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ORIGINAL ARTICLE

Study of interaction between tiagabine HCl and 2-HPβCD: investigation of inclusion process

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Abstract Tiagabine (TGB) is an antiepileptic agent enhancing the activity of GABA at neuronal and glial region. It has recently been shown that enhancement of TGB chemical stability was improved by complexation with 2-hydroxypropyl-beta-cyclodextrin (2-HPβCD). The aim of this project is to explain the improvement of the chemical stability of complexed TGB by studying the inclusion properties and factors affecting the complexation selectivity between 2-HPβCD and TGB. Analysis of the interaction between 2-HPβCD and TGB and the effect of 2-HPβCD on TGB solubility was performed by phase solubility method described by Higuchi and Connors; the complexation was followed by characterization using DSC, FTIR and NMR spectroscopy. In aqueous media, the analysis of NMR proton shift change continuous variation method (Job's plot) and the NMR diffusion-ordered spectroscopy (DOSY) measurements clearly show that TGB form 1:1 inclusion complex with 2-HPβCD with an association constant (K_a) of 3396 M^{-1}. More detailed information about the inclusion mode and the geometry of the complex was obtained by the analysis of 2D NMR NOESY experiment and molecular modelling calculations. The inclusion process indicates that A-ring, C_{10}–C_{11} double bond and the half of the B-ring of TGB molecule were located inside the cavity while the nipecotic acid part of TGB (Ring C) was exposed towards the outside of the 2-HPβCD cavity. These results suggest that the inclusion of the C_{10}–C_{11} double bond in the 2-HPβCD cavity may possibly the reason of improvement of TGB chemical stability.

Keywords Tiagabine · Cyclodextrins · Inclusion · DSC · FTIR · NMR · Docking

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Introduction

The poor physicochemical stability of a drug may limit its delivery through different routes of administration. Svensson et al. [1] pointed that the major challenge during the formulation of tiagabine HCl (TGB) in a variety of dosage forms are related to its weak stability. TGB is considered as the first antiepileptic drug that acts selectively by enhancing the GABAergic inhibition specifically by decreasing neuronal and glial uptake of GABA in the synaptic region. Another promising research area for TGB related to its possible control of cocaine dependence [2–7]. TGB is a drug composed from two parts (Fig. 1a) the first is the nipecotic acid which has a GABA inhibiting effect, and its zwitterionic structure suggested that it was incapable to penetrate the blood brain barrier. A lipophilic anchor, the second part, has been incorporated to facilitate crossing of the blood brain barrier after oral administration [8, 9]. Nahata and Morosco [10] prepared simple extemporaneous syrups of TGB 1 mg/mL in which the results indicated that TGB can tolerate up to 13-week storage at 4 °C in plastic bottles without substantial loss of potency. However, Svensson et al. [1] reported a loss of about 5% of TGB potency per storage of TGB tablets for 18 month, whereas only 2% was lost after storage with alpha-tocopherol under

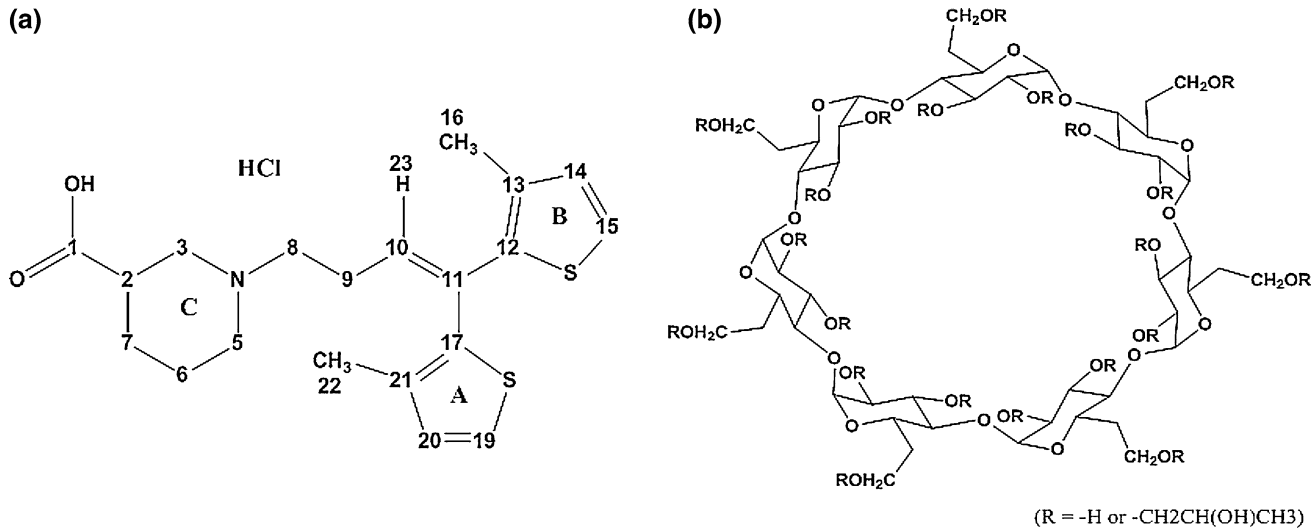

Fig. 1 The chemical structure of tiagabine HCl (**a**) and 2-HPβCD (**b**)

similar conditions. Alpha tocopherol works as an antioxidant or a stabilizing agent. Bhiwgade et al. [11] claimed an alternative method concerned with the combination of 4 stabilizing agents, butylated hydroxyl anisole BHA, butylated hydroxyl toluene BHT, propyl gallate PG and ethylene diamine tetra acetate EDTA for controlling the stability of TGB where a synergic effect of these stabilizing agents has been demonstrated. The study showed a better enhancement of TGB stability using these antioxidants than formulations prepared using alpha tocopherol alone. All described and used methods to improve the stability of TGB were based on the addition of antioxidants stabilizers [12–14]. These observations prompted us to probe the efficiency of cyclodextrins to interact and to form an inclusion complex with TGB. Complexation with cyclodextrins improved the solubility of drug substance forming non-covalent inclusion complexes [15]. Further, the resulted inclusion complex between drug (Guest) and cyclodextrins (Host) can also improve the stability and other organoleptic properties [16, 17]. We showed in a recent work that enhancement of TGB stability was noticed by complexation with 2-HPβCD [18]. In order to elucidate the improvement of chemical stability of complexed TGB, we have investigated the interaction between TGB and 2-HPβCD (Fig. 1b) to report the characterization of TGB/2-HPβCD complex by DSC and FTIR and apply NMR spectrometry and molecular calculation to investigate the TGB/2-HPβCD complex.

Materials and methods

Materials

TGB and 2-HPβCD were purchased from Molecula UK and Wacker Germany, respectively. NMR grade D_2O was obtained from Eurositop France and HPLC-grade acetonitrile and Trifluoroacetic acid (TFA) was supplied by Acros Organics France.

Phase solubility measurements

The interaction test between TGB and 2-HPβCD was performed according to the phase solubility study method described by Higuchi and Connors [19]. An excess amount of TGB was added to aqueous 2-HPβCD solution ranging in concentration from 0 to 300 mM in 10 mL capped tubes. All tubes were sealed to avoid evaporation and shielded from light to prevent any degradation. A magnetic stirring to attain equilibrium 48 h in a thermostated bath at 27 ± 0.1 °C was employed. After, suspensions were filtered through Millipores 0.45 mm filters and an aliquot from each vial was withdrawn to be assayed chromatographically by high performance liquid chromatography (HPLC) for the evaluation of dissolved amount of TGB. TGB concentrations in solution were measured by HPLC. The HPLC system comprised: a Varian system equipped with a 9050 variable wavelength UV-detector, a 9010 solvent delivery system and a 9100 auto-sampler. A Nucleosil-C18 column (125 mm×4 mm, 5 μm particle size) was used. An isocratic mobile phase was employed. The mobile phase was composed of aqueous 0.1% TFA in water and acetonitrile at a ratio 65:35% respectively. The flow rate was 1.0 mL/min, column temperature 18 ± 2 °C and injection volume 50 μL. A similar ratio of mobile phase was used to prepare standard solutions for the calibration measurements. The analysis of TGB was carried out 10 min after preparing the sample to ensure analysis of total TGB quantity. Chromatograms were collated at wavelength of 250 nm using Varian System Software. The experiment was carried out in triplicate.

Preparation of inclusion complex

The inclusion complex of TGB/2-HPβCD was prepared by freeze drying method. Equimolar quantities of TGB base and 2-HPβCD were dispersed into 50 mL Millipore water and stirred for 24 h at 27 ± 0.1 °C. In order to estimate the quantity of TGB could be weighed from the bulk, it was calculated upon the Eq. 1.

$$W_{TGB} = P\frac{412}{375.6} * \frac{100}{100 - H} \quad (1)$$

where W_{TGB} is amount of TGB could be weighed, P is amount of corresponded TGB base needed to form an equimolar complex with 2-HPβCD and H is the relative humidity. The suspension was filtered through a 0.45 mm Millipore filter, frozen and then lyophilized using freeze dryer. The heating temperature during freeze drying was 27 °C. A physical mixture was prepared by mixing equimolar quantities of TGB and 2-HPβCD with a porcelain mortar and pestle to obtain a homogeneous blend. Both prepared complex and physical mixture were refrigerated in well tight containers for further tests.

DSC and FTIR spectroscopy

DSC analysis was performed using a Perkin Elmer differential scanning calorimeter model DSC-4 USA equipped with a compensated power system. The thermal behaviours of TGB, 2-HPβCD, TGB/2-HPβCD physical mixture and TGB/2-HPβCD complex were studied by separate heating. An accurately weighed sample each equivalent to 3 mg TGB was in a sealed aluminium pan, using a sealed empty pan as reference, over the temperature range of 30–300 °C and at a rate of 10 °C/min in a dynamic nitrogen environment. Indium standard was used for calibrating the temperature. A FTIR Perkin Elmer equipped with an ATR-Ge crystal from Pike Technologies was used for the analysis of TGB, 2-HPβCD, TGB/2-HPβCD physical mixture and TGB/2-HPβCD complex. The frequency range was fixed between 4,000 and 500 cm^{-1}, at a 4 cm^{-1} resolution (20 scans).

NMR measurements

All NMR spectra have been performed on a Bruker AVI-II600 NMR spectrometer equipped with a 10 A gradient amplifier and a 5 mm CPTXI (^{1}H, ^{13}C, ^{15}N) including shielded z-gradients. The NMR spectra were recorded in D_2O solution at 298 K and all chemical shifts were measured relative to external TMS.

^{1}H NMR spectra were recorded with a 12020 Hz spectral window digitized with 32 K points. The 1D ^{13}C spectra were recorded between 0 and 200 ppm using a 25026 Hz spectral window digitized into 64 K points. Broad band proton decoupling was employed. The following parameters were used for acquiring the COSY spectra in absolute values: 256 experiments with 2048 data points and 8 scans each were recorded. HMQC spectra were measured using 512 increments of 16 scans each of 2048 complex points, with spectral widths in f_1 and f_2 of 18770 and 12020 Hz, respectively. All two-dimensional NMR data matrices were zero-filled in the f_1 dimension. Prior to Fourier transformation, a phase shifted sine bell filter function was applied. The HMQC spectra were displayed in phase sensitive mode and the COSY in absolute value. Intermolecular proximity was obtained from 2D NOESY experiment. The measuring conditions for the 2D spectra were: spectral width 12020 Hz; 512 experiments with 2048 data points; relaxation delay 3 s, and 32 scans with a mixing time of 600 ms. Phase sensitive spectra were acquired using TPPI scheme.

Diffusions coefficients were obtained from 2D DOSY (Diffusion Ordered SpectroscopY) experiments. 2D DOSY experiments were performed using a bipolar gradient LED pulse sequence [20]. The applied diffusion time (Δ) was 150 ms and the duration of gradient pulses (δ) was 2.5 ms. The maximum gradient was 5.35 G cm^{-1} A^{-1} and it took 10 different values. Therefore, the data contained 16 spectra and in each spectrum there were 8192 points on the chemical shift dimension. The DOSY processing program as implemented in TOPSPIN Software (Bruker, Germany) was used.

Determination of stoichiometry

The stoichiometry of the complex was determined by the continuous variation method. The overall concentration of the two species was kept constant ([TGB] + [2-HPβCD] = 5 mM) and the mole fraction of TGB (i.e., $r = \frac{[\text{TGB}]}{[\text{TGB}]+[\text{2-HP}\beta\text{CD}]}$) was varied from 0.1 to 0.9. This was accomplished by using the equimolar solutions of TGB and 2-HPβCD and mixing them to constant volume to the desired ratio r. Then, the quantity $\Delta\delta_{obs}^{(Hi)}$[TGB] was plotted against r. $\Delta\delta_{obs}^{(Hi)}$ is the difference between the chemical shift of free TGB and the observed value for a given ratio r.

Molecular modelling

The three-dimensional coordinates of β-cyclodextrin was taken from CSD (www.ccdc.cam.ac.uk) (Ref. code BCDEXD03) and imported into the molecular modelling program InsightII [21]. The structure was checked for chemically consistent atom and bond type assignment.

All β-cyclodextrin containing 4 or 5 hydroxypropyl group in position 2 were generated and protonated using the InsightII software. To avoid steric clashes, added hydrogen atoms were energy-minimized using the Powel algorithm, with a convergence gradient of 0.5 kcal mol^{-1}

for 1500 cycles. This procedure affected only non-experimentally detected hydrogen atoms.

AutoDock: Cyclodextrins were treated using the united atom approximation. Only polar hydrogens were added to the structure and atomic charges were then calculated using the Gasteiger method [22].

All water molecules were removed from the target structure. 60 × 60 × 60 Å affinity grids, centred on the cyclodextrin cavity with 0.375 Å spacing, were calculated by use of Autogrid 4.0 [23], for each of the following atom types: C, A (aromatic C), N, O, S, H and Cl.

Only polar hydrogens were added to the ligand and Gasteiger charges were assigned. The rotatable bonds were selected via AutoTors [23].

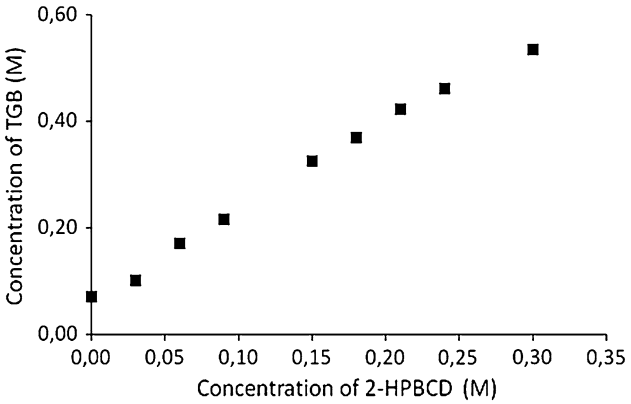

Fig. 2 The phase solubility diagram of TGB as a function of 2-HPβCD concentration

We selected the Lamarckian genetic algorithm (LGA) for ligand conformational searching. LGA adds local minimization to the genetic algorithm, enabling modification of the gene population [23]. The following docking parameters were used: trials of 100 docking, population size of 100, random starting position and conformation, translation step ranges of 1.0 Å, rotation step ranges of 1.0 Å, elitism of 1, mutation rate of 0.02, crossover rate of 0.8, local search rate 0.06 and 250,000 energy evaluations. Structures with lower energy and in agreement with the experimental NMR distances were selected.

Results and discussion

The solubilisation of TGB by the 2-HPβCD was quantitatively evaluated at pH 7.4 by Higuchi and Connors phase solubility method [19]. The solubility of TGB was increased 7.5-folds from 26.8 g L^{-1} for TGB alone to reach 201.06 g L^{-1} after complexation with 2-HPβCD. The phase solubility diagram, i.e., plots of solubility of TGB as a function of 2-HPβCD concentration (Fig. 2) shows a linear increase of TGB solubility with the increase of 2-HPβCD concentration. This linear trend can be considered as an A_L-type diagrams according to the phase solubility principle which indicate TGB/2-HPβCD interaction with a 1:1 stoichiometry.

In order to prove the formation of the TGB/2-HPβCD complex, DSC and FTIR experiments were performed on TGB, 2-HPβCD, TGB/2-HPβCD physical mixture and

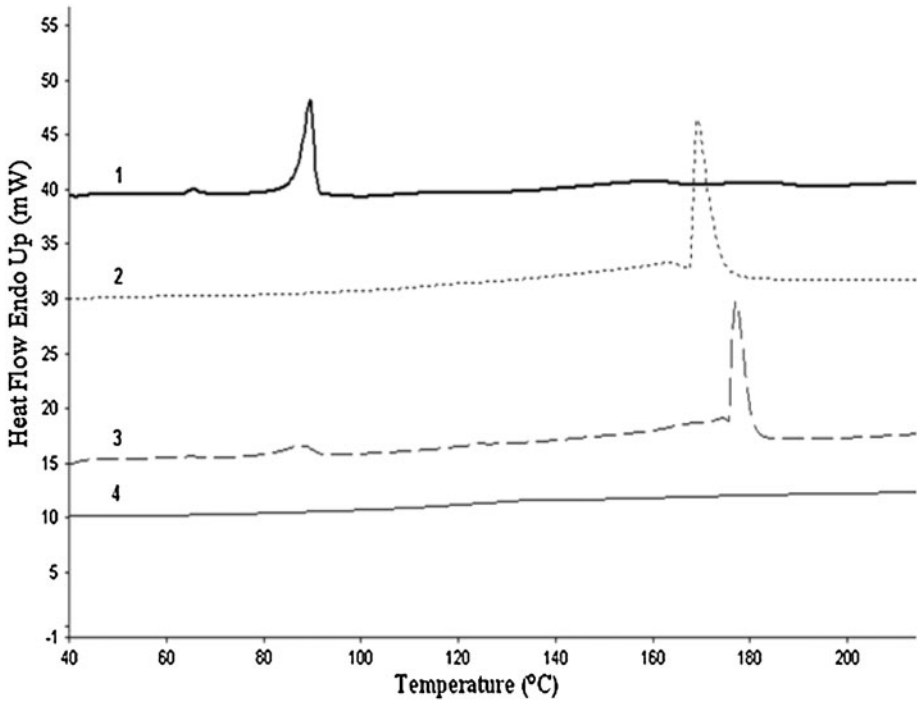

Fig. 3 DSC diagram of TGB (1), 2-HPβCD (2), physical mixture (3) and TGB/2-HPβCD complex (4)

complex. The DSC thermogram of TGB (Fig. 3.1) presents an endothermic peak at 89 °C indicating the melting point of the active substance, while the DSC curve of 2-HPβCD (Fig. 3.2) shows a melting peak of 169 °C. The DSC curve of physical mixture (Fig. 3.3) indicates that no major change was observed of the endothermic peaks of the pure TGB (89 °C) and 2-HPβCD (169 °C) suggesting that no inclusion complex was formed by physical blends.

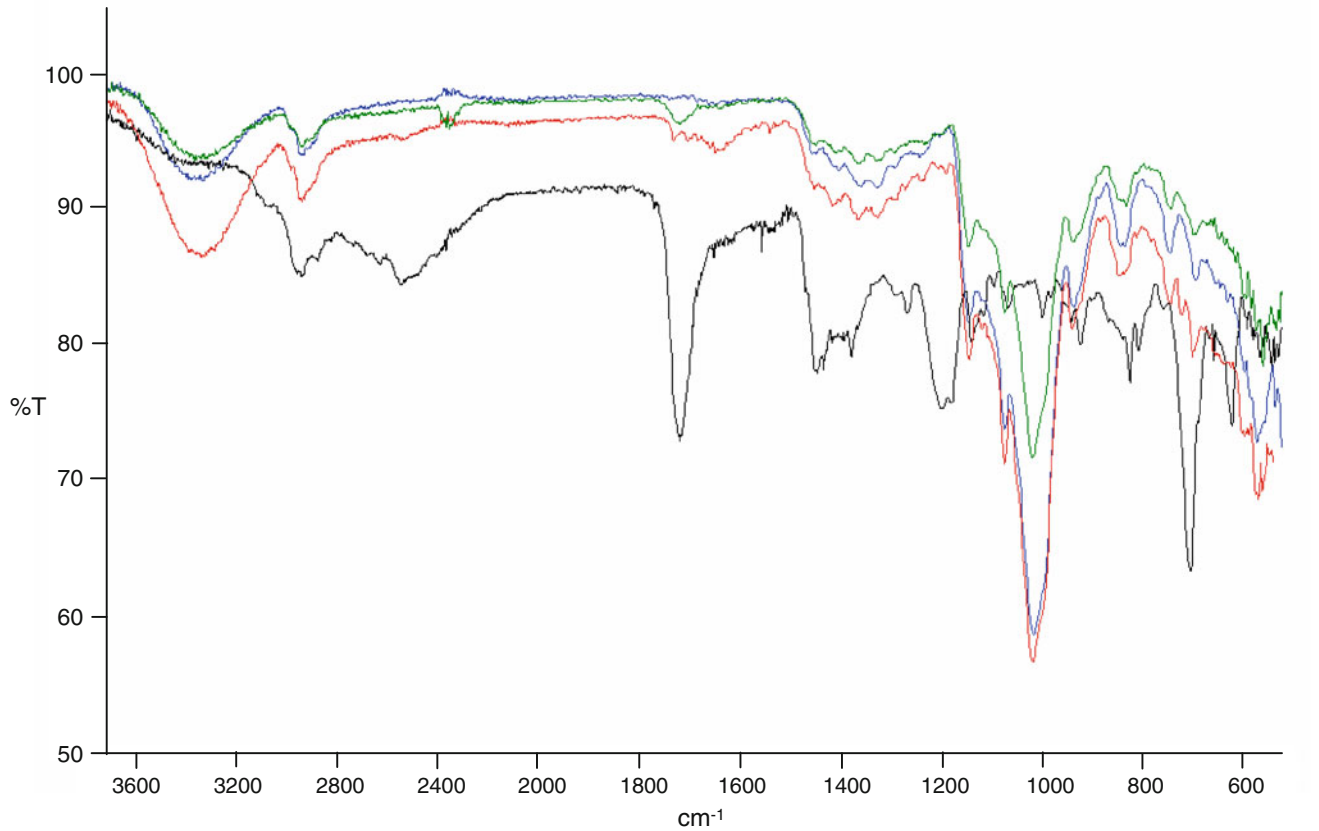

Fig. 4 Experimental FTIR–ATR spectra collected in the frequency region (4000–500 cm^{-1}) for 2-HPβCD (blue), TGB (Black), TGB/2-HPβCD physical mixture (Red) and TGB/2-HPβCD complex (Green)

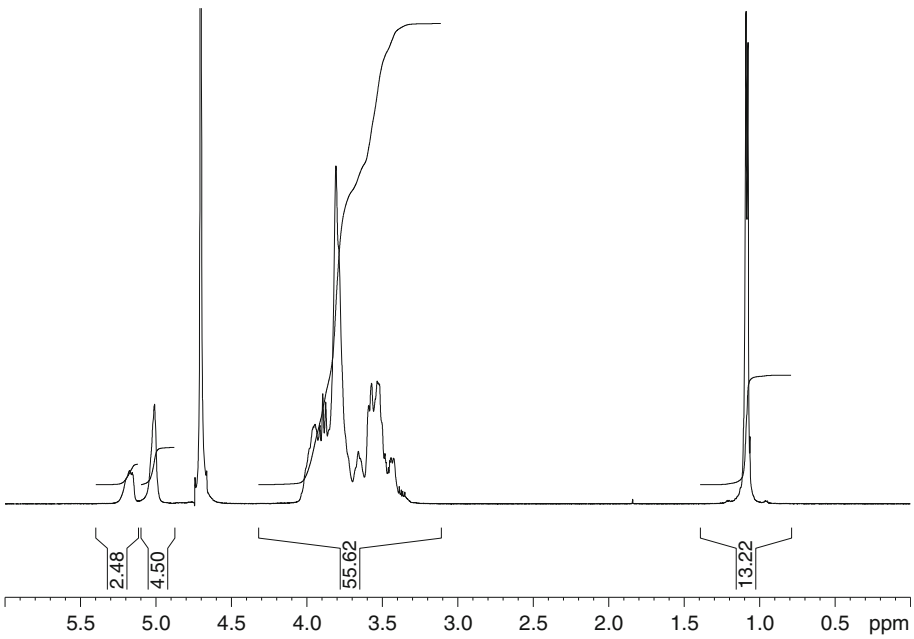

Fig. 5 1D proton NMR spectrum of 5 mM 2-HPβCD in D_2O (600 MHz, T = 298 K)

However, the absence of drug and cyclodextrin melting temperatures from the thermogrammes of TGB/2-HPβCD complex (Fig. 3.4) points toward the formation of an inclusion complex [24].

Similar to the DSC, FTIR-ATR spectrometry also allows the characterization and detection of inclusion complex in the solid state. The resulted data of FTIR confirmed the formation of the inclusion complex. The bands of the functional groups implicated in the formation of inclusion process were changed. The intensity of the very weak aromatic stretch sulphur –S–C at $\sim$650 cm^{-1} and both of the medium strength methyl R-CH_3 sym. at $\sim$1455 cm^{-1} and asymmetrical stretch at $\sim$1377.5 cm^{-1} were clearly reduced in the case of inclusion complex spectrum when compared to the other spectral bands [25, 26] (Fig. 4). However, the carboxyl group –COOH that is characterized by a strong C=O stretch band at $\sim$1712.5 cm^{-1} and medium –OH stretch at $\sim$3000, weak deformation at $\sim$1417.5 and medium deformation at $\sim$917.5 cm^{-1}, respectively, were unchanged. Likely, the tertiary amine band $(R)_3$–N that is characterized by a strong stretch C–N at $\sim$1335 cm^{-1} [27] was also unchanged indicating a partial inclusion of the heterocyclic aromatic moiety of TGB whereas the nipecotic acid part is probably exposed.

NMR is considered as the tool of choice for precise structural characterization of organic molecules in solution. In order to better characterize the tridimensional structure of the complex, a structural NMR study on TGB/2-HPβCD complex was carried out.

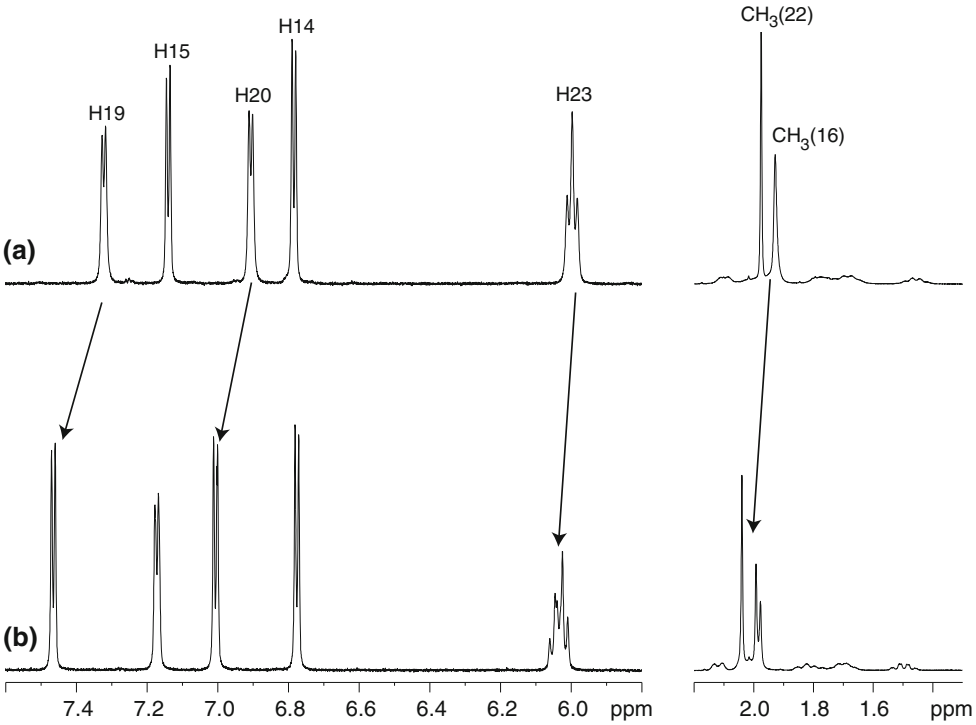

Fig. 6 Zoom of proton 1D NMR spectra (600 MHz, T = 298 K) of TGB (**a**) and TGB/2-HPβCD 1:1 complex (**b**)

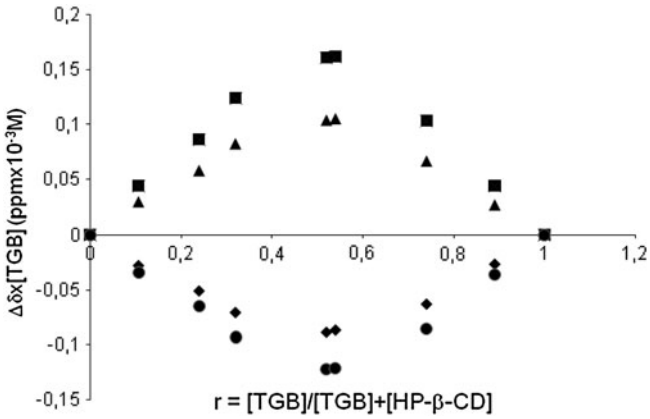

Fig. 7 Continuous variation plot of TGB/2-HPβCD, showing the observed TGB chemical shifts of H14 (*filled circle*), H15 (*filled diamond*), H19 (*filled square*) and H20 (*filled triangle*) as a function of mole fraction

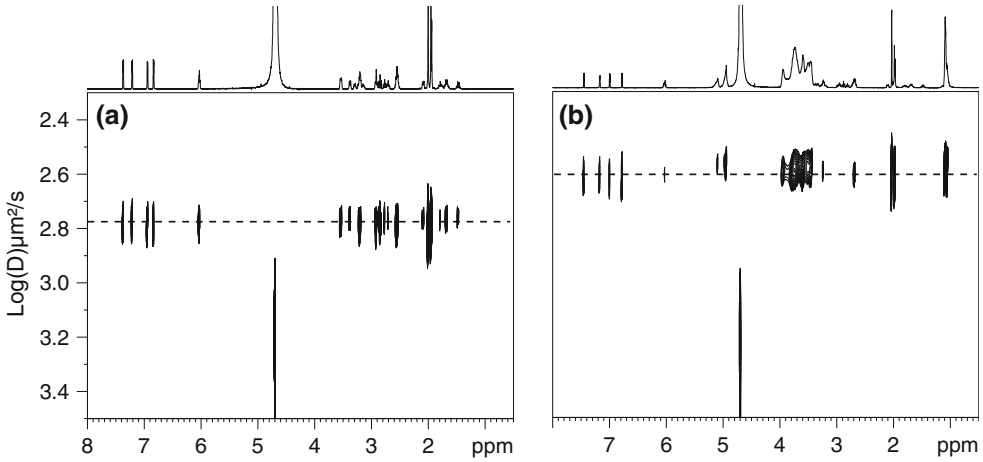

Fig. 8 2D DOSY Spectra (600 MHz, T = 298 K) of TGB (**a**) and TGB/2-HPβCD 1:1 complex (**b**)

Table 1 Diffusion coefficients determined from DOSY experiments for 2-HPβCD (D_{bound}), TGB in a complex form (D_{obs}) and in free state (D_{free})

D_{bound} (m^2/s)	D_{free} (m^2/s)	D_{obs} (m^2/s)	C_{bound}	K_a (M^{-1})
3.60×10^{-10}	6.40×10^{-10}	4.20×10^{-10}	0.785	3396

In the first step, the 2-HPβCD sample was characterized by ^{1}H NMR in order to verify the number and the position of hydroxypropyl group substitution. Assignment of ^{1}H and ^{13}C NMR spectra of the 2-HPβCD forms in aqueous solution was achieved by two dimensional ^{1}H/^{1}H COSY and ^{1}H/^{13}C HMQC and HMBC. The ^{1}H and ^{13}C assignments for all the monosaccharides derivatives are in agreement with those obtained by Zoppetti and co-workers [28]. Besides, the degree of substitution (DS), i.e., the average number of hydroxypropyl chain in D-glucose units, was quantitatively determined from the ratio of the area of peaks belonging to all the anomeric protons over those belonging to methyl protons of hydroxypropyl chain (Fig. 5). A value of DS $\approx$ 0.63 was found. These data confirm that the D-glucose residues in 2-HPβCD are unsubstituted or monosubstituted with three different substitutions: mainly hydroxypropyl group substitution in the position 2, smaller number in the position 3 and very few numbers in the position 6.

^{1}H NMR spectra of TGB and TGB/2-HPβCD solutions are shown in (Fig. 6). Here, shifted TGB NMR signals of H16, H19, H20, H22 and H23 as well as 2-HPβCD NMR signals of H3 and H5 are in accordance with a complexation involving inclusion phenomena.

To establish the stoichiometry of the complex, the continuous variation method was used to follow the changes in chemical shifts of protons H16, H19, H20 and H22 of TGB which shows the most marked variations (Fig. 7). The maximum or minimum values show insignificant deviation from r = 0.5, indicating the existence of a 1:1 stoichiometry within the range of investigated concentrations. These results were in agreement with the phase-solubility studies between TGB and 2-HPβCD. The phase solubility study indicates the existence of A_L profile from the linear regression plot of the molar concentration of TGB versus molar concentration of 2-HPβCD according to Higuchi and Connors [19].

DOSY is a two-dimensional NMR experiment, in which the idea is to correlate chemical shifts with molecular diffusion coefficients of individual molecules by using the pulse field gradient (PFG) [29–31]. The DOSY NMR experiment results in a 2D spectrum, displaying chemical shifts on one axis (f_2) and the calculated diffusion coefficients on the other axis (f_1) (Fig. 8).

In the case of complex of 1:1 stoichiometry, NMR diffusion measurements are one of the recognized methods used for determination of the association constant; particularly its convenient to indicate the binding between molecules of different size [32].

$$K_a = \frac{X_{bound}}{(1 - X_{bound})\left([\mathrm{TGB}]_0 - X_{bound}[\text{2-HP}\beta\mathrm{CD}]_0\right)} \quad (2)$$

where

$$X_{bound} = \frac{(D_{free} - D_{obs})}{(D_{free} - D_{bound})} \quad (3)$$

Association constant K_a is evaluated according to the Eqs. 2 and 3. Where X_{bound} is the fraction of TGB bound with 2-HPβCD, $[\mathrm{TGB}]_0$ and $[\text{2-HP}\beta\mathrm{CD}]_0$ are the total concentration of TGB and 2-HPβCD. D_{obs} is the observed diffusion coefficient, D_{bound} and D_{free} are the diffusion coefficient of bound and free TGB (Table 1). Based on Eqs. 2 and 3, a molar fraction of bound TGB was calculated, C $\approx$ 0.785, which corresponds to a complexation association constant of 3396 M^{-1}.

The most important information regarding the structure of the complex was given by the two-dimensional ^{1}H, ^{1}H NOESY spectrum. The NOESY spectrum of TGB/2-HPβCD complex (Fig. 9a) shows intense intermolecular

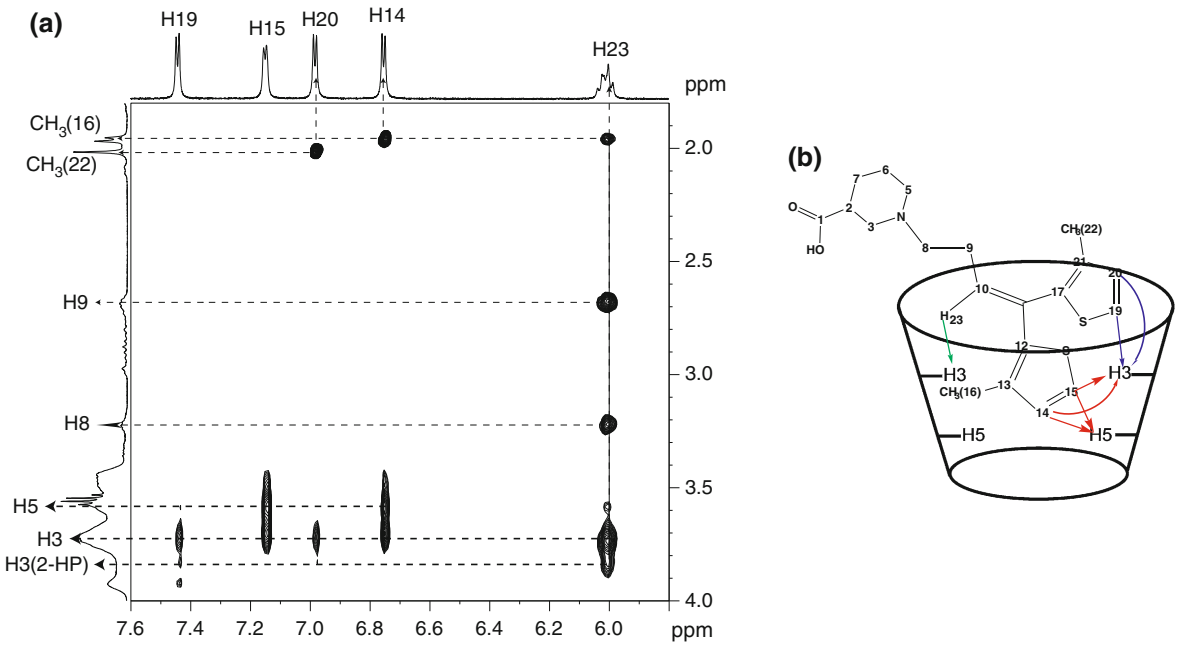

Fig. 9 Partial contour plot of the NOESY spectrum (600 MHz, T = 298 K) of TGB/2-HPβCD complex (5 mM, 1:1 M ratio) (**a**) and the conceptual design of TGB/2-HPβCD inclusion complex (**b**)

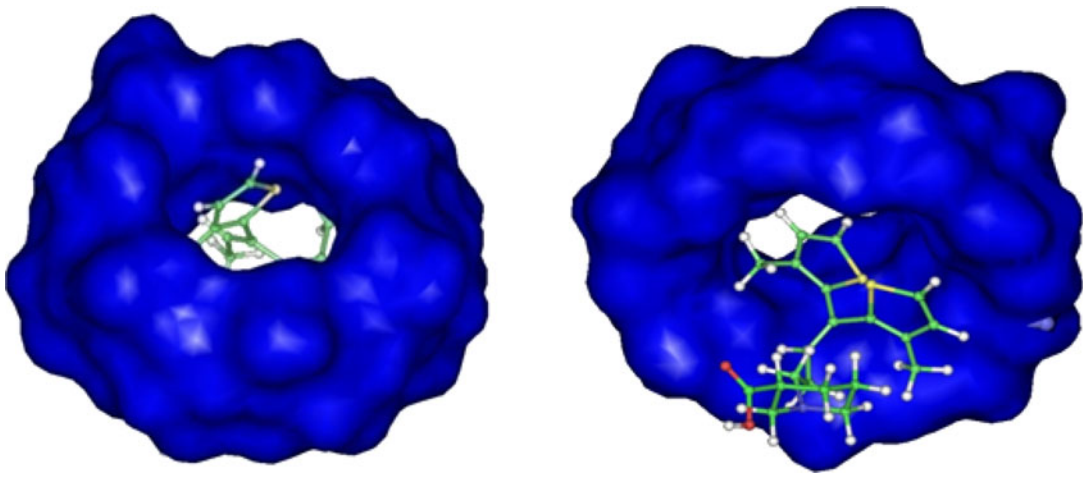

Fig. 10 Inclusion complex between TGB and 2-HPβCD obtained from molecular docking studies

correlations between protons H5, H3 of 2-HPβCD ad H14, H15 of B-ring of TGB molecule, H3 of 2-HPβCD and H23 of TGB molecule and less intense correlations between H3 of 2-HPβCD and H19 and H20 of A-ring. However, no dipolar interaction appears with H6 proton of 2-HPβCD and between the proton of the C-ring and the protons of 2-HPβCD. These results suggest that TGB is partially included (ring A and B) through the larger rim into 2-HPβCD cavity (Fig. 9b) with no favoured orientation for the nipecotic acid part of TGB molecule (ring C).

All these experimental observations are supported by the structure of the TGB/2-HPβCD complex obtained by molecular modelling (Fig. 10). This was achieved by docking of TGB molecule into 2-HPβCD cavity. The structure of the complex shows that the A-ring of TGB molecule was inside the cavity of 2-HPβCD, half of the B-ring was also located inside the cavity while C-ring of TGB molecule was oriented outside of 2-HPβCD cavity.

Conclusion

Characterization of TGB/2-HPβCD complex was carried out essentially by DSC, FTIR, NMR and molecular

modelling studies. Inclusion complex was formed successfully between TGB and 2-HPβCD with a relatively effective association constant. The inclusion process indicates that A ring, half of the B ring and the most susceptible part of TGB molecule to chemical degradation (C_{10}–C_{11} double bond) were located inside the cavity while the nipecotic acid part of TGB (Ring C) was exposed towards the outside of the 2-HPβCD cavity. The inclusion of the C_{10}–C_{11} double bond in the 2-HPβCD cavity may possibly the reason of improvement of TGB chemical stability [18].

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