Influence of the substituted β-cyclodextrins by amino groups on the complexation of antifungal drug

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Abstract: The selective functionalization on the primary face of β-cyclodextrin with amino groups is described. The inclusion complexation of griseofuvin (GSV) molecules by β-cyclodextrin and its synthesized amino derivatives has been elucidated. The stability constants of the complexes of 1:1 stoichiometry (K_{11}) have been evaluated from ^{1}H chemical shift changes of griseofuvin protons. Mono- and per-amino substuted β-cyclodextrin showed an increase of inclusion binding ability for griseofulvin guest. The fully amino substituted β-cyclodextrin at the primary face shows the strongest complexation ability towards griseofulvin molecules. A simple thermodynamic theory of the electrostatic contribution to the complexation is presented.

Keywords: Amino-β-cyclodextrins; griseofulvin, inclusion complexes, stability constant.

INTRODUCTION

GSV is an antifungal drug agent first isolated from a *Penicillium*-spp. in 1939. It is widely used for the treatment of mycotic diseases of the skin, hair and nails. It is deposited primarily in keratin cells [1]. Her very limited solubility in water (15 µg.mL-1 at 37°C) results a low bioavailability due in a little absorption from the gastrointestinal tract. Its therapeutic dose is fairly close to its toxicity limit which causes the problems with safety and efficiency. The reduction in particle size enhances the absorption of griseofulvin [2]. Indeed, it was proved that the absorption of GSV from the gastrointestinal tract may be enhanced by micronization of the drug into fine particles [3], preparation of GSV nanoparticles from water-dilutable microemulsions [4] and preparation of GSV nanosuspensions from triacetin-in-water emulsion [5], lead to the increase of its solubility and the bioavailability. Trotta and al. have previously shown that its solubility increases if solid solutions of GSV are mixed with polyethylene glycol and sodium dodecyl sulphate [6]. Nevertheless, solubilization of GSV within detergent micelles is limited [7,8] and could be require enough large concentrations of detergent at non tolerated levels. The solubilization by cyclodextrins is a good alternative since it has been shown that griseofulvin forms inclusion complexes with β-cyclodextrin [9,10].

Cyclodextrins are cyclic oligosaccharides possessing the overall shape of a hollow truncated cone, the narrow rim bearing primary hydroxyl groups and the wide rim secondary hydroxyl groups [11,12]. The most significant characteristic of cyclodextrins is their ability to include a wide variety of guest molecules into their hydrophobic cavity in aqueous solutions, without the formation

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of any covalent bond [13]. The potential utility of the inclusion complexes includes solubilization, encapsulation, and transport of small hydrophobic molecules including toxins and drugs [14]. However, among all natural CDs, β-cyclodextrin has the lowest solubility in water (18.5 $g.L^{-1}$), compared to those of counterparts α- and γ-CD (145 and 232 $g.L^{-1}$) due to the strong intramolecular hydrogen bonding of secondary hydroxyl groups [15]. As a result, the extensive investigations into the chemical modifications of cyclodextrins have been concerned primarily with influencing their solubility as well as with modifying their binding behaviors. Chemical modifications are often aimed at increasing the solubility of the β-cyclodextrin or of the inclusion complex, but the presence of substituents may also contribute to the complexation of the host.

Selective modification of cyclodextrins can concern complete set of hydroxyl groups or some of them. The glucose repeating unit of a cyclodextrin contains three different hydroxyls: one primary hydroxyl group connected to C_6 located at the narrow side of the cyclodextrin molecule, and two secondary hydroxyl groups connected to C_2 and C_3 located at the wider side (Figure 1). The primary and secondary hydroxyl groups have different reactivities. The problem of discriminating between a set of primary hydroxyl groups and that of secondary ones raises the question of the strategy to be followed.

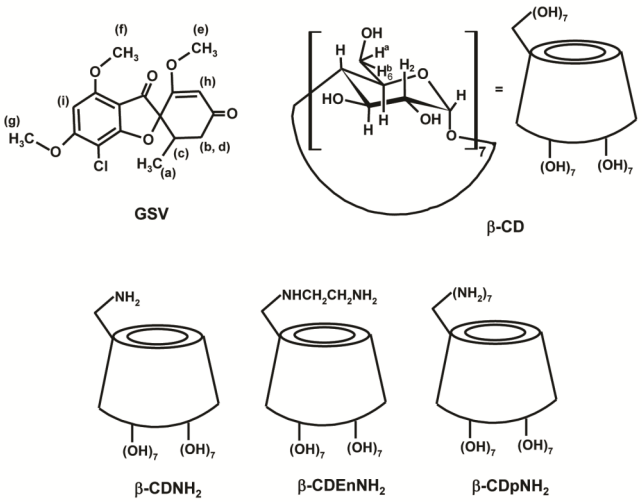

Figure 1: Structure of griseofulvin, native and amino-modified β-cyclodextrins.

The selective monofunctionalization of cyclodextrins can be carried out easier at the primary hydroxyl groups than at the secondary hydroxyl ones. This is due to the more nucleophilic primary hydroxyls character over the more acidic secondary hydroxyl ones. Several different methodologies are known for the selective introduction of one sulfonyl group at the primary hydroxyl face of cyclodextrin [16]. Monotosylation of β-cyclodextrin (β-CD) is important since a good leaving group is introduced, which can be easily substituted by other functional groups. Thus, the synthesis of cyclodextrins singly or fully substituted on primary hydroxyls with amino groups has been reported in the literature [17,18]. The general synthetic strategy is to generate the amino derivatives from the corresponding β-cyclodextrin mono-tosylate (for the mono-functionalization of primary hydroxyls) or β-cyclodextrin per-iodide (for the per-functionalization of primary hydroxyls) via nucleophilic displacement of the sulfonate or iodine atoms by azide ion or ethylenediamine. The azido derivatives are subsequently converted into amino compounds by reduction [19].

In this study, the objective is to elucidate the formation of complexes between amino-modified β-cyclodextrins and griseofulvin molecule.

In this study, the objectives are firstly, of elucidate the formation of complexes between amino-modified β-cyclodextrins and griseofulvin molecule and secondly, to evaluate the electrostatic contribution of amino-groups at the complexation of GSV guest by cyclodextrins hosts. A comparative study of the complexation processes and of the stability of formed complexes was done through the use of ^1H NMR chemical shift changes.

EXPERIMENTAL SECTION

1. Materials and methods

β-cyclodextrin was generously provided by Roquette (Dunkerque, France) and was dried at 120 °C overnight under vacuum before use. Triphenylphosphin (99%, Aldrich), potassium iodide (99%, Aldrich), sodium azide (99%, Aldrich), ammonium hydroxide (P.A., 28-30 wt% solution of NH_3 in water), *p*-toluenesulfonyl chloride (99%, across), 1,2-diaminoethane (98%, Riedel deHaen) and potassium bromide were used without purification. DMF was dried over $CaCl_2$ and distilled. ^1H and ^{13}C NMR spectra were

determined with Bruker DRX300 spectrometer working at 300 MHz and 75 MHz for ^{1}H and ^{13}C nuclei respectively. The superscript A stands for nuclei of the functionalized glucopyranose units. Fourier transform infrared (FTIR) spectra of each β-cyclodextrin were recorded with a Perkin Elmer 1000 spectrometer using discs of each β-cyclodextrin and previously prepared KBr containing a 0.01 g sample in 0.1 g of potassium bromide between wavelengths 400-4000 cm^{-1}.

2. Synthesis of amino-modified β-cyclodextrins

2.1. Mono-6-amino-6-deoxy-cyclomaltoheptaose (β-CDNH$_2$)

β-CDNH$_2$ was synthesized as described in a previous work [19]. In summary, one of the seven primary hydroxyl groups of β-cyclodextrin was first tosylated using p-toluenesulfonyl chloride. The regioselective substitution of cyclodextrins has been performed according to a process taken in the literature [20]. Substitution of the tosyl group by azide leads to mono-6-azido-6-deoxy-β-cyclodextrin that was reduced via the Staudinger reaction, in DMF with triphenylphosphinfollowed by treatment with aqueous ammonia to give the mono -amino cyclodextrin with 76 % yield.

The disappearance of organic azide was controlled with IR (ν = 2100 cm^{-1}). Compound **3** was recovered with 95 % yield.

R_f : 0.28 (butanol /methanol /water / NH_3(aq) 4:3:2:3).

Mass E.S (m/z) : 1155.9 $[M+Na]^+$; calcd for $C_{42}H_{71}NO_{34}$: M = 1133.6 g/mol.

^{1}H NMR (D_2O) δ: 2.90-3.15 (m, 2H, H_6^A); 3.44-3.97 (m, 42H, H_2, H_3, H_4, H_5, H_6a, H_6b); 5.07 (d, 7H, J=2.8Hz, H_1).

^{13}C NMR (D_2O): 47.3 (C_6^A); 64.8 (C_6); 74.1 (C_2^A); 78.8-80.7 (C_2, C_3, C_5); 81.24 (C_4^A); 82.8 (C_4); 100.1 (C_1^A); 102.3-103.1 (C_1).

IR (cm^{-1}): 3500-3300 (OH and NH_2) ; 2920 (C-H).

2.2. Mono-(6-aminoethylamino-6-deoxy)-cyclomaltoheptaose (β-CDEnNH$_2$)

The mono ethylenediamine β-cyclodextrin (β-CDEnNH$_2$) was synthesized following Matsui's procedure [21]. The tosyl group of mono-6-(*p*-toluenesulfonyl)-cyclomaltoheptaose was displaced by reaction in ethylenediamine, used as solvent, at 70 °C. β-CDEnNH$_2$ was recovered with 76 % yield.

R_f : 0.26 (1,4-dioxane /NH_3(aq) 10:7).

Mass E.S (m/z) : 1199.5 $[M+Na]^+$; calcd for $C_{44}H_{76}N_2O_{34}$: M = 1176.4 g/mol.

^{1}H NMR (D_2O): 2.97 (m, 2H, CH_2-NH); 3.11 (m, 2H, CH_2-NH_2); 3.18-3.33 (m, 2H, H_6^A); 3.76 (t, 1H, H_4^A); 3.85 (t, 6H, H_4); 3.92 (m, 7H, H_5); 3.8-4.09 (m, 26H, H_2, H_3, H_6); 5.36 (d, 7H, J=2.8Hz,H_1).

^{13}C NMR (D_2O): 40.5 (CH_2NH_2); 46.4 (CH_2-NH-); 61.1 (C_6); 70.1 (C_2^A); 72.9-74.4 (C_2, C_3, C_5); 81.9 (C_4^A); 84.3 (C_4); 102.8 (C_1).

IR (cm^{-1}): 3460-3338 (OH and NH_2); 2977 (C-H).

2.3. Heptakis(6-amino-6-deoxy)-cyclomaltoheptaose (β-CDpNH$_2$)

The preparation of per-aminoβ-CD was done in our previous work [19]. In the first step, β-CD was converted to heptakis(6-iodo-6-deoxy)-β-CD as previously described [22] Thereafter, it was reacted with sodium azide in DMF to afford heptakis(6-azido-6-deoxy)-cyclomaltoheptaose that was reduced to amino Compound via Staudinger reaction with 95 % yield.

Mass E.S (m/z) : 1151.1 for $[M+Na]^+$; calcd for $C_{42}H_{77}O_{28}N_7$: M = 1128 g/mol.

^{1}H NMR (D_2O): 3.25 (dd, 7H, H_6a); 3.44 (dd, 14H, H_2, H_4); 3.72 (m, 14H, H_3, H_5); 3.88 (m, 7H, H_6b); 4.14 (d, 7H, J=3Hz, H_1).

^{13}C NMR (D_2O): 51.3 (C_6); 70.4-72.6 (C_2, C_3, C_5); 83.2 (C_4); 102.1-102.9 (C_1).

I.R. (KBr, ν cm^{-1}): 3399 (OH and NH_2); 2924 (C-H); disappearance of organic N_3 (ν = 2100 cm^{-1}).

3. Characterization of inclusion complexes

^{1}H NMR spectra (400 MHz) were recorded on a JEOL JNM-A500 NMR spectrometer. Two categories of samples containing GSV and native and amino-β-CDs were prepared in a mixture of 50 % (v/v) D_2O/DMSO-d_6 due to the weak solubility of GSV in water. At First, continuous variation Job's method [23] was used to determine the stoichiometry of formed complex between GSV and native or amino-β-cyclodextrins. The total concentration of the two substances, host [H] and guest [G], was kept constant for each solution at constant volume ($[H]_t + [G]_t$ = 10 mM.) and the mole ratio r (r = [G]/([G]+[H]) was varied in the range 0-1 (0 < r < 1). In all cases, the mixture of guest and host were stirred during 24h. Secondly, to determine stability constant K_{mn} of native and amino-β-cyclodextrins with GSV, spectra of solutions at constant concentration of amino-β-cyclodextrins (10 mM) and varying concentration of GSV were recorded. The concentration of guest was varied from 2.5 to 20 mM. Only the chemical shifts of GSV protons (H_a, H_b, H_c, H_d, H_e, H_f, H_g,

H_h and H_i) were used for this purpose, due to the overlapping of ^1H NMR picks in the spectra of the amino-β-CD complexes.

RESULTS AND DISCUSSIONS

1. Inclusion complex by amino β-cyclodextrins

^1H NMR spectroscopy is often used to obtain additional information about formation of GSV/amino-β-CDs inclusion complexes and also to determine the stability constants. The chemical shifts of both host and guest molecules could provide evidence of the formation of inclusion complexes in solution, since significant changes in the environment are known to occur between the free and bound states.

In order to determine the stoichiometry and its stability constant of the inclusion complexes of GSV with native or amino-β-cyclodextrins (β-CDNH$_2$, β-CDEnNH$_2$ and β-CDpNH$_2$), inclusion complexes were prepared in mixture of 50 % (v/v) D_2O/DMSO-d_6 and investigated by means of ^1H NMR to explain the structure of the host-guest complex. The comparison of inclusion complexes GSV/β-CD spectra at different molar ratios (from 0.5 to 4) with the corresponding uncomplexed native or amino-β-CDs and GSV recorded in the same conditions, allows to show the variation of chemical shift (Figure 2). The chemical shift values of amino-β-CD protons in presence and absence of GSV are shown in Figure 2, where $\Delta\delta = \delta_{complex} - \delta_{free}$. Positive and negative signs indicate a downfield and upfield shifts, respectively. From Figure 2, it could be seen that the H_3 and H_5 protons, which located in the inner cavity of amino-□-CDs show significant changes. It is noteworthy that the chemical shift change for H_3 was larger than H_5 after the formation of the inclusion complex. Such changes of this proton shifts clearly support the idea that GSV penetrate into the β-CDs cavity through its secondary side. Also, the downfield shifts of H_1 equatorial protons and H_2 and H_4 axial protons, located outside the cavity, were observed. This shift of external protons is caused by the interaction of GSV molecules with the exterior surface of the torus via the moiety hanging outside of the torus. It is assumed that GSV penetrate into the CD cavity from the narrow side.

Furthermore, some GSV in ^1H NMR recorded spectra shifted upon complexation as result of local environment changes. The ^1H NMR of GSV (20 mM in D_2O/DMSO-d_6) presented in Figure 2 showed well-resolved signals. It can be found that some chemical shift values of GSV protons (H_b, H_d, H_e and H_c) were masked by CDs.

The singulet centered at 6.41, 3.98 and 3.88 ppm assigned to the H_i, H_g and H_f protons of aromatic ring and methoxy groups linked to aromatic ring respectively, present the most chemical shift changes. Addition of native β-CD to GSV solutions gives rise to a low shift compared to that observed in presence of amino-β-CD. Complexation with β-CDpNH$_2$ have induced an important downfield shift for H_i, H_g and H_f protons resulting from the formation of GSV/β-CDpNH$_2$ inclusion complex. It can be also noticed that the order of chemical shift variation was as follows: $\Delta\delta H_i > \Delta\delta H_g > \Delta\delta H_f$ for all GSV/β-CDs complexes. Also, the highest chemical shift changes of H_i, H_g and H_f protons indicate that aromatic moiety of GSV molecule is inside the CDs cavity. Figure 2, show negligible chemical shift change for signals (H_h and H_a) cyclohexan ring which suggest that probably cyclohexan ring is located at outside the cavity.

2. Determination of the complex stoichiometry

Continuous variation or Job's method [23] was used to determine the stoichiometry of GSV/amino-β-CDs complexes. The method is based on the analysis of ^1H NMR spectra for a serie of GSV/β-CDs mixtures in different portions keeping the same final volume. In general, the inclusion complex with cyclodextrin is a reversible process and for griseofulvin host it can be described by the following equation:

$$mGSV + nCD \overset{K_{mn}}{\rightleftharpoons} GSV_m : CD_n$$

Where *m* and n are the stoichiometry coefficients and K_{mn} is the stability constant.

Taking into account the mass balance for GSV and CD, the stability constant can be writing as (1):

$$K_{mn} = \frac{[GSV_m : CD_n]}{[GSV]^m [CD]^n} = \frac{[GSV_m : CD_n]}{([GSV]_t - m[GSV_m : CD_n])^m ([CD]_t - n[GSV_m : CD_n])^n} \qquad (1)$$

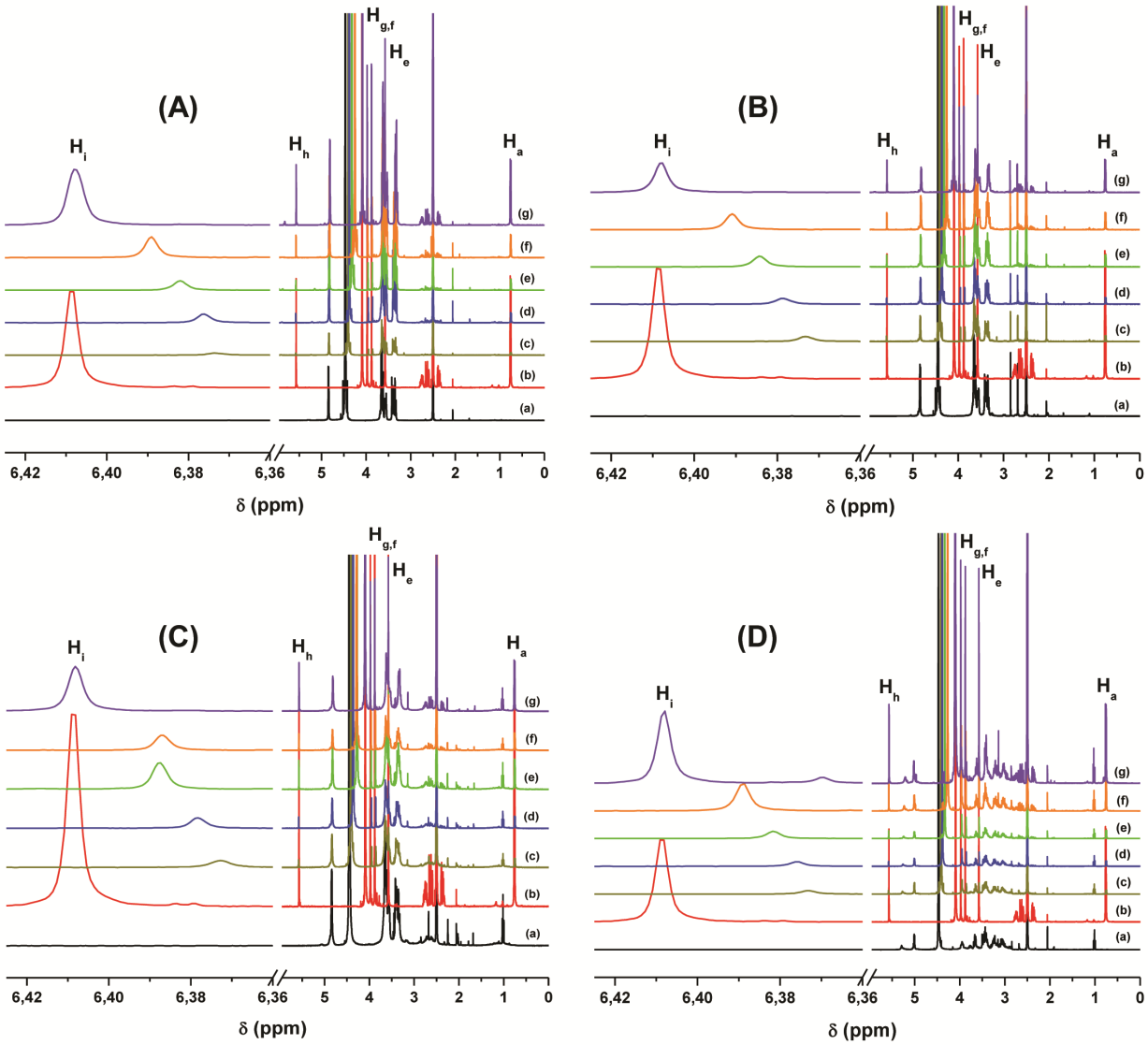

Figure 2. ^{1}H NMR spectra in mixture of 50 % (v/v) D_2O/DMSO-d_6 as a function of molar ratios of (A) GSV/ β-CD, (B) GSV/ β-CDNH$_2$, (C) GSV/ β-CDEnNH$_2$ and (D) GSV/ β-CDpNH$_2$ with (a) native or modified β-cyclodextrin alone, (b) GSV alone (c) 0.25/1, (d) 0.5/1, (e) 1/1, (f) 1.5/1 and (g) 2/1.

Where $[GSV]_t$ and $[CD]_t$ are the total concentrations of GSV molecule and amino β-CDs and $[GSV]$, $[CD]$ are the concentration of the free species, whereas $[GSV_m : CD_n]$ is the concentration of complex in solution.
The molar ratio *r* of the GSV guest is defined as:

$$r = \frac{[GSV]_t}{[GSV]_t + [CD]_t} = \frac{n}{m+n} \quad (0 < r < 1) \quad (2)$$

Due to the complexity of NMR spectra, GSV aromatic peaks were considered for determination of inclusion complexes stoichiometry. Job's plot, established on the basis of the variation of chemical shift of the H_i, H_f and H_g protons of GSV (Figure 3), show a maximum at molar ratio r = 0.5, traducing the formation of a 1:1 stoichiometry complexes for all β–cyclodextrins.

3. Determination of stability constant

Considering the formation of 1:1 GSV/amino-β-CDs complexes between guest (GSV) and host (CD), the formation equation and the stability constant would be:

$$GSV + CD \overset{K_{11}}{\rightleftharpoons} GSV{:}CD$$

$$K_{11} = \frac{[GSV\ :\ CD]}{[GSV][CD]} = \frac{[GSV\ :\ CD]}{([GSV]_t - [GSV\ :\ CD])\,([CD]_t - [GSV\ :\ CD])} \qquad (3)$$

Taking into account the mass balance equation and law of mass action, the concentrations of the different species can be given as a function of complex concentration in solution $[GSV : CD]$, by resolution of equation (4).

$$[GSV : CD]^2 - \left([GSV]_t + [CD]_t + \frac{1}{K_{11}}\right)[GSV : CD] + [GSV]_t\,[CD]_t = 0 \qquad (4)$$

Subsequently, equation (4) can be solved to give equation (5):

$$[GSV{:}CD] = \frac{1}{2}\left\{\left([GSV]_t + [CD]_t + \frac{1}{K_{11}}\right) - \sqrt{\left([GSV]_t + [CD]_t + \frac{1}{K_{11}}\right)^2 - 4\,[GSV]_t\,[CD]_t}\right\} \qquad (5)$$

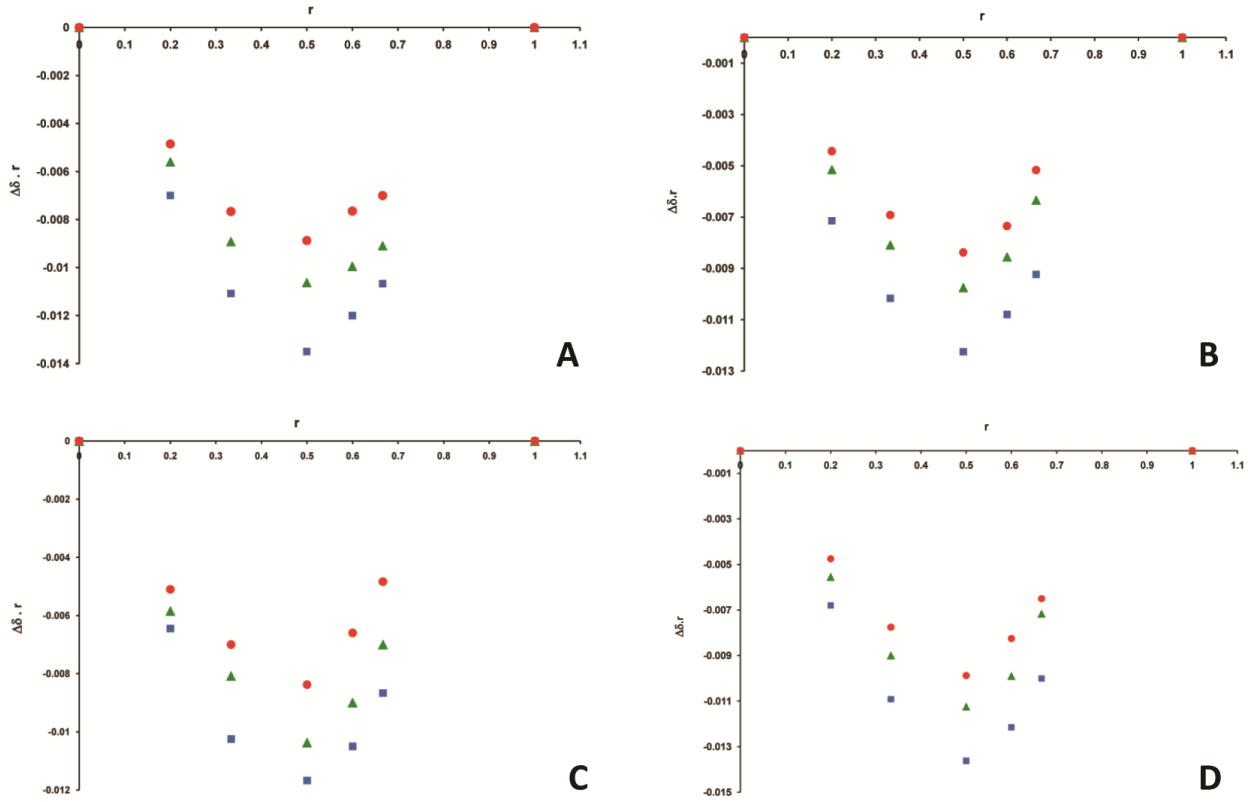

Figure 3. Job's plots corresponding to the chemical shift displacement of protons of (■) H_i, (▲) H_g and (●) H_f protons of (A) GSV/β-CD, (B) GSV/β-$CDNH_2$, (C) GSV/β-$CDEnNH_2$ and (D) GSV/β-$CDpNH_2$ complexes.

The chemical shift δ_{GSV} was calculated according to the total concentration of griseofulvin $[GSV]_0$ for a fixed CD concentration $[CD]_0$. The values of the pair of variables (K_{11}, $\delta_{GSV:CD}$) were adjusted to obtain the best fitting curve of the observed chemical shift data.

Figure 4 gives a typical curve-fitting plot for chemical shifts of GSV protons (H_i, H_g and H_f) which shows the good accordance between the experimental and calculated data and accounting for a 1:1 stoichiometry. The obtained stability constants are summarized in Table I.

The obtained results show the enhancement of the complexes stability for the systems formed by amino-β-cyclodextrins in comparison with that formed with native β-cyclodextrin. The obtained values of K_{11}, increase in the order GSV/β-CD < GSV/β-$CDNH_2$ < GSV/β-$CDEnNH_2$ < GSV/β-$CDpNH_2$. This tendency is correlated with the attached amino groups to β-cyclodextrin which contributes to the enhancement of the complex stability. Thus, the increase in K_{11} value by increasing the number of amino groups is reasonable since the additional grafted amino groups can efficiently interact with aromatic group of GSV resulting in stronger binding ability of amino-β-cyclodextrins towards GSV.

A simple model of electrostatic interaction is evaluated for all charged amino-β-cyclodextrins. Thus, the standard free energy of complexation (Table I) is calculated from the stability constant K_{11} according to equation (6):

$$\Delta_{comp}G^{o} = -RTln(K_{11}) \qquad (6)$$

The total free energy of complexation can be written as a sum of two terms corresponding to

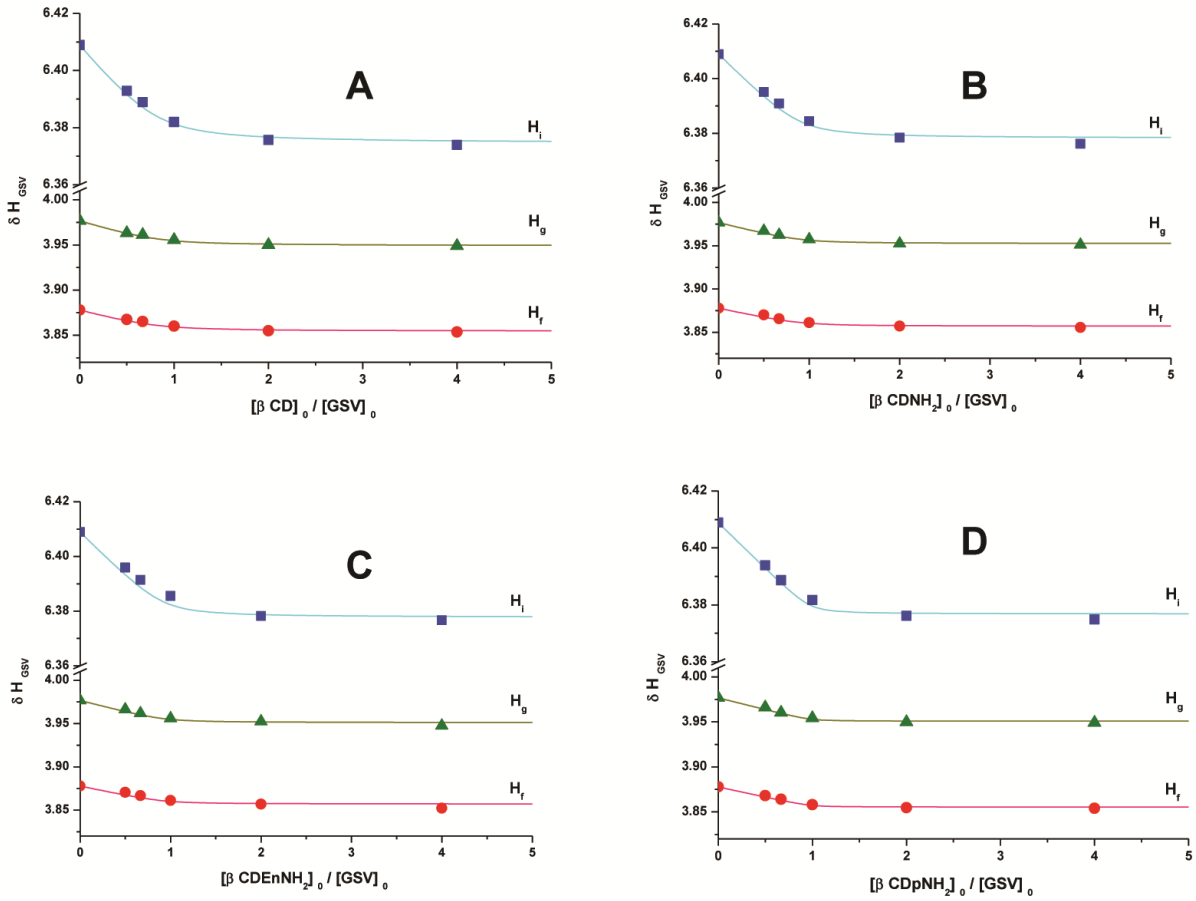

Figure 4. Chemical shift of GSV protons (H_i, H_g and H_f) as a function of molar ratio of CD/GSV (A) GSV/β-CD, (B) GSV/β-$CDNH_2$, (C) GSV/β-$CDEnNH_2$ and (D) GSV/β-$CDpNH_2$ complexes, solid line: best fit of the 1:1 complexation equilibrium with K_{11} given in table 1.

Table I: Thermodynamic parameters of GSV/CD host-guest associations.

Name of Guest:Host complex	Stoichiometry of the complex	$\Delta_{comp}G^0$ (kJ.mol^{-1})	Stability constant K_{11} (M^{-1})
GSV/β-CD	1:1	-15.69	1000
GSV/β-CDNH$_2$	1:1	-17.26	2000
GSV/β-CDEnNH$_2$	1:1	-17.77	2500
GSV/β-CDpNH$_2$	1:1	-20.91	10000

electrostatic and non-electrostatic contribution, equation (7):

$$\Delta_{comp}G^o = \Delta_{comp}G^o(elec) + \Delta_{comp}G^o\ (non–elec) \quad (7)$$

The non-electrostatic contribution is the standard free energy of complexation of the neutral native b-CD. The electrostatic contribution is the work for approaching the aromatic ring from an infinite distance to the closest approach distance (*r)* following equation (8).

$$\Delta_{comp}G^0(elec) = \frac{1}{4\pi\varepsilon_0\varepsilon}\frac{ne^2}{r} \quad (8)$$

Where *n* is the number of amino groups per b-CD and *e* is the dielectric permittivity at the complexing site.

The results indicate that the stability of the formed complexe is largely affected in some cases as it can be seen from the very close values of standard free energy or the corresponding stability constants. The enhancement of stability is traduced by a reduction of the standard free energy or the increase of the corresponding stability constant (Table I). Although it is quite a crude modeling, its overall agreement with the ideas presented above provides more confidence with the effect of electrostatic contribution for the enhancement of the complexation.

CONCLUSION

Our study demonstrated the formation of inclusion complexes between GSV (acting as guest) and β-cyclodextrin modified by attachment of amino groups on their primary face on the bases of ^{1}H NMR shifts measurements. β-CDNH$_2$ and β-CDpNH$_2$ as well as β-CDEnNH$_2$ were prepared and used as complexing macromolecule in this study. A comparative study of host-guest binding ability of amino-β-cyclodextrin towards GSV molecule was examined. The results show the formation of complexes of 1:1 stoichiometry as evidenced by specific changes in chemical shift of GSV as well as β-CDs protons. The stability constants were strongly influenced by the number of grafted amino groups at β-CD. The interactions between the amino-β-cyclodextrins and the GSV were interpreted in terms of hydrophobic effect assisted by electrostatic interactions between aromatic ring and ammonium groups. A simple thermodynamic theory provides an account of the variation of stability constants as a function of the number of ammonium groups.

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