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^{1}H-NMR NOE and molecular modelling to characterize thymol and carvacrol β-cyclodextrin complexes

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Abstract

In this study the structural conformations of the complexes between β-cyclodextrin and the geometrical isomers, thymol and carvacrol, were investigated using ^{1}H-NMR and molecular modelling techniques. The NOE experiments were performed in aqueous solutions and showed the presence of only one complex with a well-defined orientation either for thymol or for carvacrol. Molecular modelling investigations were useful to clarify the structural conformation of the supramolecular edifice. To develop graphic simulations closer to the experimental conditions, the studies were performed in dynamic at 300 K with and without evaluating the dielectric constant, D. The best agreement of ^{1}H-NMR NOE findings with the theoretical data was obtained after the evaluation of the dielectric constant parameter and these results confirmed the inclusion of thymol and carvacrol inside the βCD hydrophobic cavity.

Keywords: Thymol; Carvacrol; β-Cyclodextrin; ^{1}H-NMR NOE; Dynamic molecular modelling

1. Introduction

This work is part of a wider project focused on obtaining an improvement of the active vegetable molecule formulations by complexation with cyclodextrins (CDs). The aim is the optimization of the biopharmaceutical properties in order to improve the molecule stability and to increase the potential fields of application. CDs are known for their ability to bind organic molecules in aqueous medium by non-covalent interactions and the complexation driving forces have been attributed to hydrophobic interactions, van der Waals-London dispersion forces, and hydrogen bonds. It is well-known that the internal diameter of the βCD cavity and the size of the guest molecules are of primary importance for the formation of the inclusion complexes. In order to investigate molecular complexes with CDs it has been found that NMR NOE and molecular modelling studies represent very useful tools in obtaining, respectively, direct evidence of the complexation and a better description of the supramolecular assemblies in solution.

In recent years more ^{1}NMR NOE studies were developed to investigate the structure of the complexes between CDs with several drugs (Redenti et al., 1992) or with natural compounds (Nimmagadda et al., 1991; Soundar, 1993). Recently also,

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high resolution NMR has appeared to be a suitable and indispensable technique to clarify structure and conformation of some complexes (Amato et al., 1992; Berthault and Perly, 1993; Amato et al., 1993). Molecular modelling investigations are generally associated to NMR studies because they represent a complementary method in rationalizing the experimental information (Cabral Marques et al., 1990; Amato et al., 1992, Mulinacci et al., 1993).

In this paper the structural conformation of the complexes between the two geometrical isomers thymol and carvacrol with βCD, has been studied using ^{1}H-NMR and molecular modelling techniques. Thymol and carvacrol were chosen as guest molecules because they are suitable geometrical isomers for evaluating the influence of the hydroxyl group position on the aromatic ring, with respect to the complexation process.

The coupling of these techniques permitted us to compare and to integrate the theoretical findings obtained in the vacuum (molecular modelling) with the experimental data (^{1}H-NMR NOE) in order to clarify the real conformation of the complexes in aqueous medium.

2. Materials and methods

2.1. Materials

β-cyclodextrin (βCD) was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and used without any further purification. Thymol and carvacrol were purchased from Fluka Chemie AG (Buchs, Switzerland). The complexes were obtained by co-precipitation, adding equimolar concentrations of thymol and carvacrol to saturated solutions of βCD in distilled water, after stirring for 24 h. The precipitates were filtered, dried and utilized for the NMR experiments.

2.2. Methods

2.2.1. ^{1}H-NMR Studies

Chemical shifts were measured relative to the peak of the solvent D_2O (4.74 ppm) with a Bruker AMX 600 at 600 MHz in a Fourier transform mode. The NOE experiments were performed on a Varian Gemini 200 at 200 MHz with a Varian NOE DIFF program, version 6.3 A. All spectra were recorded with a 5 mm tube at the probe temperature (25°C) in D_2O, without degassing.

The NOE measurements were obtained with steady state experiments.

Table 1
Chemical shifts (ppm) of thymol and βCD protons in free and complex states in D_2O solution

Protons	Free state ppm	Complex state ppm	$\Delta\delta_{\text{free-complex}}$
Thymol			
H1	1.197	1.235	−0.038
H2	3.187	3.241	−0.054
H3	7.236	7.080	0.156
H4	6.771	6.736	0.035
H5	6.841	6.671	0.170
H6	2.267	2.267	0.000
βCD			
H1	5.115	5.068	0.047
H2	3.693	3.651	0.042
H3	3.996	3.942	0.054
H4	3.630	3.599	0.031
H5	—	3.797	—
H6	—	3.816	—

Table 2
Chemical shifts (ppm) of carvacrol and βCD protons in free and complex states in D_2O solution

Protons	Free state ppm	Complex state ppm	$\Delta\delta_{\text{free-complex}}$
Carvacrol			
H1	1.203	1.269	−0.066
H2	2.853	2.829	−0.024
H3	6.827	6.691	0.136
H4	6.853	6.713	0.140
H5	7.163	7.034	0.129
H6	2.188	2.202	−0.022
βCD			
H1	5.115	5.068	0.047
H2	3.693	3.652	0.041
H3	3.996	3.919	0.077
H4	3.630	3.601	0.029
H5	—	3.736	—
H6	—	3.818	—

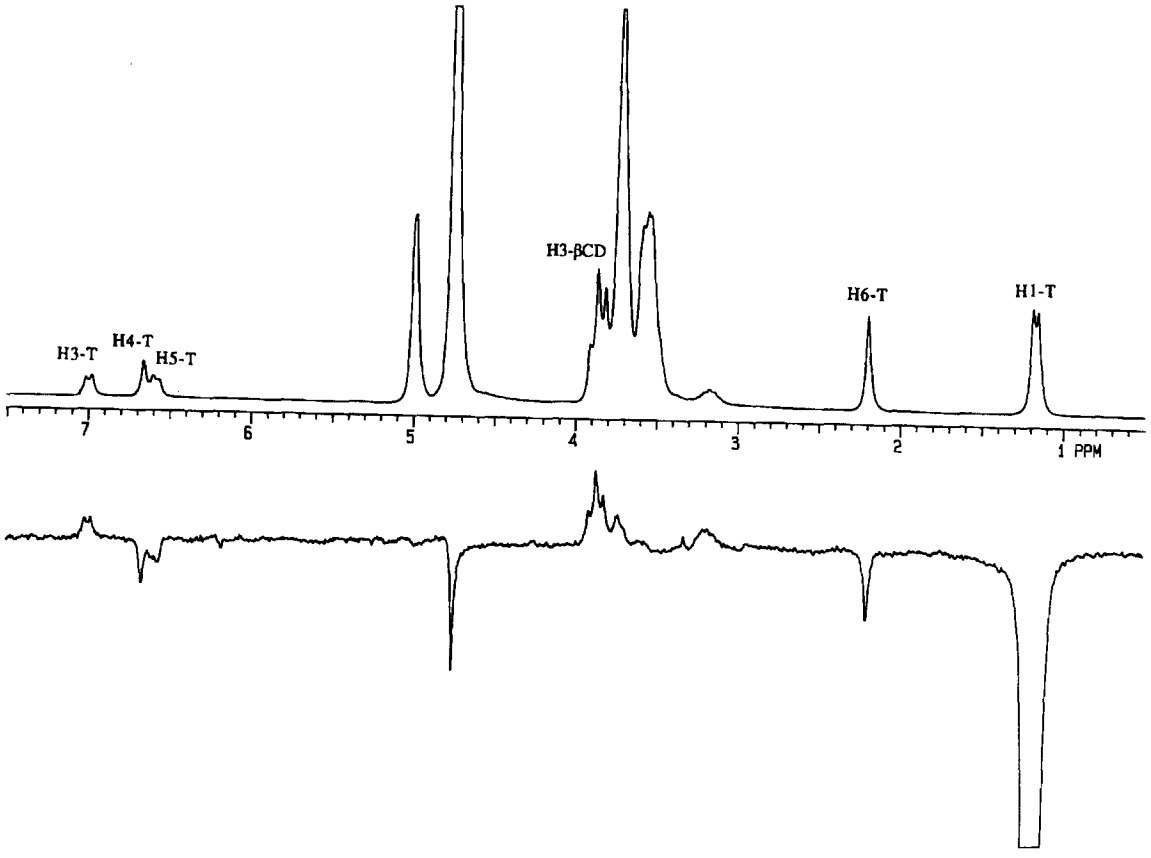

Fig. 1. ^{1}H-NMR spectra of thymol-βCD complex in D_2O solution and NOE experiment; NOE when H1 protons of thymol had been presaturated.

2.2.2. Computer graphics

All molecules were constructed and minimized using the Insight II program, version 2.0.0 and Discover program, version 2.7.0 from Biosym Technologies (San Diego, CA, USA), run on the Personal Iris from Silicon Graphics; the molecular dynamic method of calculation was consistence valence force fields (CVFF).

The first conformational structure minimization of each complex was performed at 0 K and the molecular dynamic simulations were evaluated at 300 K with equilibration time of 0.1 ps and total simulation time of 10 ps. The memorization range for each conformation was every 100 steps. In the CVFF calculations the dielectric constant parameter (D) was applied with a value of 4.

For both thymol and carvacrol two dummy atoms were defined to represent the protons of the isopropyl group (H-1), and one dummy atom was defined to represent the protons of methyl group (H-6). The evaluation of the intermolecular distance between H-1 and H-6 of the guest molecules and the H-3 and H-5 of βCD was performed using the dummy atoms described above and the real protons of βCD.

Each atomic distance represents the average of 31 different conformations obtained in the range between 5 and 8 ps.

3. Results and discussion

The experiments performed with ^{1}H-NMR NOE and molecular dynamic simulations showed

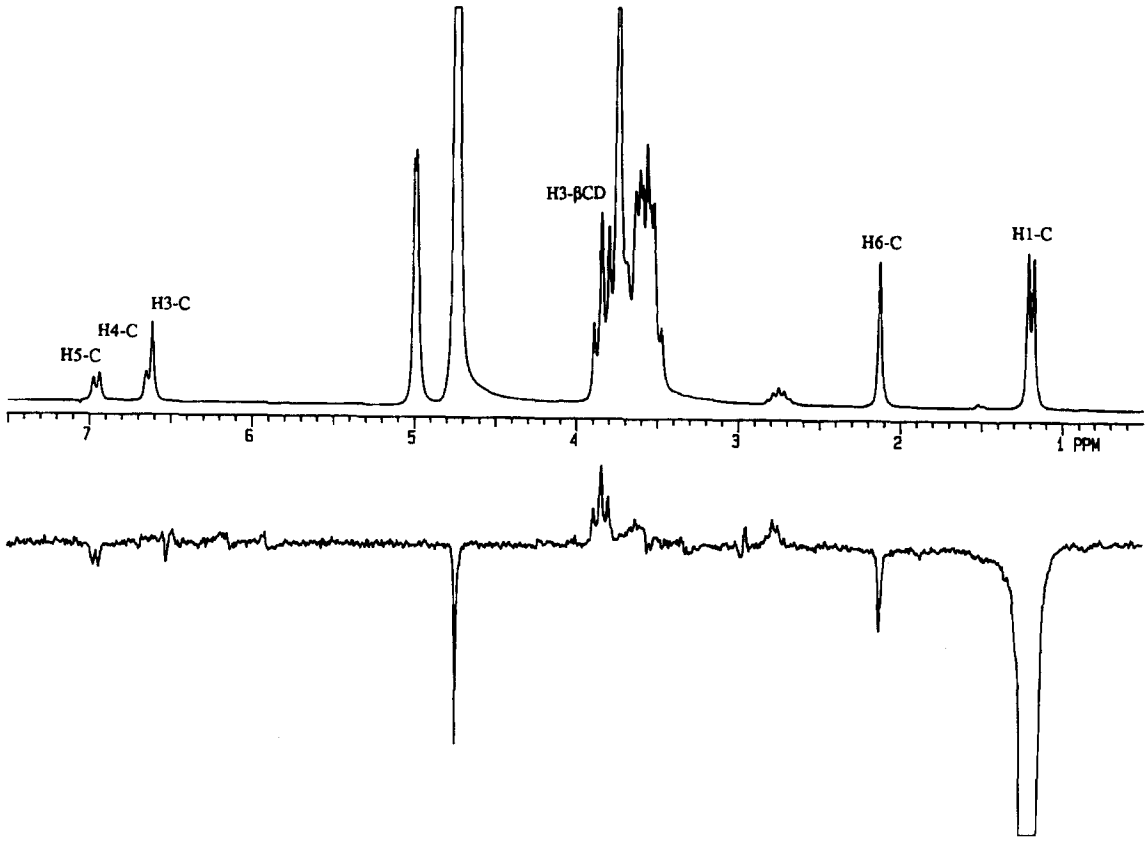

Fig. 2. ^{1}H-NMR spectra of carvacrol-βCD complex in D_2O solution and NOE experiment; NOE when H1 protons of carvacrol had been presaturated.

that thymol and carvacrol are really included inside the lipophilic βCD cavity. Other techniques, like DSC, IR, UV-Vis, are able to either suggest or establish if the guest molecules form a complex or not, but they are unable to give any sure finding, neither on the kind of complex (if inclusion or adsorption) nor on the structural conformation of the molecules.

On the basis of ^{1}H-NMR experiments it was possible to define the stoichiometry of the complexes: for either thymol or carvacrol the ratio was 1:1. Table 1 and Table 2 report the chemical shift values, respectively, of thymol, carvacrol and βCD protons in the free and complex state in D_2O solution, as well as the differences between the signals of the free and included molecules. As for thymol and βCD, the main differences between complex and free state regarded the aromatic proton signals; the H-3 and H-5 protons showed, respectively, a $\Delta\delta$ value of 0.156 ppm and 0.170 ppm (Table 1). Carvacrol also showed the same behaviour, in fact a downfield shift, between free and complex state of aromatic protons, H-3, H-4 and H-5 was observed with $\Delta\delta$ respectively of 0.136, 0.140 and 0.129 ppm (Table 2). A diagnostic downfield shift was observed also for the βCD protons signals (H-3 and H-5) between the complex and free state. The main difference was reported about the H3-βCD protons, with a $\Delta\delta$ of 0.054 ppm for thymol complex and 0.077 ppm for carvacrol complex. In light of these findings, it may thus be hypothesized that the protons of the aromatic rings are "immersed" in the hydrophobic cavity of βCD, either for thymol or for carvacrol complexes.

Table 3
The average distances (Å), and the docking energy (kcal/mol) of complexes A and B were evaluated with molecular dynamic simulations. The increments of the signals (NOE %) were evaluated with the NOE diff program

	Complex A		Complex B		tS[a]	Docking energy				NOE %
	Distances	S.D.	Distances	S.D.		A	S.D.	B	S.D.	
Thymol										
H3βCD-H1	5.11	1.35	5.81	1.22	8.0	−23.7	1.4	−21.2	1.7	2.17
H3βCD-H6	6.95	1.09	6.03	1.70	2.5					<0.1
H5βCD-H1	6.32	1.17	4.70	1.20	20.2					<0.1
H5βCD-H6	5.17	1.10	7.03	2.20	11.1					0.24
Carvacrol										
H3βCD-H1	5.23	1.40	6.40	1.20	13.2	−23.03	1.1	−23.3	1.3	1.30
H3βCD-H6	7.60	1.10	5.30	1.00	22.8					<0.1
H5βCD-H1	5.80	1.30	4.90	1.17	17.7					<0.1
H5βCD-H6	5.53	0.90	7.00	0.70	18.9					<0.1

[a]tS = t Student.

The ^{1}H-NMR spectra only indicated that the aromatic ring of the guest molecule lay inside the cavity of βCD, but gave no clear indication concerning the real position of the whole molecule inside the βCD cavity. On the other hand, with NOE experiments, it was possible to identify the geometry of the complexes in solution and especially the interactions between thymol and carvacrol protons and H-3 and H-5 βCD protons, respectively, were investigated. Fig. 1 shows the ^{1}H-NMR spectrum of thymol-βCD complex in comparison with a NOE spectrum obtained after presaturation of H-1 thymol protons. A significant nuclear Overhauser enhancement was observed between H-1 thymol and H-3 βCD with a signal increase of 2.17%. The same results for thymol βCD complex were obtained for carvacrol complex as well; in fact Fig. 2 shows an intense nuclear Overhauser enhancement between H-1 carvacrol and H-3 βCD protons by presaturating the H-1 carvacrol protons with a signal increment of 1.30%. Table 3 shows the percentage signal increase related to the more significant nuclear Overhauser enhancements between the protons of the guest molecule and those of βCD.

Depending on either the asymmetry of thymol and carvacrol or on the truncated cone shape of βCD, it was possible to hypothesize about the existence of two complexes with different orientation for each guest molecule investigated and so the conformational analysis studies were performed using the reference structures A and B reported in Fig. 3. The results obtained from the dynamic simulations confirmed those of NOE only for thymol-βCD complex while for carvacrol-βCD complex an anomalous result relating to the distances between the H-1 carvacrol and H-3 βCD protons was registered. This value was the same for complex A and complex B in spite of the different geometry of the supramolecular systems (Fig. 3).

It is important to underline that with this kind of calculation the theoretical findings are strictly related to the complexes in the vacuum and not in solution. Therefore we have, at least partially, evaluated the influence of water during the complexation process. For this reason a new parameter, the dielectric constant (D), was inserted in the CVFF calculations. The minimum value of D was imposed because it was reasonable to suppose only a weak effect of water molecules especially around the edge of the hydrophobic cavity of βCD. With this parameter the distances between some key protons of βCD and of guest molecules were re-calculated and these findings were compared with the intermolecular nuclear Overhauser enhancements. Observing the data in Table 3, the average distances between H-3βCD/H-1 and H-5βCD/H-6 for thymol and carvacrol complexes agree with the corresponding intensity of NOE

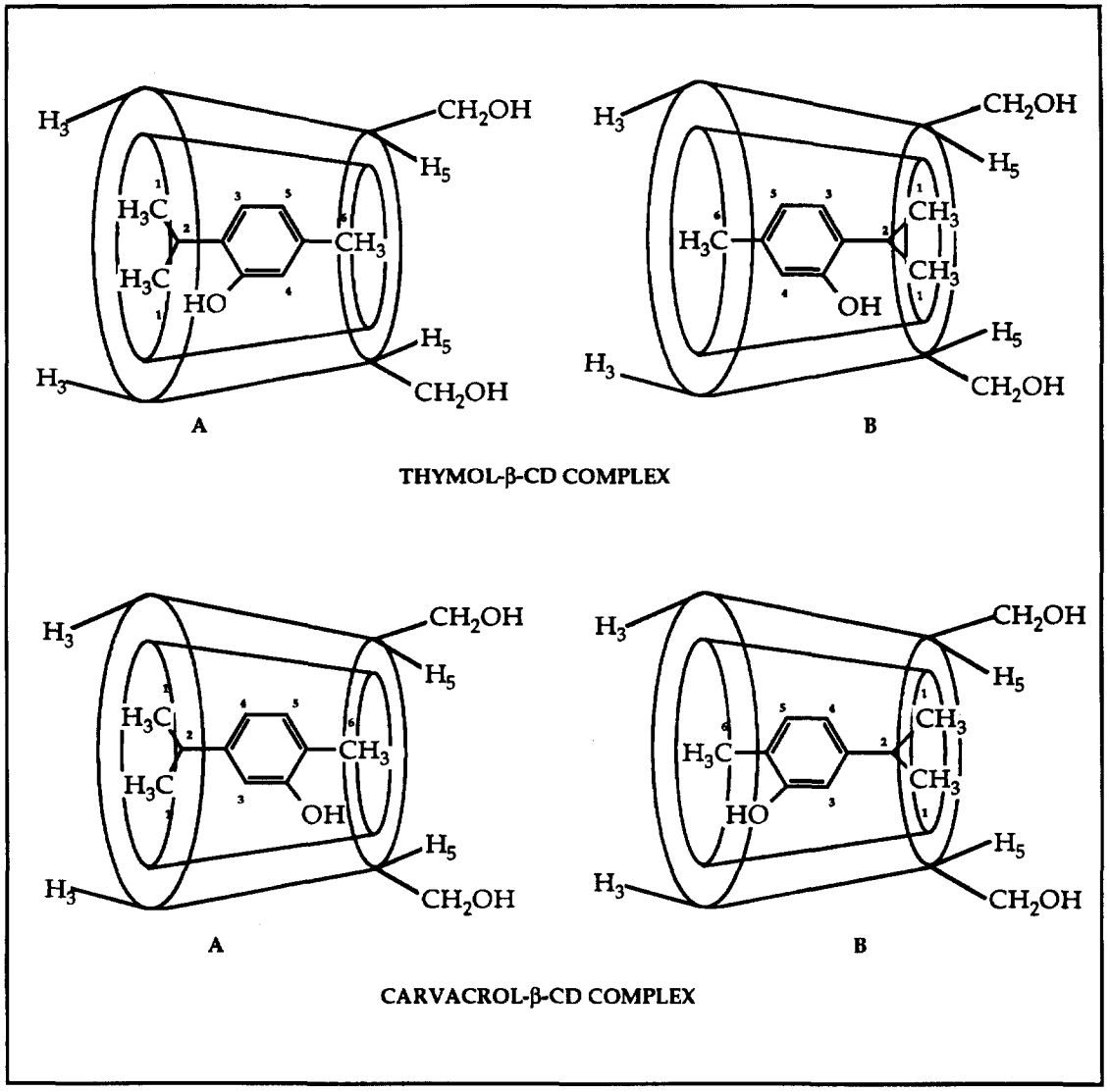

Fig. 3. Scheme of the two possible complexes, A and B, between βCD and thymol and carvacrol respectively.

enhancements. Besides, observing the values of Students t reported in Table 3, it was clear that the two structures, A and B, were significantly different.

For complex A the three dimensional structures of thymol and carvacrol βCD complexes are reported respectively in Fig. 4 and Fig. 5. Considering only these theoretical findings it was impossible to establish which of the two complexes, A or B, really exist in aqueous solution, since all four hypothesized structures showed approximately the same values either for the conformational energy or for the docking energy. On the other side, the experimental data showed how the geometry of the two supramolecular systems was the same in aqueous solution with the complex A as the real complex in aqueous medium either for thymol or carvacrol; the protons of isopropyl group of the guest molecules lay closer to the larger side of the hydrophobic cavity of βCD.

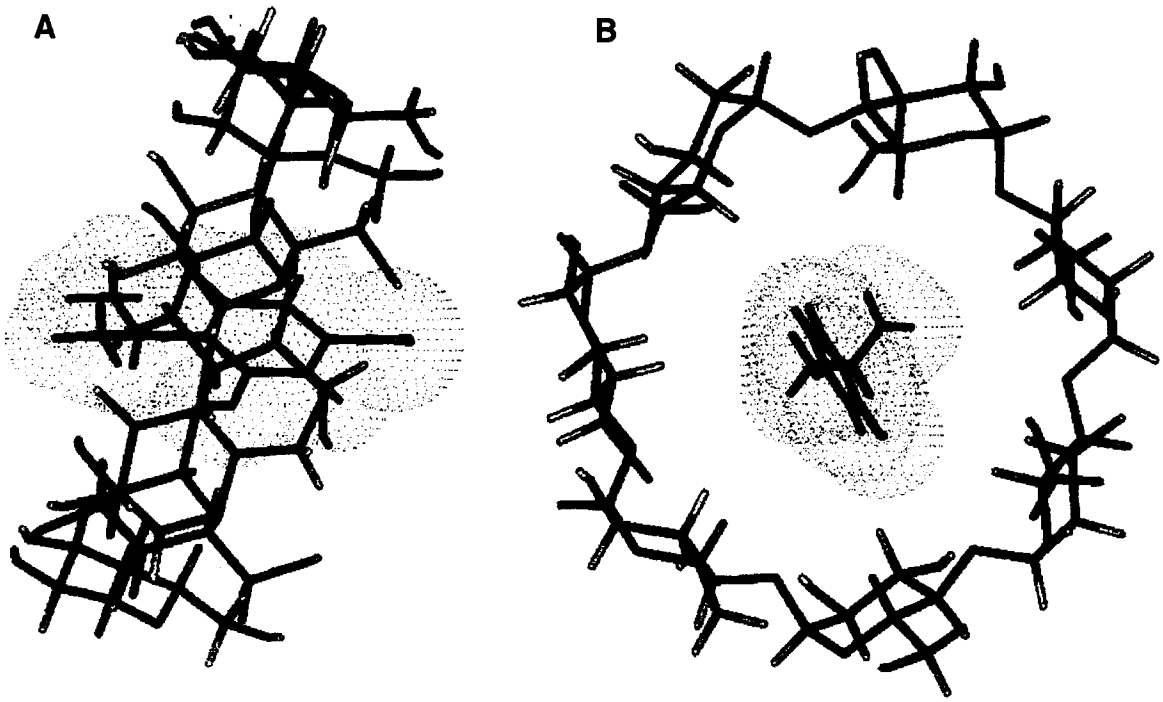

Fig. 4. (a,b) Relative position of thymol and βCD in complex A. Minimized structure with molecular dynamic simulations (see Methods section).

Therefore the different position of the hydroxyl substituent on the aromatic rings of the guests has not substantially modified the conformation of the complexes.

Besides comparing the intensity of the NOE interactions between thymol complex and carvacrol complex, it was possible to hypothesize only a different "depth of immersion" of the two guest molecules into the cavity of βCD.

In this study the ^{1}H-NMR NOE represented an important tool to investigate the real structure of the complex in solution. Molecular graphic was utilized to obtain a better knowledge of the topology of the interaction between βCD and the guest molecules. Dynamic calculations at 300 K, evaluating also the dielectric constant parameter D, were performed in order to develop graphic simulations closer to the experimental conditions. This method was combined with ^{1}H-NMR NOE because dynamic calculations were insufficient alone to describe the real structure of the complexes in solution. The interactions between these two techniques allowed us to establish that only one kind of complex existed in aqueous solution, either for thymol or for carvacrol, and the orientation of this complex was not modified by the different position of the hydroxyl substituent on the aromatic ring of the guest. Certainly the interactions between theoretical findings (dynamic simulations) and the experimental data (NMR) lead to more complete structural information which is closer to the reality.

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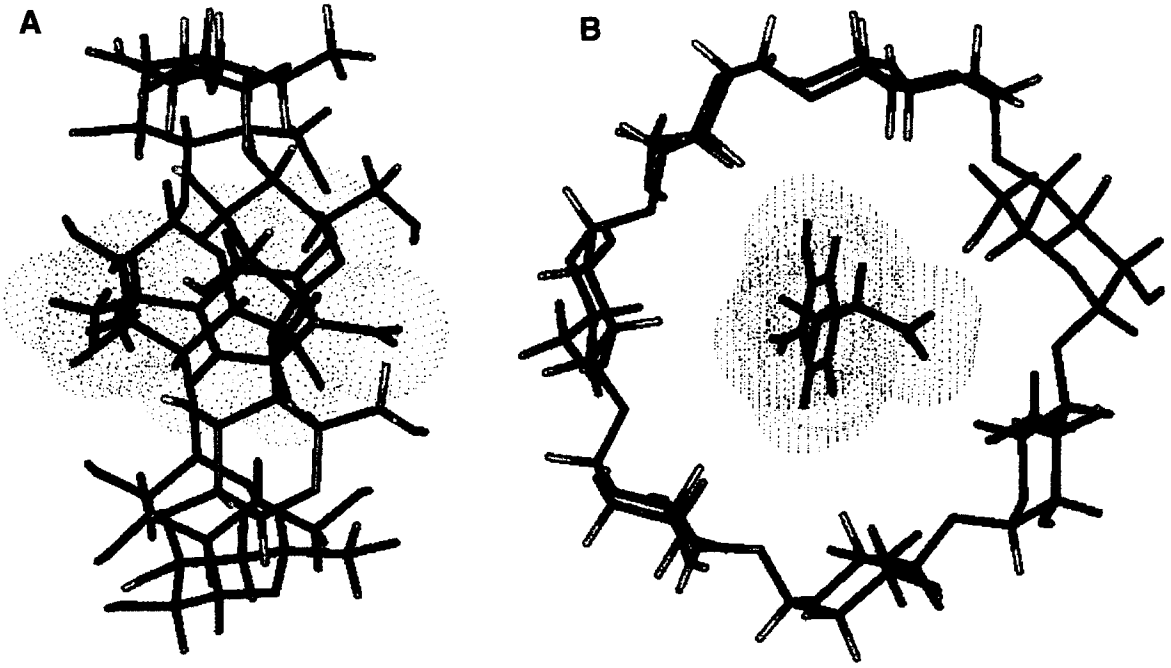

Fig. 5. (a,b) Relative position of carvacrol and βCD in complex A. Minimized structure with molecular dynamic simulations (see Methods section).

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