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Physicochemical Study of Ribavirin Complexes with α-, β- and γ-Cyclodextrins

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Abstract

The aim of this work was to characterise interactions between ribavirin (RBV) and native cyclodextrins (CDs). The extent of complexation in solution has been evaluated by high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR). Thermogravimetry (TG), differential scanning calorimetry (DSC) and infrared spectroscopy (FT-IR) were used to characterise the solid state of all the binary systems. Complexation of RBV with α-, β-, and γ-CDs was proved by FT-IR, HPLC and thermal analysis. The 1:1 stoichiometry for the complexes was obtained by HPLC. The stability constants for RBV with α-, β- and γ-CD were determined to be 1493, 2606, and 1179 M^{-1}, respectively. Consequently β-CD was the most suitable of the three complexing agents since it showed the highest stability constant. RBV appears not included inside the cavity of the CD because H-3 and H-5 protons were not shifted in the presence of the molecule as proved by NMR. The 2D ROESY spectra did not show any dipolar proton interaction of the RBV with the CDs. Thus the complexation does not seem to be a host–guest inclusion complex but an external intermolecular complex. FT-IR spectral changes due to the RBV carboxamide group vibrations with the CDs confirm this association.

Introduction

Ribavirin (RBV) is a synthetic guanosine analogue, licensed in aerosol form for the treatment of *Human Respiratory Syncytial Virus* and orally to treat hepatitis C in combination with interferon-α or pegylated interferon-α [1, 2]. Intravenous RBV is the sole treatment of infections caused by haemorrhagic fever viruses [3]. However, the therapeutic use of RBV is restricted by its toxic effects: anaemia and teratogenicity.

In order to enhance RBV bioavailability, activity and tolerance of the treatment, different carriers have already been tested. Lactosaminated poly L-lysine has been conjugated with RBV to target the liver of mice infected with the murine hepatitis virus. The conjugate was found to be active at a dose two or three times lower than that of the free RBV. However, lactosaminated poly L-lysine RBV is expensive to synthetise [4]. The lipophilic dihydropyridine (DHP)-hydrophilic pyridinium salt was used as chemical delivery system with RBV to target the central nervous system. The RBV-DHP has been tested on mice infected with Japanese encephalitis virus resulting in 40–50% survival, whereas RBV or the vehicle alone were inefficient [5, 6]. Liposome-encapsulated RBV has led to contradictory results. With an encapsulation efficacy of 20%, liposomal RBV has been more efficient than free RBV in the treatment of mice infected with Rift Valley fever, Influenza or Herpes simplex viruses [7, 8]. On the contrary, and despite an encapsulation efficiency of 90%, a study on kittens infected with Feline infectious peritonitis virus has shown that liposome-encapsulated RBV was less effective than the free RBV at the same concentrations. This poor efficacy has been related to low stability in the bloodstream and passive targeting of the reticuloendothelial system [9, 10].

The use of cyclodextrins (CDs) to complex RBV might be an alternative to these carriers. The most common native CDs are α-, β- and γ-CD constituted by 6, 7 or 8 α-1,4-linked glucopyranose units, respectively. Complexation with a CD improves the solubility, the stability as well as the bioavailability, and facilitates absorption of the invited molecule [11]. These carriers have been widely applied as multi-functional pharmaceutical excipients due to their remarkable molecular complexation properties with many drugs such as flavonoids [12], ciprofloxacin [13], sparfloxacin [14], antisense DNA [15], ganciclovir [16, 17], modifying their physical, chemical and biological properties [18].

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A wide variety of techniques have been used to study the complexes obtained with CDs including UV–Visible spectroscopy [19], circular dichroism [20], solubility [21], NMR [22], liquid chromatography [23], infrared spectroscopy [24], X-ray diffractometry [25] and differential scanning calorimetry (DSC) [26].

In order to determine the stability constant of complexes, all these techniques can be used, but the advantage of the high performance liquid chromatography (HPLC) method is that K_C values could be rapidly obtained with a minimum quantity of the drug. And we do know that reproducibility and accuracy were not below those obtained with other methods such as spectroscopy [27].

The aim of this work was to characterise the interactions between RBV and native CDs. The extent of complexation in solution has been evaluated by HPLC and nuclear magnetic resonance (NMR). Thermogravimetry (TG), DSC and infrared spectroscopy (FT-IR) were used to characterise the solid state of all the binary systems.

Materials and methods

Materials

The RBV is the 1-β-D ribofuranosyl-1-H-1,2,4-triazole-3-carboxamide (Figure 1a). It was purchased from ICN Biomedical Inc. (Aurora, Ohio, USA) and used as received. The native CDs (Figure 1b) were given by Wacker-Chemie GmbH (Lyon, France). The deuterium oxide (D_2O, deuterium content 99.9%) was purchased from Eurisotop (Gif-sur-Yvette, France). All solutions were prepared using deionised water filtered by an Elgastat UHQ system (Elga, Decines, France). Other chemicals and solvents were of analytical grade or HPLC grade and were used as received.

Preparation of the complexes

The RBV-CD complexes were obtained according to the conditions described by Higuchi and Connors [28]. RBV and CD molecules were dissolved in deionised water at a

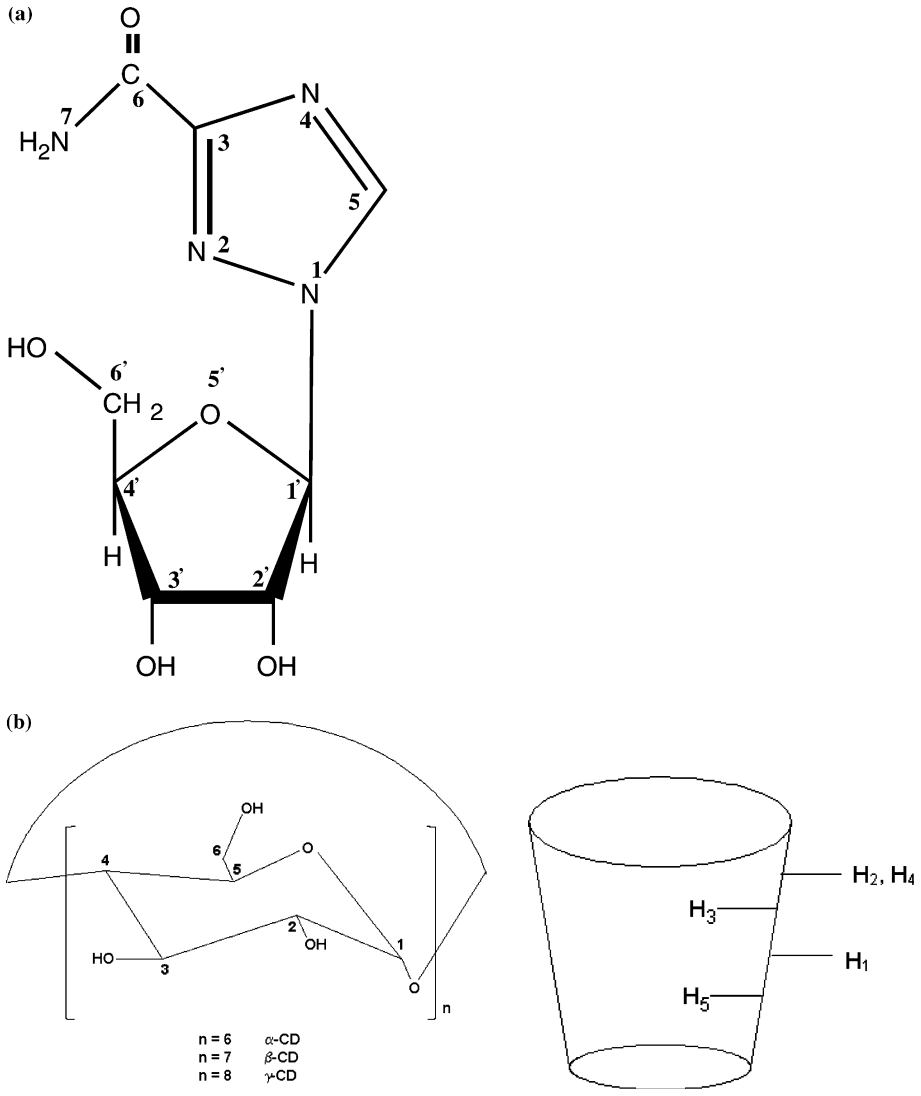

Figure 1(a). IUPAC numbering scheme of ribavirin. (b) Chemical structures of native cyclodextrins.

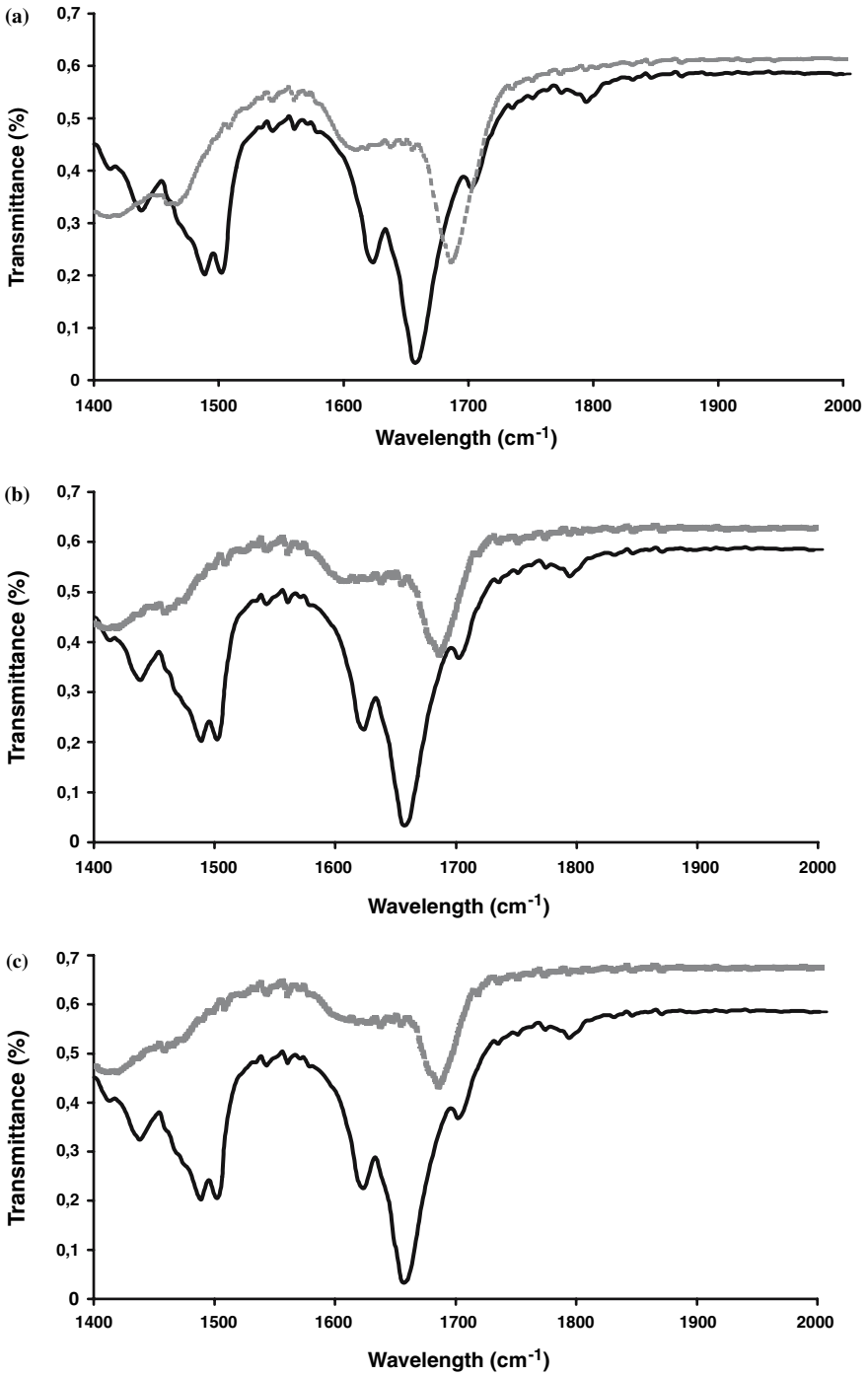

Figure 2. FT-IR spectra of RBV/CD binary systems. (a) RBV (—) and RBV/α-CD system (- - -), (b) RBV (—) and RBV/β-CD system (- - -), (c) RBV (—) and RBV/γ-CD system (- - -).

molar ratio of 1:1. The solution was equilibrated overnight at 22 °C under stirring at 250 rpm with a Certomat®M apparatus (B. Braun Biotech, Plaisance du Touch, France) and was then freeze-dried for the FT-IR and calorimetric experiments.

Complex characterisation

FT-IR

FT-IR spectra were obtained with a Bruker Vector 22 spectrophotometer (Wissembourg, France). The spectra were registered in potassium bromide pellets (1%) (w/w) between 400r0 and 450 cm^{-1}.

NMR

The CDs and the RBV as powder were dissolved in deuterated water. RBV and different native CDs were mixed in D_2O at a molar ratio 1:1. ^1H and ^{13}C NMR measurements were performed at 300 K on a Bruker DRX-400 spectrophotometer (Wissembourg, France) at 400 and 100 MHz, respectively. The 2D ROESY (intermolecular nuclear Overhauser enhancement in the rotating frame) spectra were acquired with number of

Table 1. Proton chemical shifts (ppm) of the RBV, native CDs and putative complexes of RBV with α-, β-, and γ-CD (Δδ = δ complex- δ free)

Protons of CD	α-CD			α-CD x5		
	δ free	δ complex	Δδ	δ free	δ complex	Δδ
1	4.984	4.983	−0.001	4.984	4.983	0.000
2	3.571	3.570	−0.001	3.571	3.571	0.000
3	3.911	3.911	0.000	3.911	3.911	0.000
4	3.515	3.515	0.000	3.515	3.515	0.000
5	3.775	3.773	−0.002	3.775	3.776	0.001
6	3.817	–	–	3.817	–	–
	β-CD			γ-CD		
	δ free	δ complex	Δδ	δ free	δ complex	Δδ
1	5.001	5.001	0.000	5.042	5.042	0.000
2	3.588	3.588	−0.001	3.593	3.593	0.001
3	3.896	3.895	−0.001	3.866	3.867	0.000
4	3.563	3.563	0.000	3.568	3.568	0.001
5	3.797	3.796	−0.001	3.792	3.793	0.001
6	–	–	–	–	–	–

scans = 32, number of dummy scans = 16, recycle delay = 1.5 s, acquisition size 1k × 512, processing size 2k × 2k and mixing time 400 ms [29].

Thermogravimetry (TG), differential scanning calorimetry (DSC) and mass spectrometry (MS)

Mass changes (TG), calorimetric effects (DSC) and mass spectrometry (MS) were determined on the same sample under identical conditions using the Skimmer Coupling System (Netzsch, Gerätebau, Germany). This system combines the simultaneous thermal analysis instruments (STA 409C) for TG and DSC and quadrupole mass spectrometer for detection and analysis of the reaction gases. All instruments were calibrated before use.

The thermal behaviour was studied by heating 5 mg of sample in aluminium open crucible in argon atmosphere (flow of 50 ml min^{-1}) with a heating rate of 10 K min^{-1} over the temperature range 30–400 °C.

Determination of stability constants by HPLC

HPLC was used to determine the stability constants between RBV and native CDs (α-,β- or γ-CD). HPLC experiments were carried out using a Thermo-Finnigan (San Jose, CA, USA) liquid chromatograph equipped with a vacuum degasser SCM1000 and a narrow-bore quaternary Spectra System P1000XR gradient pump with a loop of 20 μl. A stainless steel column Hypersil 120-3 ODS (150 length × 4.6 mm i.d.) Macherey-Nagel was used and thermostated at 18 °C with a column temperature controller 560-CIL (Cluzeau Info Labo, Puteaux-la-Defense, France). The effluents were monitored with a double-beam spectrophotometric detector (SpectraSystem UV1000) at 235 nm.

The mobile phase is constituted of increasing concentrations of α-, β- or γ-CD (0, 0.4, 0.8, 1.6, 3.2, 4.0 mM) dissolved in an aqueous monobasic potassium phosphate buffer (pH 4.6, 0.02 M) and then filtered through a Millipore membrane (0.45 μm). The flow rate of eluent was set at 0.5 ml min^{-1}. The retention times of the drug (final concentration of 16×10^{-6} M) in the absence and in the presence of excess amounts of CDs ($0.4–4 \times 10^{-3}$ M) in the mobile phase were measured.

The stability constants (K_C) were determined by the retention method as described by Uekama *et al.* [18]:

$$\frac{(CD)_m}{T'_o - T_{obs}} = \frac{1}{T'_o - T_c}(CD)_m + \frac{1}{K_C(T'_o - T_c)} \quad (1)$$

where $(CD)_m$ is the concentration of CD in the mobile phase and T_o', T_c and T_{obs} are retention times of RBV,

Table 2. Carbon chemical shifts (ppm) of the RBV, native CDs and putative complexes of RBV with α-, β-, and γ-CD (Δδ = δ complex- δ free)

Carbons of RBV	δ free	α-CD		β-CD		γ-CD	
		δ complex	Δδ	δ complex	Δδ	δ complex	Δδ
3	156.841	156.834	−0.007	–	–	156.859	0.018
5	146.521	146.485	−0.036	146.496	−0.025	146.528	−0.007
6	163.127	163.109	−0.018	–	–	–	–
1′	91.898	91.924	0.025	91.931	0.032	91.902	0.004
2′	74.909	74.920	0.011	74.927	0.018	74.909	0.000
3′	70.453	70.460	0.007	70.464	0.011	70.460	0.007
4′	85.402	85.406	0.004	85.413	0.011	85.413	0.011
5′	61.563	61.570	0.007	61.563	0.000	61.563	0.000

Carbons of CD	α-CD			β-CD			γ-CD		
	δ free	δ complex	Δδ	δ free	δ complex	Δδ	δ free	δ complex	Δδ
1	101.782	101.763	−0.018	102.218	102.211	−0.007	102.016	102.012	−0.004
2	72.064	72.056	−0.007	72.168	72.154	−0.014	72.667	72.650	−0.016
3	73.674	73.663	−0.011	73.436	73.421	−0.014	73.288	73.280	−0.007
4	81.586	81.568	−0.018	81.488	81.477	−0.011	80.788	80.780	−0.007
5	72.392	72.356	−0.036	72.432	72.414	−0.018	72.132	72.125	−0.007
6	60.794	60.761	−0.032	60.642	60.624	−0.018	60.588	60.577	−0.011

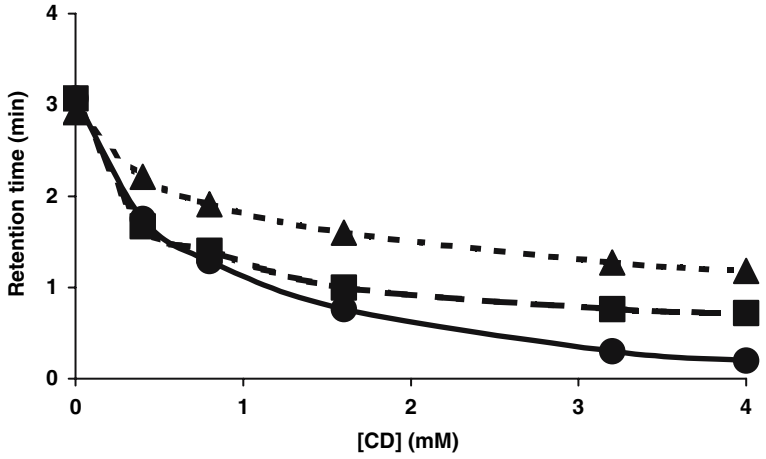

Figure 3. Observed HPLC retention times for RBV with varying concentrations of CD in the mobile phase (0.02 M phosphate buffer, pH = 4.6). — α-CD - - - β-CD ---- γ-CD

of RBV-CD complex and of RBV at a given concentration of CD, respectively. A plot of the left hand term *versus* $(CD)_m$ gives both the K_c and T_c values from the linear relationship by slope on intercept.

Results and discussion

FT-IR results

FT-IR is not suitable for detection of inclusion compounds if the resulting spectra present a superposition of host and guest bands. Fortunately, RBV exhibits some characteristic IR absorption bands in the spectra region where α-, β- or γ-CD have a weak one, making this region suitable for detecting host–guest interactions.

The FT-IR spectra in the region 2000–1400 cm^{-1} of RBV and RBV-CDs complexes, of molar ratio 1:1, are shown in Figure 2a–c. In a RBV spectrum of the free ligand, characteristic band of *v*(C=O) and *v*(N–H) appeared at 1658 and 1620 cm^{-1}, respectively. This result is in accordance with the literature [30, 31]. Absorption bands of RBV in the spectra of binary systems RBV-CDs are affected by complexation. The frequencies of the RBV absorption bands are shifted by 29 cm^{-1} approximately to higher frequencies upon complexation in comparison with the free RBV spectrum. Bratu [24] explains that the shift of the stretching vibration to higher frequency is generally the result of the interaction between the guest molecule and host CD. This shift was explained by the breakdown of the intermolecular hydrogen bond between the molecules and the establishment of a stronger binding in the complex system [32, 33]. As the spectral changes are due to atom group vibrations directly involved in interaction, this effect could be attributed to the interaction between the carboxamide group of RBV and the CDs.

NMR results

All the measurements were performed in D_2O due to the good solubility of the RBV, native CDs and the three binary systems RBV-CDs in this solvent.

Proton and carbon chemical shifts of the RBV, CD and putative complexes of RBV with α-, β- or γ-CD are collected in the Tables 1 and 2, respectively. Complexation of RBV with α-, β- and γ-CD, even with a molar ratio RBV-CD of 1:5, did not cause any significant chemical shift variations of either CD inner protons H-3 and H-5 or ribofuranosyl protons (see Figure 1 for atom numbering). The H-5 proton of the triazole shifted downfield but not significantly. These results better

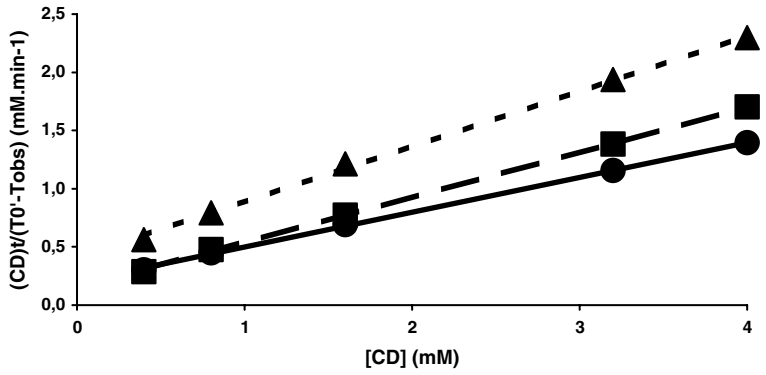

Figure 4. Determination of Kc of RBV-CD binary systems according to Equation (1). — α-CD - - - β-CD ---- γ-CD

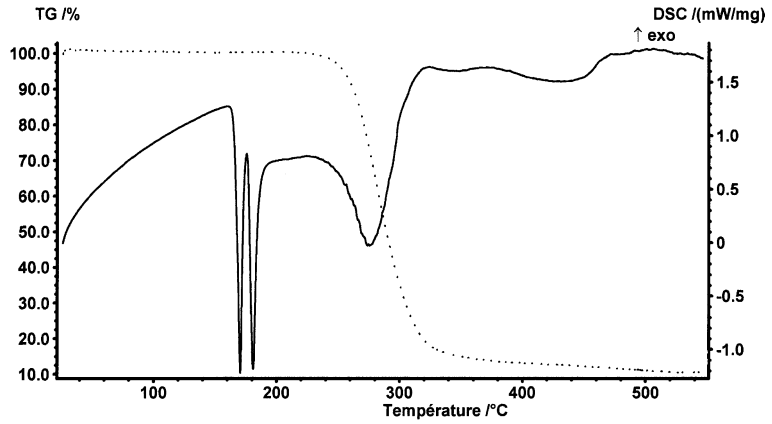

Figure 5. Mass change (.....) and DSC Thermogram (——) of RBV.

indicate a probable external association complex existence than a true host–guest complex formation between RBV and CDs. This assumption is corroborated by the high hydrophilicity of RBV which is not in favour of an inclusion in the cyclodextrin hydrophobic cavity.

On the other hand, the carboxamide protons easily exchangeable with deuterium did not give any signal on the ^{1}H NMR spectrum. In order to observe a potential interaction between carboxamide and native CDs, ^{13}C NMR study was conducted. The ^{13}C NMR chemical shifts observed for the RBV matched those obtained by Dea *et al.* [34] and Di Stephano *et al.* [4]. Also no chemical shifts of either triazole (C-3, C-5) or carboxamide (C-6) carbon atoms are observed for the binary systems RBV-native CDs. The RBV atoms do not interact with the electromagnetic environment of the H-3, H-5 protons of native CDs. No interaction has been found between RBV and H-3, H-5 CD protons. As it was early demonstrated by ^{1}H NMR in the case of doxorubicin with β- and γ-CD [35, 36], it was observed here that no interaction exists between internal H-3 and H-5 protons of the CDs and those of RBV. Consequently, the best hypothesis generally assumed is to consider the occurrence of an interaction different from the host–guest classical inclusion.

The 2D ROESY spectra, according with above results, did not show any other dipolar proton interaction of the RBV with the CDs (spectra not shown), confirming the above hypothesis. The lack of any other visible correlation in the ROESY spectra would suggest that no defined molecular association was observed in solution. Nevertheless, as fast intermolecular exchange to the NMR scale probably exists in solution and prevent the observation of such molecular association, the latter assumption should be considered with care.

Stability constants determined by HPLC

In order to determine stability constants of RBV-CDs complexes, we used HPLC. Several assays for RBV using this method have been developed for pharmacokinetic studies in biological fluids [37–39]. In this work, we have developed a simple and rapid technique on anion exchange support with an aqueous mobile phase. Potassium dihydrogenophosphate buffer was used as mobile phase since phosphate anions cannot interfere

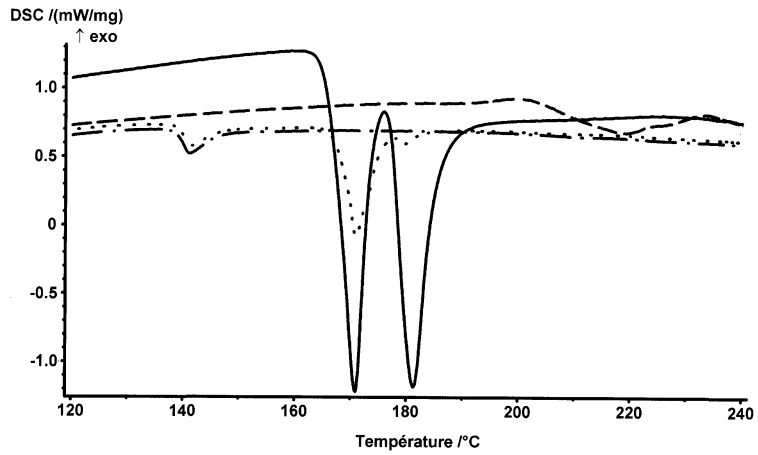

Figure 6. Thermoanalytical profile of RBV (——), α-CD (–··-), physical mixture RBV and α-CD (.....) and RBV/αCD complex (– –).

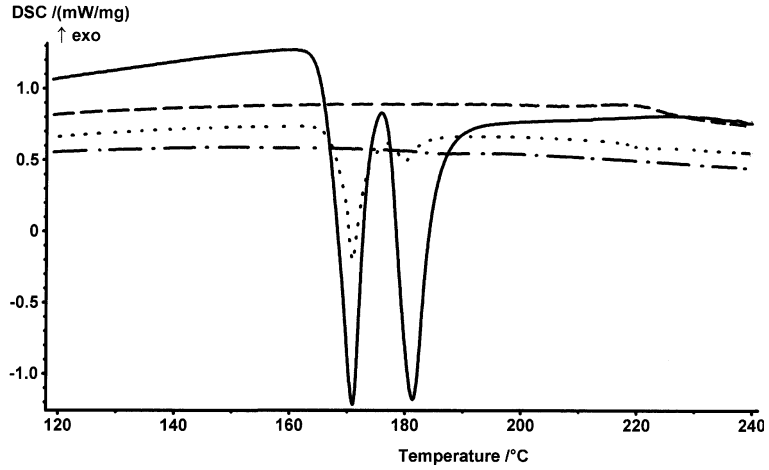

Figure 7. Thermoanalytical profile of RBV(——), β-CD (– ·-), physical mixture RBV and β-CD(·····) and RBV-βCD complex (– –).

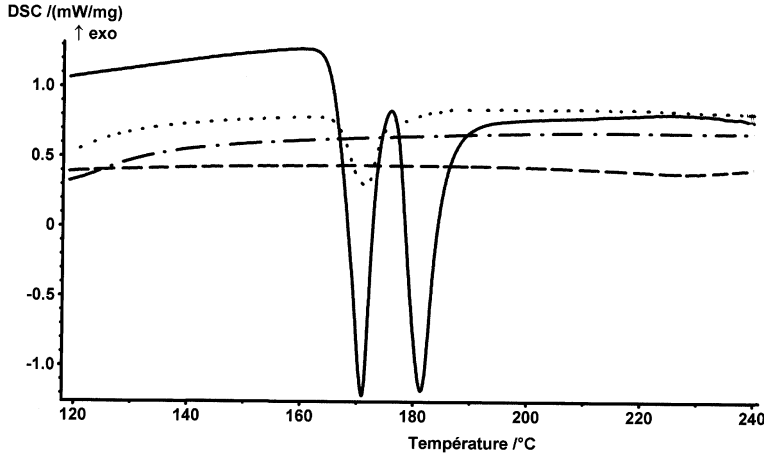

Figure 8. Thermoanalytical profile of RBV(——), γ-CD (– ·-), physical mixture RBV and γ-CD (·····) and RBV-γCD complex (– –).

with the complexation process. The column void volume was determined by elution of a thiourea solution (0.273 μg ml^{-1}). This volume was systematically controlled three times after column equilibration. Retention times obtained for RBV were the difference between mean of retention times of RBV and thiourea.

Significant RBV decreasing retention times (Figure 3) occur by increasing concentrations of α-, β-

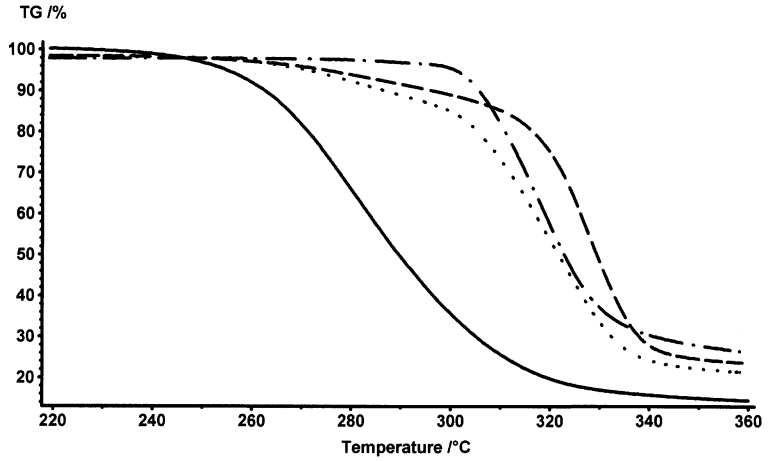

Figure 9. Mass changes of RBV (——) α-CD (– ·-), physical mixture RBV and α-CD (·····) and RBV/αCD complex (– –).

and γ-CD into the phosphate buffer, indicating an enhancement in the solubility of RBV by the binding to CDs. Figure 4 shows the determination of K_C from the retention time data of RBV-CDs complexes according to the Equation 1. As shown in Figure 4, linear plots were obtained for both of the α-, β- and γ-CD, verifying 1:1 stoichiometry. Whatever the CD (α,β or γ) the RBV was complexed with, the R values were higher than 0.99 showing a good linear correlation. The equations of the lines for RBV with α-, β- and γ-CD were respectively:

$$y = 0.2992x + 0.2004 \times 10^{-3} (R = 0.9996)$$
$$y = 0.3881x + 0.1489 \times 10^{-3} (R = 0.9997)$$
$$y = 0.4779x + 0.4053 \times 10^{-3} (R = 0.9992)$$

Accordingly, the calculated RBV-α, -β and -γ-CD complex stability constants are of 1493, 2606, and 1179 M^{-1}, respectively. Like the limits reported by Szetjli [40], the stability constants of the complexes of RBV with the native CDs would be suitable for pharmaceutical use.

DSC results

As a preliminary consideration, it should be pointed out that thermal analysis represents a very popular and widespread analytical approach to the characterisation of multicomponent systems such as inclusion compounds in the solid state.

DSC thermogram of RBV from 20 to 400 °C in the Figure 5 exhibits two sharp endothermic peaks corresponding to the two polymorphic forms of RBV [41]: one, the low melting form II, with a melting point at the onset 166.2 °C ($\Delta H = 68$ J·g^{-1}) and another, the high melting form I, with a melting point at the onset 177.2 °C ($\Delta H = 62$ J·g^{-1}). Thermal degradation of RBV starts above 260 °C in solid phase at first and continues in liquid state after fusion which occurs approximately at 278 °C.

Thermoanalytical profile of α-CD (data not shown) presents a water loss between 45 °C and 110 °C with dehydration energy of 40.83 J·g^{-1} and a variation mass of 2%. After its water loss, a small endothermic peak appeared at the onset 138.8 °C which was attributed to a phase transformation in different marketed α-CD [42, 43]. A small mass loss was observed at 259.8 °C which can be attributed to the loss of very tightly bound water ($\Delta H = 0.95$ J·g^{-1}). Beta-CD (data not shown) lost its water from ambient temperature up to 120 °C ($\Delta H = -26.5$ J·g^{-1}), an endothermic transition at 186°C and the degradation between 303 and 338 °C. These results are in accordance with the literature [44].

In the case of γ-CD (data not shown), the loss of water appeared in two waves between 35 °C and 125 °C ($\Delta H = 87.21$ J·g^{-1}). Its fusion/decomposition appeared at the onset 296.4 °C with a maximum at 316.8 °C. Since degradation of RBV occurred before degradation of native CDs, then thermal analysis can be used for examination of host–guest binary systems.

Comparison of the thermal behaviour with of single components (RBV and native CDs), their physical mixture and the inclusion compound candidate are presented in Figures 6–8 for α-, β-, and γ-CD, respectively.

In all cases, DSC thermograms of the physical mixtures were the combination of the DSC thermograms of the components analysed separately with little difference in the peak of the form II which was neglected. Drug endothermic maxima peaks can be observed at 170.9 and 179.9, 171.0 °C and 177.8, 171.3 and 179.2 °C, respectively for the systems RBV-α-CD, β-CD and γ-CD. Between 30 and 260 °C, TG curves of physical mixtures show exactly the same profile as the CDs-TG curves with mass variation corresponding to the lost water. Then after 260 °C, the lost mass of RBV fusion degradation was observed in all cases. DSC measurements of the putative inclusion compound RBV-α-CD in the ratio 1:1 show the disappearance of the endothermic peak at 138.8 °C corresponding to α-CD. Then the curve was flat between 120 and 200 °C. On the other hand, new endothermic transitions are observed which can correspond to the RBV peaks even though a very important size reduction and broadening of the RBV fusion peaks with a concomitant shift to higher temperatures (onset 203 and 216 °C) was observed. This effect could be ascribed to some drug-α-CD close interaction but was not sufficient to assess an inclusion [21].

The complete disappearance of the RBV endothermic peaks was observed for the lyophilised systems RBV-β-CD and RBV-γ-CD in the ratio 1:1. Generally, the flattening of the DSC profile in the melting region of the crystalline guest is taken as conclusive evidence of the molecular encapsulation of the drug inside the β-CD cavity [21, 44, 45]. But in our case RBV, with regards to the NMR results, should not be included inside the cavity of the CD because H-3 and H-5 protons were not shifted in the presence of the molecule. Nevertheless, this phenomenon indicates a stronger interaction between RBV and β- or γ-CD than between RBV and α-CD, in the solid state.

The thermogravimetric result of the RBV-α-CD system (Figure 9) presents a mass variation between 260 and 300 °C corresponding to the degradation of the recrystallised RBV. Indeed, this mass variation was not observed on the two other systems: RBV-β-CD and RBV-γ-CD. Consequently, the TG curves of the three lyophilised systems confirmed our DSC conclusions.

Conclusion

In conclusion, complexation of RBV with α-, β-, and γ-CDs was proved by FT-IR, HPLC and thermal analysis. The 1:1 stoichiometry for the complexes was obtained by HPLC experiments. It should be pointed out that β-CD shows the best interaction with RBV regards to its higher stability constant. As the IR results show spectral

changes due to the interaction of RBV carboxamide group with CD macrocycle, one can imagine the RBV carboxamide group was in close contact with hydroxyls of the CD ring. Concerning the NMR results, they did not show any visible interaction between RBV and CDs.

Consequently it may be advanced, this complexation is closer an external molecular association than a true host–guest inclusion. In this situation, this association probably involve hydrogen bonds between peripheral CD OH groups and RBV. Finally, the high solubility of the RBV in aqueous medium is probably at the origin of the observed RBV preferential non-inclusion in a CD hydrophobic cavity.

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