Journal of Pharmaceutical and Biomedical Analysis 51 (2010) 1064–1068

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Physico-chemical characterization of an amphiphilic cyclodextrin/genistein complex

C. Cannavà[a], V. Crupi[b], P. Ficarra[a], M. Guardo[a], D. Majolino[b], A. Mazzaglia[c,*], R. Stancanelli[a], V. Venuti[b,*]

[a] Dipartimento Farmaco-Chimico, Università di Messina, Viale Annunziata, 98168 Messina, Italy
[b] Dipartimento di Fisica, Università di Messina, and CNISM, UdR Messina, C.da Papardo, S.ta Sperone 31, P.O. BOX 55, 98166 S. Agata, Messina, Italy
[c] Istituto per lo Sudio dei Materiali Nanostrutturati ISMN-CNR, c/o Dipartimento di Chimica Inorganica, Chimica Analitica e Chimica Fisica, Università di Messina, 98166 Messina, Italy

ARTICLE INFO

Article history:
Received 7 September 2009
Received in revised form
24 November 2009
Accepted 24 November 2009
Available online 29 November 2009

Keywords:
Genistein
Amphiphilic cyclodextrin
Inclusion complexes
UV–vis spectroscopy
FTIR–ATR spectroscopy

ABSTRACT

Specific recognition of cell-targeting systems as host-carriers modified with receptor targeting groups, is a major ambition in the application of supramolecular science to medicine and life science. Genistein (Gen), an isoflavone belonging to the class of phytoestrogens, is of great interest because it has been considered as potential remedy for many kinds of disease. In this work, Genistein in aqueous medium and in the presence of an host nanocarrier as amphiphilic cyclodextrin (CyD) modified in the upper rim with oligoethylene hydroxyl groups [(2-oligo(ethyleneoxide)-6-hexylthio)-β-CyD, SC6OH] at 1:1 molar ratio, has been firstly investigated by UV–vis measurements coupled with circular dichroism data, in order to characterize the drug/macrocycle binding affinity through the formation of the complex.

Furthermore, FTIR–ATR technique has been used to detect the complex formation in solid phase and to characterize the functional groups responsible of the solid Gen/SC6OH complex stability. The infrared absorbance spectra of the complex, collected in a wide range of wavenumber and around the physiological temperature, have been analysed and compared with the spectra of the pure compounds and their physical mixture. By monitoring the most significant changes in the shape and position of the absorbance bands of the Gen functional groups, we showed that the formation and/or modification of polar bonds play the main role in the interaction of the drug with the amphiphilic CyD. From the results, Gen is shown to be entangled in SC6OH nanoaggregates, establishing hydrogen bonding with the hydrophilic PEG chains.

1. Introduction

Recently, much interest has been focused on cyclodextrins (CyDs), because of their remarkable ability to form, via noncovalent interactions, "host–guest" inclusion complexes with a wide variety of molecules [1–3], altering the physico-chemical characteristics of the guest [4].

Many studies have been devoted to the characterization of CyDs alone and their inclusion complexes with non-polar drugs, both in solution and in solid phase. X-ray, neutron diffraction investigations, conductometric and NMR measurements, ultraviolet and visible absorption spectroscopy, fluorescence techniques, are only some examples [5–9]. In particular, Fourier transform Raman and infrared studies (FT-Raman and FTIR) [10–14] on solid complexes are aimed at showing the "host–guest" interactions at molecular level that drive the complexation process, by monitoring the spectral changes suffered by the main vibrational bands of the guest molecules upon complexation, through a comparison with the vibrational features of the physical mixture, a pure blend of the drug with the macrocycle, and the single components.

Non-ionic amphiphilic CyDs (ACyDs) modified with small portions of PEG at the wider rim and hydrophobic chains of intermediate length at the narrower rim, named (2-oligo-ethyleneoxide-6-hexylthio)-β-CyD, SC6OH (see Fig. 1(a)) are highly versatile towards both encapsulation of drug with different polarity [15,16] and controlled covalent modifications with receptor targeting groups [17]. The special benefits [18] of using this kind of ACyDs include, among the others, the formation in aqueous medium of stable, potentially low immunogenic, nanoaggregates with an outer hydrophilic surface, that can direct the drug to the site of actions (i.e. solid tumors and cellular membranes).

These nanoaggregates are, in particular, able to entrap Genistein (4′,5,7-trihydroxyisoflavone, Gen) (Fig. 1(b)) an isoflavone

* Corresponding authors. Tel.: +39 090 6765010; fax: +39 090 395004.
E-mail addresses: antonino.mazzaglia@ismn.cnr.it (A. Mazzaglia), vvenuti@unime.it (V. Venuti).

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doi:10.1016/j.jpba.2009.11.025

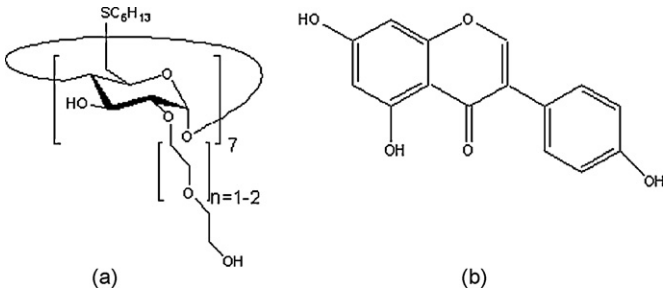

Fig. 1. Structure of SC6OH amphiphilic CyD (a) and Genistein (Gen, 4′,5,7-trihydroxyisoflavone) (b).

contained in soy beans, well known for its antioxidants and anticarciogenic activities [19,20]. Due to its chemical structure Gen shows, however, poor solubility in water, that of course drastically reduces its bioavailability.

Recently, nanoaggregates of Gen/SC6OH were prepared by emulsification–diffusion method [16], and their self-assembly properties have been studied, in aqueous medium, at different host/guest molar ratio [21]. Gen turned out to be complexed starting from 1:1 molar ratio. The occurred Gen/ACyD interaction, which increases isoflavone solubility in water from 3 μM to, at least, 30 μM, is suggested by UV–vis measurements through the shift of the absorption spectra with respect to free Gen, whereas size, charge and structure of aggregates and their complexes with Gen are measured by means of Static and Quasi-Elastic Light Scattering, and electrophoretic mobility measurements. It has been shown that nanocarrier size is not affected by the formation of the complexes with Gen, which is likely located in the surface of the ACyD aggregates.

On the bases of the aforementioned results, nanoaggregates of Gen/SC6OH complex at 1:1 molar ratio have been prepared by emulsification–diffusion method and studied, in water, by UV–vis and circular dichroism. Samples have been freeze-dried and a detailed spectroscopic investigation has been performed by FTIR–ATR absorption, in order to emphasize the functional groups involved in the supramolecular interactions, that we believe to be responsible, in solid phase, of the observed complex stability at different temperatures around the physiological one.

2. Experimental

2.1. Materials and samples preparation

The following reagents were used: powder of crystalline isoflavone Genistein (Gen, 4′,5,7-trihydroxyisoflavone, $C_{15}H_{10}O_5$, FW ≈ 270.24) in native form purchased from Sigma–Aldrich Chemie® (Genay, France); a viscous oil of fully hydrated amphiphilic cyclodextrin (2-oligo(ethyleneoxide)-6-hexylthio)-β-CyD (SC6OH, FW ≈ 2722) synthesized according to general procedures [22].

Nanoaggregates dispersions of SC6OH and Gen/SC6OH complex were prepared by an emulsification–diffusion method: an amount of SC6OH in $CHCl_3$ (30 μM) and an equimolar amount of Gen in ButOH were mixed and drop-wise dispersed in a stirred aqueous phase to obtain the complex Gen/SC6OH at 1:1 molar ratio. The dispersions of SC6OH, Gen/SC6OH and free Gen were sonicated 3 min by ultrasound bath (Bandelin RK 514, Berlin, Germany) and evaporated by stirring for about 12 h at room temperature. Thereafter, they were freeze-dried (VirTis BenchTop K Series Freeze Dryers, USA) in order to obtain powders and to eliminate most of the organic solvent. Hereafter, the powders were re-dispersed in water (2 mL) for UV–vis and dichroism circular analysis. In the case of FTIR–ATR measurements, the same procedure for sample preparation has been followed, but starting from an increased amount of SC6OH in $CHCl_3$ and Gen in ButOH (300 μM).

A physical mixture consisting of Gen and SC6OH cyclodextrin in the same 1:1 molar ratio as the complex was also prepared. Gen and SC6OH were admixed together in a agate mortar and pestle to obtain homogeneous blend.

All the solvents used for the preparation of the samples were filtered through 0.22 μm Millipore® GSWP filters (Bedford, USA) and dried by standard techniques. All other reagents were of the highest commercial grade available and were used as received or were purified by distillation or recrystallization when necessary.

2.2. UV–vis and circular dichroism measurements

UV–vis absorption spectra were performed on free Gen and Gen/SC6OH complex (1:1 molar ratio) by using a Hewlett Packard mod. HP 8453 diode array spectrophotometer. The pathlength of the quartz cell (Hellma) was 1 cm. All measurements were run at least three times at 25.0 ± 0.1 °C.

The circular dichroism spectra (c.d.) were collected on Gen/SC6OH complex (1:1 molar ratio) using a JASCO J-500A spectropolarimeter equipped with a 150 W Xenon lamp. The instrument was interfaced with a PC for CyD signals reading. The measurements were performed at 25.0 ± 0.1 °C and the samples were contained in rectangular quartz cuvettes of 1 cm pathlength.

2.3. FTIR–ATR absorption measurements

We performed FTIR–ATR absorbance measurements in the wide wavenumber range (3800–600) cm^{-1}, on the Gen/SC6OH 1:1 complex, the 1:1 Gen + SC6OH physical mixture and the corresponding single components. The data were collected around the physiological temperature, from 23 to 40 °C, through a Bomem DA8 Fourier transform spectrometer which used a Globar lamp as source, in combination with a KBr beamsplitter and a DTGS/KBr detector. The powders of the samples were contained in a Golden Gate diamond ATR system, based on the well-known attenuated total reflectance (ATR) technique [23]. One of the main advantages of this configuration, besides the usual non-destructiveness of the technique in itself, is that it requires only few micrograms of the sample avoiding the possible saturation of the absorbance signal. All the data were collected in dry atmosphere so minimizing unwanted dirty contributions, adding 100 repetitive scans using a resolution of 2 cm^{-1}. All the FTIR–ATR spectra were normalized in order to take into account the effective number of adsorbers. The data showed a good signal to noise ratio and no mathematical correction, such as smoothing, was necessary, while baseline adjustment and normalization were performed through the Spectracalc software package GRAMS (Galactic Industries, Salem, NH, USA).

3. Results and discussion

3.1. UV–vis and circular dichroism measurements

The formation of the complex in aqueous dispersion was investigated by UV–vis measurements. Fig. 2 shows UV–vis absorption spectra of free Gen dissolved in H_2O/ButOH mixture (a) and in the presence of SC6OH (b), respectively. Both spectra were collected on the sample prepared after re-hydration of freeze-dried powders. As well known, no contributions are expected from SC6OH in the UV–vis absorption range of Gen, so eventually detected spectral changes can be directly related to different environments in which Gen is involved. As can be seen, in absence of the amphiphilic CyD the absorption band of Gen is centred at 260 nm, while in the presence of SC6OH (at 1:1 molar ratio), it is batochromically shifted at 268 nm. This observed shift suggests the capacity of Gen to interact

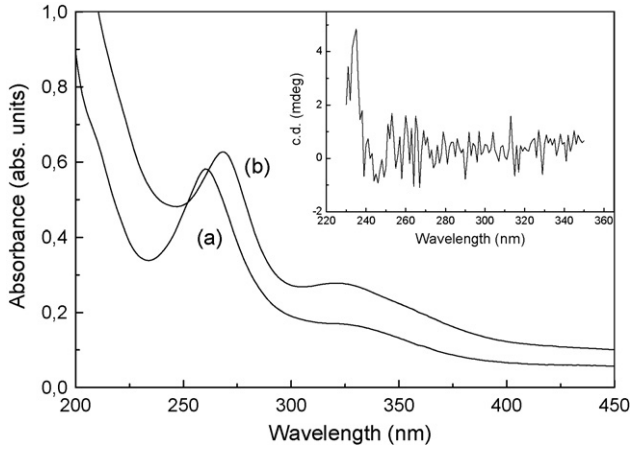

Fig. 2. UV–vis spectra of Gen (30 μM) in water/ButOH (96:4 v/v) (a) and in the presence of SC6OH ([SC6OH]/[Gen] = 1:1) (b), both collected after re-dispersion in H_2O from freeze-dried powder. In the inset the circular dichroism spectrum of Gen/SC6OH complex at 1:1 molar ratio is also reported.

with SC6OH. As already evidenced by dynamic light scattering data [21], the system prepared with emulsification–diffusion method turns out to be stable, since the size of the nanoaggregates remains unchanged for about 30 days. The "neat" absorbance of Gen, as estimated by subtracting, from both curves of Fig. 2, the corresponding scattering contribution, in the presence of SC6OH resulted diminished, indicating an entangling of Gen in CyD nanoaggregates.

The circular dichroism spectrum obtained for Gen/SC6OH system is reported in the inset of Fig. 2. The very low signal around the absorption wavelength of Gen (~260 nm) is probably due to the absorption of the drug. Actually, we did not observe any circular dichroism induced by the cavity, so we can hypothesize that, in solution, Gen is not included inside the SC6OH cavity. The signal at ~230 nm (even if very noisy) was tentatively ascribed to the amphiphilic cyclodextrin.

3.2. FTIR–ATR absorption measurements

We analysed and compared the FTIR–ATR spectra of Gen/SC6OH complex with those of the pure compounds and the corresponding physical mixture, all in solid phase. In Figs. 3 and 4, we report the absorbance spectra of Gen (a), SC6OH (b), their physical mixture (c) and complex (d) in the (3700–2500) cm^{-1} and (1800–1200) cm^{-1} wavenumber ranges, at $T = 30\,^{\circ}C$, as example.

As can be seen, the infrared spectrum of Gen shows several characteristic peaks, the most prominent being at ~3404 cm^{-1} and ~3080 cm^{-1}, representing O–H and C–H stretching vibrations, respectively [11,24]. Going to lower wavenumbers, the vibrational C=O stretching frequency appears at ~1650 cm^{-1}, the C=C stretching at ~1615 cm^{-1}, the C–O–C stretching in the range ~(1320–1150) cm^{-1}, and the C–O stretching in the range ~(1260–1000) cm^{-1} [25].

By comparing the spectra illustrated in Figs. 3 and 4, one can see that the infrared spectrum of the physical mixture exhibits unaltered features of each component, indicating that both the pure substances maintain their crystal structures intact, suggesting no or little interaction between them. On the other side, the spectral variations (broadening, shifts, variations in relative intensities) observed passing from physical mixture to the complex indicate changes, upon complexation, in bond strengths and lengths, due to the activation of some "host–guest" interactions.

As general result, we observe that the FTIR–ATR spectra of Gen/SC6OH complex appear quite different from those of the corresponding macrocycle. This evidence, revealed at all the analysed

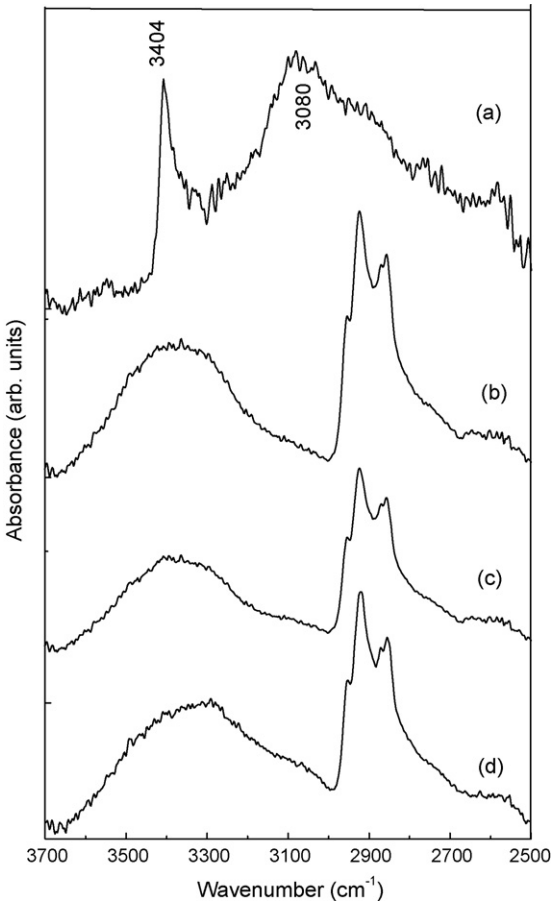

Fig. 3. FTIR–ATR spectrum in the (3700–2500) cm^{-1} region of Gen (a), SC6OH (b), their physical mixture (c) and complex (d) at $T = 30\,^{\circ}C$.

temperatures, allowed us to make some considerations. It has been already reported [10,11] that the inclusion inside CyDs cavity originates a sort of "shield" effect of the guest [26]. When encapsulated, the guest molecule rearranges its crystal structure inside the host, giving rise to a final configuration quite similar to that of the pure macrocycle. As a consequence, the quite complete disappearance of guest signals should be expected in the complex spectrum, that hence should result similar to that of the cyclodextrin, and this can be considered a confirmation of the formation of the inclusion complex. This similarity should be also emphasized by considering the difference in molecular weight of the two components: being the molecular weight of SC6OH (FW ≈ 2722) almost ten times higher than that of Gen (FW ≈ 270.24), the characteristic absorption bands of SC6OH should be supposed to overlap those typical of Gen, and the spectrum of the inclusion complex should closely recall the one of cyclodextrin.

On the basis of the aforementioned considerations, the revealed difference in the spectra of SC6OH and Gen/SC6OH complex let us hypothesize that Gen is entangled in amphiphilic portions of SC6OH nanoaggregates, and not included in the cavity, giving probably rise to supramolecular interactions with the external chains of the modified macrocycle. These experimental evidences are in agreement with those obtained by circular dichroism, already discussed in the previous paragraph. A deeper analysis of the changes in the peculiar vibrational bands of the involved functional groups can support our hypothesis.

In the high-frequency region, the broad O–H stretching band (3700–3000) cm^{-1} of Gen + SC6OH physical mixture and Gen/SC6OH complex reflects the contributions to the O–H stretch-

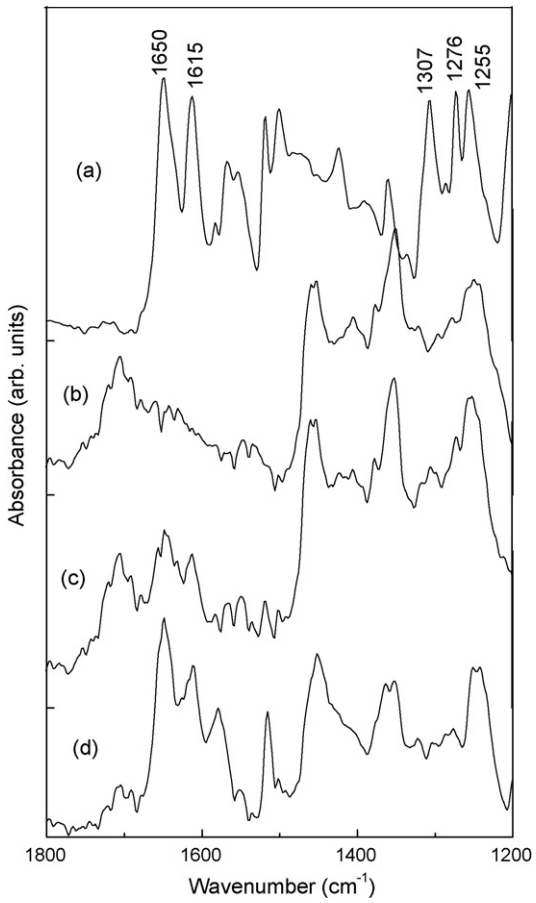

Fig. 4. FTIR–ATR spectrum in the (1800–1200) cm^{-1} region of Gen (a), SC6OH (b), their physical mixture (c) and complex (d) at $T = 30\,^{\circ}C$.

ing vibration coming from different O–H groups. Based on the results of previous FTIR–ATR studies on similar systems [11,26], these contributions have been ascribed, in particular, to come from primary (at ~3525 cm^{-1}) and secondary (at ~3280 cm^{-1}) OH groups of SC6OH, clusters of water molecules inside the hydrophobic SC6OH cavity (at ~3580 cm^{-1}), H_2O molecules in the interstices among different SC6OH molecules linked to them via hydrogen bond (at ~3410 cm^{-1} and at ~3174 cm^{-1}), hydroxyl groups of PEG (at ~3000 cm^{-1}, expected to be overlapped with the C–H stretching vibrations) and Gen (at ~3340 cm^{-1}, as already reported, expected to be downshifted with respect to pure Gen, being the O–H vibration more hindered as a consequence of the activation of some supramolecular interactions). The spectral modifications, passing from physical mixture to complex, of this band suggest that, upon complexation, new hydrogen bonds are formed between Gen and SC6OH, reasonably involving, in particular, the hydrophilic PEG chains, and the water molecules redistribute among the different hydrogen bonds sites. To support this interpretation, we observe that, in the case of Gen-loaded PEG microparticles, the establishment of hydrogen bonding between drug and the polymer matrix was already put into evidence by FTIR–ATR spectroscopy [26]. Again, these changes in the H-bond environment should imply, as a consequence of the establishment of the "host–guest" interactions upon complexation, an increased cooperativity involving longer lifetimes, as indicated by the low-frequency enhancement and the broadening of the OH band shape of the complex with respect to physical mixture. In the 3000–2700 cm^{-1} range one sees the C–H stretching bands, which are almost unchanged upon complexation. The C–H functional groups result, then, not involved in supramolecular interactions, and on this basis we can exclude an interaction of Gen with the hydrophobic alkyl chains of SC6OH.

Further information on the molecular state of the drug upon complexation are given by the analysis of the lower spectral region from 1800 to 1200 cm^{-1}, displayed in Fig. 4.

The characteristic peak of the C=O stretching vibration (at ~1650 cm^{-1}) in free Gen reveals, in the physical mixture, well-defined side-peaks that enlarge and overlap in the inclusion complex spectrum, indicating an interaction of this molecular part of Gen and SC6OH. We also observe, passing from physical mixture to complex, evident changes in the C=C stretching vibration, at ~1615 cm^{-1}, and, in particular, in the C–O–C stretching mode at ~1307 cm^{-1} and in the two peaks corresponding to the C–O stretching at ~1276 cm^{-1} and ~1255 cm^{-1}, that appear particularly bumped and of decreased relative intensity in the solid complex. The band-enlargement suggests an amorphization of the Gen/SC6OH system, usually associated to complexation [12]. The reduction of the bands intensities can be interpreted as a decrease of the dipole moment of the CO group that, in the solid complex, is involved in new hydrogen bonded environments that restrict the corresponding stretching vibrations. Being the C–O–C and C–O groups characteristic only of the hydrophobic cavity of SC6OH and of its hydrophilic PEG chain (they are not present in the hydrophobic alkyl chain), as well as of Gen molecule, their remarkable changes with respect to the other low-frequency vibrational modes, indicates an involving of these functional groups in the activated "host–guest" interactions, allowing us to hypothesize an interaction, upon complexation, between Gen and the hydrophilic PEG chains, confirming the results obtained in the high-frequency region. These results are in agreement with the measurement of ζ potential in solution indicating that Gen affects the surface charge of the nanoaggregate and consequently is probably located close to the outer casing of the amphiphile [21].

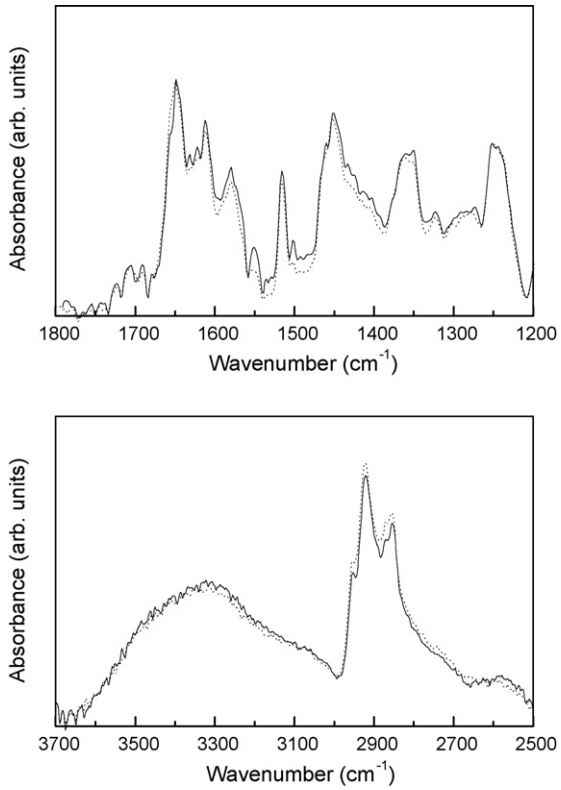

Fig. 5. FTIR–ATR spectra in the (3700–2500) cm^{-1} and (1800–1200) cm^{-1} region of Gen/SC6OH complex, at $T = 23\,^{\circ}C$ (solid line), and $40\,^{\circ}C$ (dotted line).

No relevant effects of temperature are revealed around the physiological temperature, as turned out by comparing the FTIR–ATR spectra collected for the Gen/SC6OH complex in the (23–40) °C range. Fig. 5 displays the absorbance spectral profiles of the complex at the two extreme temperatures, as example. They appear almost completely overlapped. This indicates a thermal stability of the solid system, that we can hypothesize to be induced by the functional groups responsible of the aforementioned interactions.

4. Conclusions

In the present paper, the formation of the complex, prepared with the emulsification–diffusion method, constituted by Genistein with the modified amphiphilic cyclodextrin SC6OH at 1:1 molar ratio in water medium was shown through UV–vis and circular dichroism. Gen molecules are shown to be not included inside the SC6OH cavity, but to interact with the side chains of SC6OH nanoaggregates.

The lack of inclusion of the guest molecules inside the host macrocycle was confirmed, in solid phase, by monitoring the significant differences in the spectral features of the FTIR–ATR spectrum of complex with respect to that of the physical mixture, revealed in the O–H, C=O, C=C, C–O–C, and C–O stretching regions, that allowed us to put into evidence the functional groups involved in the activation of the host–guest interactions driving the complexation process. The FTIR–ATR studies suggested hydrogen-bonding interactions between the drug and the hydrophilic PEG chains of the macrocycle. These host–guest interactions are hypothesized to be responsible of the thermal stability of the complex, observed in the physiological temperature range. These evidences shed light on the complexation of drugs in host nanocarriers at solid phase and open the way to detect the supramolecular interactions in complexed species host/drug and host/drug/receptor in targeted drug delivery.

Acknowledgments

This research was supported by FUSINT (CNR-CSGI) project and MIUR (NANOMAC project).

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