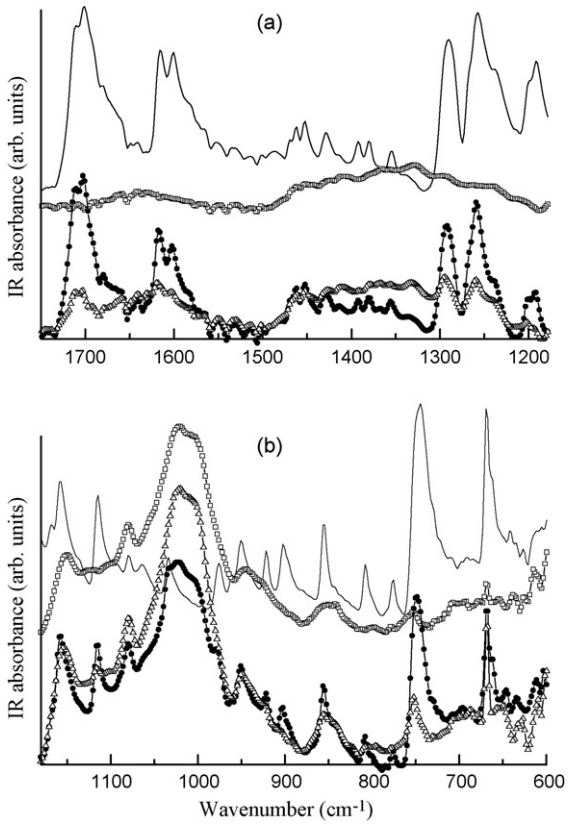

Fig. 4. 1750–1180 cm^{-1} (a) and 1180–600 cm^{-1} (b) FTIR-ATR spectra of AG11 (solid line), HP-β-CyD (open squares), AG11+HP-β-CyD physical mixture (closed circles), and AG11/HP-β-CyD complex (open triangles).

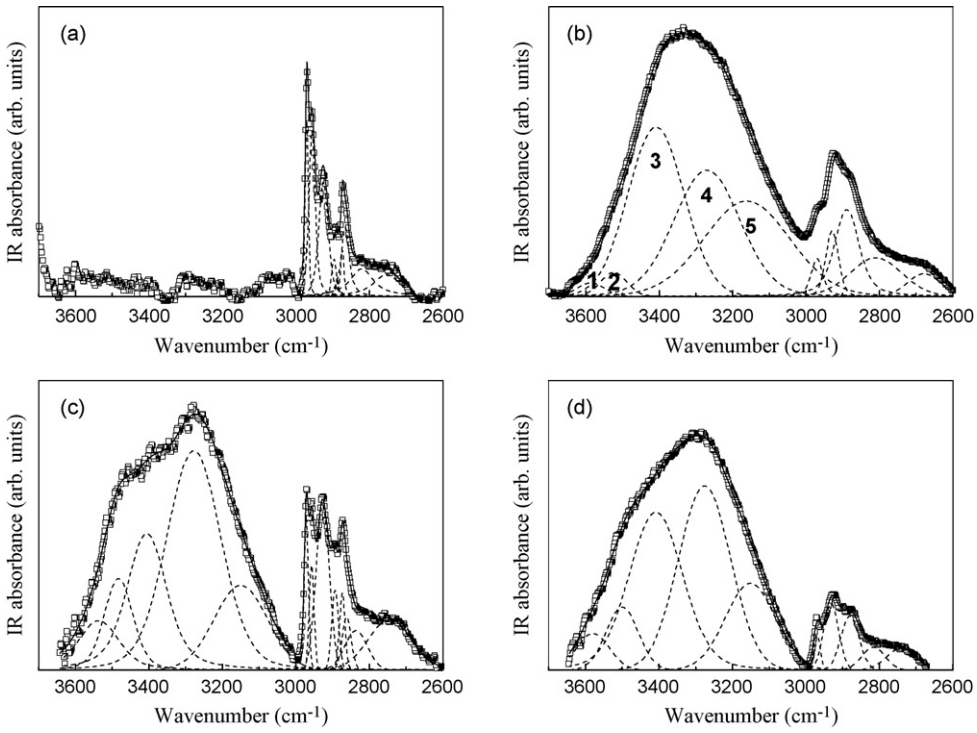

Fig. 5. The fit of the FTIR-ATR bands in the O—H/C—H stretching region for AG11 (a), HP-β-CyD (b), AG11+HP-β-CyD physical mixture (c), and AG11/HP-β-CyD complex (d). In particular, the sub-bands ω_i (i = 1–5) revealed in the O—H stretching region have been labeled.

uations) from parent spectra, i.e. drug and cyclodextrin, and from the simple physical mixture spectrum, indicative of the existence of the complex as a new compound with different spectroscopic bands. In particular, the characteristic absorption bands of HP-β-CyD are superposed over the AG11 ones. This phenomenon is due to the differences between molecular weights of the components.

Information concerning host–guest interactions are first of all provided by the analysis of the O—H and C—H stretching vibrations. In this region, we used the curve-fitting technique in order to separate the contribution of the individual vibrations to the experimental spectra. The obtained sub-bands are shown in Fig. 5(a–d) for AG11, HP-β-CyD, AG11 + HP-β-CyD physical mixture and AG11/HP-β-CyD complex, respectively. The detailed assignment of all the observed sub-bands is reported in Table 1. It is worth remarking that, from a general point of view, the well-known difficulties of uniquely fitting IR band profiles must lead to caution against over interpretation of the data. Nevertheless, we remark that the procedure we adopted here uses the minimum number of parameters and, at the same time, it furnishes extremely good fits to the data. The best-fit is, in fact, characterized by $r^2 \sim 0.9999$ for all the investigated systems. Furthermore, as previously demonstrated in the case of Genistein [17], this procedure provides a sound tool to quantitatively account for

the changes in H-bond scheme upon complexation with β-CyDs. In particular, five Voigt peaks has been used to fit the wide FTIR-ATR band corresponding to the O—H stretching vibrations. We remark that, other than being five the minimum number of parameters that well reproduces the experimental O—H spectra, the presence of five sub-bands with the assigned centre-frequencies was also suggested by the analysis of the second derivative profiles, not reported here, that showed five minima approximately corresponding to the maxima of each band component, according to a well-established procedure already used for a variety of systems [17–20]. The interpretation of the different contributions revealed for the unresolved O—H stretching band has been performed on the basis of previous studies [17,21–23], according to which this region is a superposition of the O—H stretching vibrations corresponding to inter- and intramolecular water molecules, primary and secondary OH groups of the cyclodextrin.

The O—H band of the inclusion complex shows, if compared with that of physical mixture, a high-frequency shift of the peaks corresponding to the O—H stretching vibration of intra-cavity water molecules and primary hydroxyl groups of HP-β-CyD. This was already observed for other systems [17] and explained in terms of a reduction, upon complexation, of the degrees of hydrogen bonding of the arrangements in which these OH oscillators are engaged. Again, the respective fractions I_i/I_{OH} (i = 1–5, expressed in % in Table 2) of the different OH band Voigt areas reveal, as main results, a reduction of H_2O molecules inside the torus together with a dramatic increase of interstitial H_2O molecules. During complexation the drug penetrates the host cavity, forcing the water molecules present inside to change their environment and, generally, altering all the others hydrogen bonding schemes by favouring interstitial H-bonded clusters.

The changes induced by complexation in the C—H stretching region, where in particular the disappearance of the sub-bands at $\sim 2957\,cm^{-1}$ (connected to the C—H stretching vibration of the groups belonging to the AG11 rings), $\sim 2893\,cm^{-1}$ and $\sim 2862\,cm^{-1}$ (assigned to the C—H stretching vibration of the alkylic chain of AG11 and/or HP-β-CyD) observed in the physical mixture is revealed, let us hypothesize an interaction, involving these functional groups, between guest and host as a consequence of encapsulation.

Further information can be obtained by looking at the low-frequency region of the spectra. Passing from physical mixture

Table 1
Main fitting parameters, i.e. wavenumber (ω, cm^{-1}) and percentage intensity (I, %) of the FTIR-ATR sub-bands in the O—H/C—H stretching (O—H/C—H str.) region of AG11, HP-β-CyD, physical mixture (pm) and inclusion complex (co)

AG11		HP-β-CyD		pm		co		Vibrational assignment
ω (cm^{-1})	I (%)	ω (cm^{-1})	I (%)	ω (cm^{-1})	I (%)	ω (cm^{-1})	I (%)	
		3576.9	1.0	3536.8	6.4	3579.0	4.0	O—H str. intra-cavity H_2O
		3511.8	3.4	3482.8	7.9	3499.7	6.8	O—H str. primary OH groups
		3398.2	25.3	3405.9	17.5	3407.8	30.6	O—H str. interstitial H_2O
		3269.7	22.5	3277.9	34.5	3275.5	32.4	O—H str. secondary OH groups
		3172.5	28.6	3150.4	12.2	3151.3	14.7	O—H str. interstitial H_2O
2970.6	10.9	2970.1	1.7	2971.9	1.9	2970.0	0.9	C—H str. (ring)
2956.2	20.6			2957.3	1.4			C—H str. (ring)
2925.3	20.5	2929.0	2.7	2927.4	5.9	2931.3	3.4	C—H str. (ring)
2896.4	2.9	2890.8	6.9	2893.2	1.5			C—H str.
2887.2	0.9							C—H str.
2874.1	3.3			2874.0	1.2	2880.1	3.3	C—H str.
2865.9	12.2			2861.9	1.4			C—H str.
2821.1	15.4	2809.7	5.7	2836.6	2.4	2816.1	1.8	C—H str.
2753.2	13.3	2678.9	2.2	2745.9	5.8	2741.4	2.1	C—H str.

Their vibrational assignment is also reported.

Table 2
Main fitting parameters, i.e. wavenumber (ω_i, cm^{-1}, i = 1–5) and percentage intensity (I_i/I_{OH}, %, i = 1–5) of the FTIR-ATR sub-bands in the O—H stretching region of AG11 + HP-β-CyD physical mixture (pm) and AG11/HP-β-CyD inclusion complex (co)

	ω_1 (cm^{-1})	I_1/I_{OH} (%)	ω_2 (cm^{-1})	I_2/I_{OH} (%)	ω_3 (cm^{-1})	I_3/I_{OH} (%)	ω_4 (cm^{-1})	I_4/I_{OH} (%)	ω_5 (cm^{-1})	I_5/I_{OH} (%)
AG11 + HP-β-CyD pm	3536.8	8.1	3482.8	10.1	3405.9	22.3	3277.9	43.9	3150.4	15.5
AG11/HP-β-CyD co	3579.0	4.5	3499.7	7.7	3407.8	34.6	3275.5	36.6	3151.3	16.6

to complex, the characteristic carbonyl stretching band of drug appeared broadened and reduced in intensity, and its low-frequency shoulder at ~1691 cm^{-1} became an evident peak. Analogous changes have been already observed for other inclusion complexes [23,24]. The observed broadening and decreasing in intensity of the band can be probably connected to the restriction of this stretching vibration of the AG11 molecule, because of the cyclodextrin cavity. The enhancement of the low-frequency contribution, indicative of a weakening of the carbonyl radical double bond, may be associated to the establishment, as a consequence of complexation, of intermolecular hydrogen bonds between AG11 molecule, inserted more or less deeply in the host cavity, and the hydroxyl groups of cyclodextrin or the water molecules also present inside it. The main signal due to the phenyl stretching vibration, still sharp in the physical mixture, reduces its intensity and sharpness in the complex, revealing the restriction of this group due to the inclusion within the cavity of HP-β-CyD. Furthermore, the comparison of physical mixture and inclusion complex spectra evidences, in this last case, the broadening of the bands associated to CH rocking vibrations of the rings, indicative of an interactions between these molecular part of AG11 and HP-β-CyD. This occurrence let us hypothesize the AG11 aromatic rings as groups of inclusion inside the HP-β-CyD cavity, in agreement with the results obtained from the analysis of the C—H stretching region. Going on, the signals relative to C—O stretching vibration, even overlapped by the strong HP-β-CyD signal in this region, undergo spectral modifications when passing from physical mixture to inclusion complex. In particular, the peak at ~1190 cm^{-1} in the AG11 spectrum, almost unaltered in the physical mixture, reduces in intensity, enlarges and up-shifts to ~1204 cm^{-1} in the complex. The intensity of the peak at ~1114 cm^{-1} in the AG11 spectrum, almost unchanged in the physical mixture, dramatically reduces in the complex. Finally, in the aromatic out-of-plane C—H bending range, the peak at ~745 cm^{-1}, still distinctly present in the physical mixture, strongly diminishes in intensity in the co-precipitated compound. A hindering of this bending vibration, due to the close fitting into the cavity, can justify this occurrence.

From the analysis of the spectral changes revealed in the FTIR-ATR spectra we have been able to confirm the existence, in solid phase, of the AG11/HP-β-CyD inclusion complex, putting into evidence the functional groups mainly involved in the complexation process. These data can be also very useful for understanding the geometry of the complex, by using, for example, molecular dynamics simulation.

4. Conclusions

Solubility profile of complex of 9-fluorenonic derivative using HP-β-CyD as complexing agent in a molar ratio 1:1 by co-precipitation method indicated that the aqueous solubility of compound was enhanced considerably by formation of an inclusion complex with CyD.

Thus, the derivative CyD may be useful in improving the dissolution and the bioavailability of AG11 in pharmaceutical formulation.

In addition, FTIR-ATR spectroscopy has been used to study the complex formed between AG11 and HP-β-CyD in solid phase, by comparison with physical mixture and pure substances. The use of mathematical procedures such as baseline corrections, second derivative computations and curve fitting revealed essential to investigate interactions at molecular level in these solid samples. As main results, different O—H stretching vibrations have been observed and assigned to water molecules involved in various hydrogen bonds environments, giving quantitative information on their rearrangement upon complexation. The changes evidenced in the C—H stretching region revealed the existence of interactions among the rings and the alkyl chain of the AG11 molecule with the HP-β-CyD. This let us hypothesize an inclusion of these groups inside the HP-β-CyD cavity, and the broadening of the CH rocking vibrations of the rings reinforces this hypothesis. Remarkable modifications have been also detected in the carbonyl stretching vibration region, explained in terms of different degrees of association via hydrogen bond. Evidences are observed of the formation of the complex also in the C=C, C—O stretching and aromatic out-of-plane C—H bending vibrations.

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