Utility of Nuclear Magnetic Resonance Spectroscopy to Characterize the Structure of Dexamethasone Sodium Phosphate Inclusion Complexes with Cyclodextrins in Solution and to Analyze Potential Competitive Effects

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ABSTRACT: The interaction between dexamethasone sodium phosphate (DSP) and four cyclodextrin (CyD) derivatives [2,6-di-*O*-β-cyclodextrin (DIMEB), γ-cyclodextrin (γ-CyD), and hydroxypropyl-β-cyclodextrin with either 2.7 or 4.6 degrees of substitution (HPβCyD 2.7 and HPβCyD 4.6, respectively)] was investigated by proton nuclear magnetic resonance spectroscopy (^{1}H NMR). The data suggested the formation of inclusion complexes in solution in which B and C rings of the molecule are located inside the cavity. Nevertheless, the structure, in terms of depth within CyD, depends on the derivative considered. Molecular mechanics calculations of DSP complexes with DIMEB and γ-CyD support the NMR results. The potential displacement of DSP from the CyD cavity by usual ophthalmic drugs (e.g., polymyxin B, trimethoprim, and benzalkonium chloride) was determined by NMR. The technique has been found useful to analyze this problem in pharmaceutical preparations. © 2002 Wiley-Liss, Inc. and the American Pharmaceutical Association J Pharm Sci 91:1536–1547, 2002

Keywords: glucocorticoids; cyclodextrins; ophthalmic solutions; nuclear magnetic resonance spectroscopy; molecular modeling; micelles

INTRODUCTION

Dexamethasone [pregna-1,4-dieno-3, 20-dione, 9-fluoro-11,17, 21-trihidroxi-16 methyl, (11β, 16α)-] is a synthetic glucocorticoid that is broadly used for the treatment of asthma, allergy, and rheumatic and inflammatory processes.[1,2] It has low water solubility and, therefore, its liquid pharmaceutical dosage forms, commonly used in parenteral and ophthalmic administration, are very problematic. Different alternatives have been used to overcome this problem, such as the synthesis of chemical derivatives (mainly, esters of the acetic, phosphoric, or succinic acids).[3,4] In particular, dexamethasone sodium phosphate (DSP) is the derivative more frequently incorporated in ophthalmic solution formulations. The formation of inclusion complexes with cyclodextrins (CyDs) is another possible approach.[5,6] The use of CyDs in pharmaceutical technology is very extensive because they improve water solubility, oral or ophthalmic bioavailability, as well as the stability of many drug molecules by the formation of inclusion complexes.[7–9] These complexes are formed spontaneously in aqueous solution because the cavity of the CyD contains a variable number of water molecules in an unfavorable energy

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state (i.e., a hydrophobic environment) that can be substituted by a molecule or a hydrophobic group.[10,11]

Multiple drug formulations are very frequent in parenteral and ophthalmic formulations and the design and elaboration of the formulation becomes a complicated problem. In ophthalmic dosage forms, the application of antiinflammatory, antibiotic, and conservative drugs together is very common. The physical and chemical compatibility of the components as well as the absorption of certain drugs into the surface of the containers have to be studied. DSP is ionized in aqueous solution, and the formation of insoluble complexes with basic molecules has been already described.[12] This interaction reduces the availability of the drug from the formulation. To avoid these incompatibility problems, several technological alternatives can be considered. Among them, the protection of one of the drugs by the formation of inclusion complexes with CyDs is very promising.

In CyD complexation technology, it is very important to analyze the interaction of the drug molecules present with the elected CyD derivatives because if there is competition for the cavity, the more stable inclusion complex will form, and this complex may not be desirable.[13–15] To prevent such undesirable competitions, knowledge of the stability constants of the complex in the formulation conditions is very useful. These stability constants can be determined by different procedures described in the literature, such as nuclear magnetic resonance spectroscopy (NMR),[16] spectrophotometric techniques based on the method described by Benesi and Hildebrand,[17] and liquid chromatography.[18] However, the universal method used when the molecule is slightly soluble in water is that described by Higuchi and Connors[19] in which the calculations are made with data from phase solubility diagrams.

The purpose of this investigation is to characterize the structure of the inclusion complexes of DSP with four CyD derivatives in solution [2,6-dimethyl-β-cyclodextrin (DIMEB), γ-cyclodextrin (γ-CyD), and two hydroxypropyl-β-cyclodextrins with 2.7 and 4.6 degrees of substitution (d.s.; HPβCyD2.7 and HPβCyD4.6, respectively)]. This characterization was made using NMR spectroscopy [proton NMR (^{1}H NMR) and two-dimensional rotating frame ^{1}H–^{1}H nuclear Overhauser effect (2D ROESY)]), the Job method of continuous variation,[20] and molecular mechanics modeling (MM2) calculations (made with DIMEB and gamma-CD) in vacuum. ^{1}H NMR was used to analyze the influence of drugs included in ophthalmic formulations (i.e., benzalkonium chloride, trimethoprim, and polymyxin B) on the complexation with DSP. The results show that NMR can be used to elucidate the competition of two drug molecules for the CyD cavity.

EXPERIMENTAL SECTION

Materials

Dexamethasone sodium phosphate (DSP; Velezfarma, Spain), polymyxin B, trimethoprim, and benzalkonium chloride were donated from Laboratorios Cusí, S.A., Spain. 2,6-di-*O*-β-Cyclodextrin (DIMEB), hydroxypropyl-β-cyclodextrin (d.s., 2.7; HPβCyD2.7), and hydroxypropyl-β-cyclodextrin (d.s., 4.6; HPβCyD4.6) were purchased from Cyclolab, Ltd (Hungary), and γ-cyclodextrin (γ-CyD) was purchased from Wacker Biochemie Inc. (Germany). All chemicals were of an analytical reagent grade.

Methods

^{1}H NMR

^{1}H NMR spectra were recorded on a Brücker WN 300 spectrometer (Brücker Anal.) at 25°C. DSP (15.50 mM) and CyDs (7.70–46.00 mM) were dissolved in deuterated water (Sigma Chemical, Spain). The internal reference was a peak due to small amounts of DHO and H_2O, present as impurities (assigned a value of $\delta =$ 4.6 ppm).

2D ROESY experiments were performed for the solution with a molar ratio of 1:1 (1.50 mM) using a mixing time of 227 ms and a spectral width of 4504.5 Hz.

The stoichiometry of the complexes was determined by the continuous variation method:[20] 4.65 mM solutions of CyD derivatives and DSP were mixed in deuterated water solution, keeping total concentration fixed. The changes in the most sensitive NMR signal were noted.

The effects of polymyxin B, trimethoprim, and benzalkonium chloride were analyzed by ^{1}H NMR. These experiments were performed with a fixed concentration of those drugs (0.84 mM polymyxin B; 34.10 mM trimethoprim, and 3.00 mM benzalkonium chloride) corresponding to a DSP–drug molar ration of 1:1.

Critical Micelle Concentration (cmc) of DSP

Surface tension measurements were made by the platinum ring method with a Lauder Tensiometer TD1 (Postfach, Germany) applying the needed density corrections. The cmc was identified in the concentration range with the minimal values of surface tension.

Molecular Modeling

Molecular modeling was carried out on an IBM Pentium III 700 MHz personal computer. The molecular mechanics MM2 force-field method implemented in Hyperchem software was used for molecular modeling calculations. The geometry parameters of DIMEB,[21] γ-CyD.[22] and DSP[23] were taken from X-ray diffraction data deposited in the Cambridge Crystallographic Databank. The calculations were made by docking the optimized structure of DSP into the CyDs cavity and allowing for full geometry optimization. Different orientations of DSP into the cavity of γ-CyD were tested, taking into account the different groups of DSP that can be included in the CyDs cavity by both the narrower and wider sides. The complexes formed by these different orientations of DSP into CyD cavity are illustrated in Figure 7. Consideration of the aqueous solvent used in the calculations was made by assuming a box of water molecules with the following dimensions: $x = 22.0$ Å, $y = 17.0$ Å, and $z = 22.0$ Å. The minimum distance between solvent and solute atoms was fixed at 2.3 Å. Molecular graphics shown in this work were build by RasWin Molecular Graphics software.

RESULTS AND DISCUSSION

Structural Characterization of the Inclusion Complexes DSP–CyD

NMR has shown the potential to provide almost complete information on guest–host interactions (stoichiometry, binding constants, energy of the complexation process, and structure of the complexes) in solution and in solid state.[24] This information may be obtained mainly using ^{1}H NMR experiments based on the chemical shifts that show the protons of the drug and the CyD when the inclusion occurs. In this paper, ^{1}H NMR was used to characterize the interaction in water of DSP with four CyD derivatives: DIMEB, γCyD, and two HPβCyD derivatives with two different degrees of substitution (namely, 2.7 and 4.6). These derivatives were chosen because of the complexation capacity of DIMEB and the potential use in ophthalmic formulation of γ-CyD and HPβCyD.[25] Chemical shift changes of the protons of DSP (assigned according to Cohen[2]) in increasing concentrations (1:0 to 1:3 mol/mol DSP–CyD) of the CyD derivatives were analyzed.

The induced chemical shift changes for the hydrogen atoms of DSP whose signals were not masked by the CyD signals as a function of the CyD concentration are shown in Figures 1 and 2. The negative sign of Δ (ppm; i.e., the difference in DSP chemical shifts in the absence and presence of CyD) indicates a downfield displacement and the positive sign indicates an upfield one. Downfield shifts of the protons of DSP are caused by variations of the local polarity due to the inclusion in the CyD cavity. These chemical shifts are dissimilar in the presence of the four derivatives, indicating that the structures of the derivatives, in terms of depth of the molecule in the cavity, may differ. In fact, the four derivatives have a different structure: γ-CyD has the biggest cavity, but β-CyD derivatives, especially DIMEB, show a longer cavity because of lengthening due to the methyl substitution in this CyD derivative.

The protons of DSP move in a different way depending on the CyD considered. $C_2-\underline{H}$ as well as $C_4-\underline{H}$ show upfield shifts, whereas these protons move downfield with γ-CyD. The $C_1-\underline{H}$ proton shifts upfield with γ-CyD and DIMEB, but with the hydroxypropyl derivatives, these protons show downfield shifts. On the other hand, methyl protons from carbons $C_{16}-C\underline{H}_3$, $C_{18}-C\underline{H}_3$, and $C_{19}-C\underline{H}_3$ moved downfield, probably due to their situation inside the cavity. To clarify this point, 2D NMR spectra of the complexes were studied by the 2D ROESY method. The 2D ROESY spectra for all the CyDs show cross-peaks for the B and C rings protons ($C_{11}-\underline{H}$, $C_{19}-C\underline{H}_3$) with protons inside the cavity (H_3, H_5, and H_6). The 2D ROESY results for DSP–γ-CyD are shown in Figure 3. The following cross-peaks between DSP and γ-CyD are evident: $C_{11}-\underline{H}$ (4.19 ppm) with protons H_3, H_5, and H_6 (3.6–3.8 ppm); $C_{19}-C\underline{H}_3$ (1.57 ppm), with protons H_6 orientated in the narrow part of the cavity and H_2 and H_4 outside of the edge; and $C_{18}-C\underline{H}_3$ (0.98 ppm) with protons H_2 and H_4. These results suggest that in the complexes, the orientation of the protons is as follows: $C_{11}-\underline{H}$ is in the interior of the CyD, and $C_{19}-C\underline{H}_3$ is outside but near the narrow edge. In the case of DSP–DIMEB, the 2D ROESY cross-

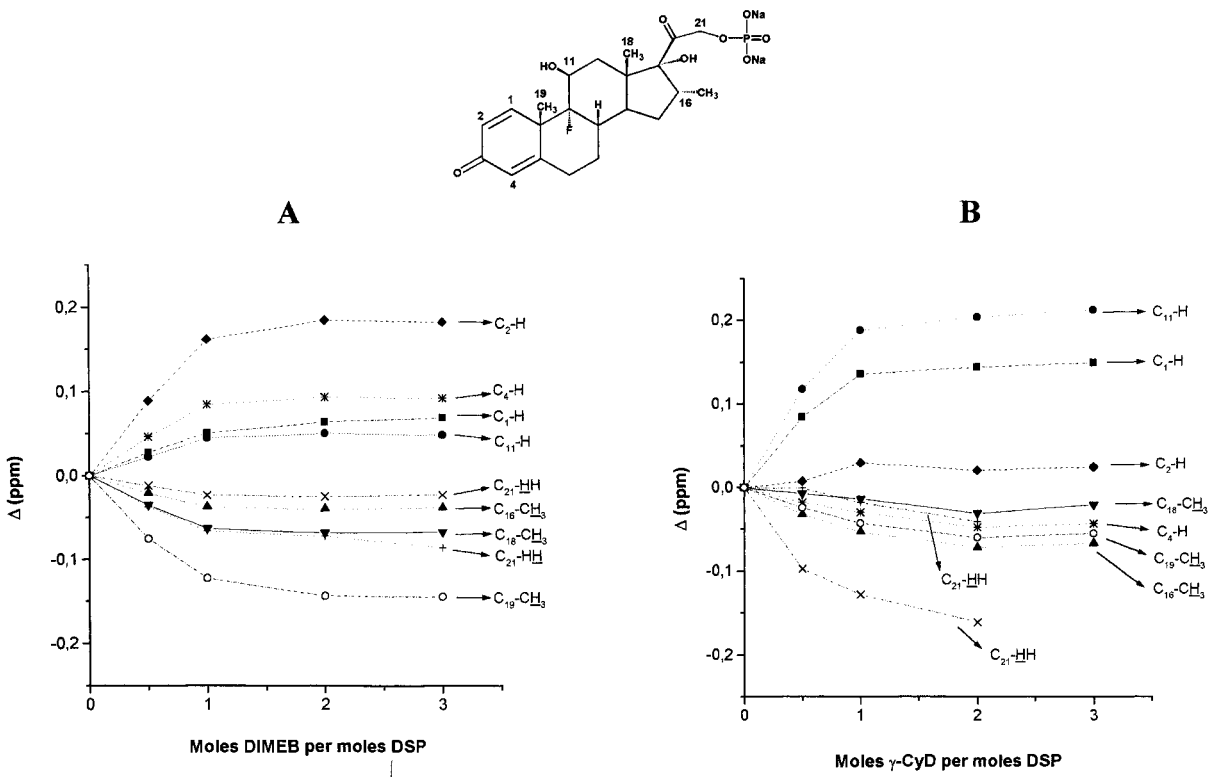

Figure 1. Chemical shifts of DSP protons in the presence of (A) DIMEB and (B) γ-CyD.

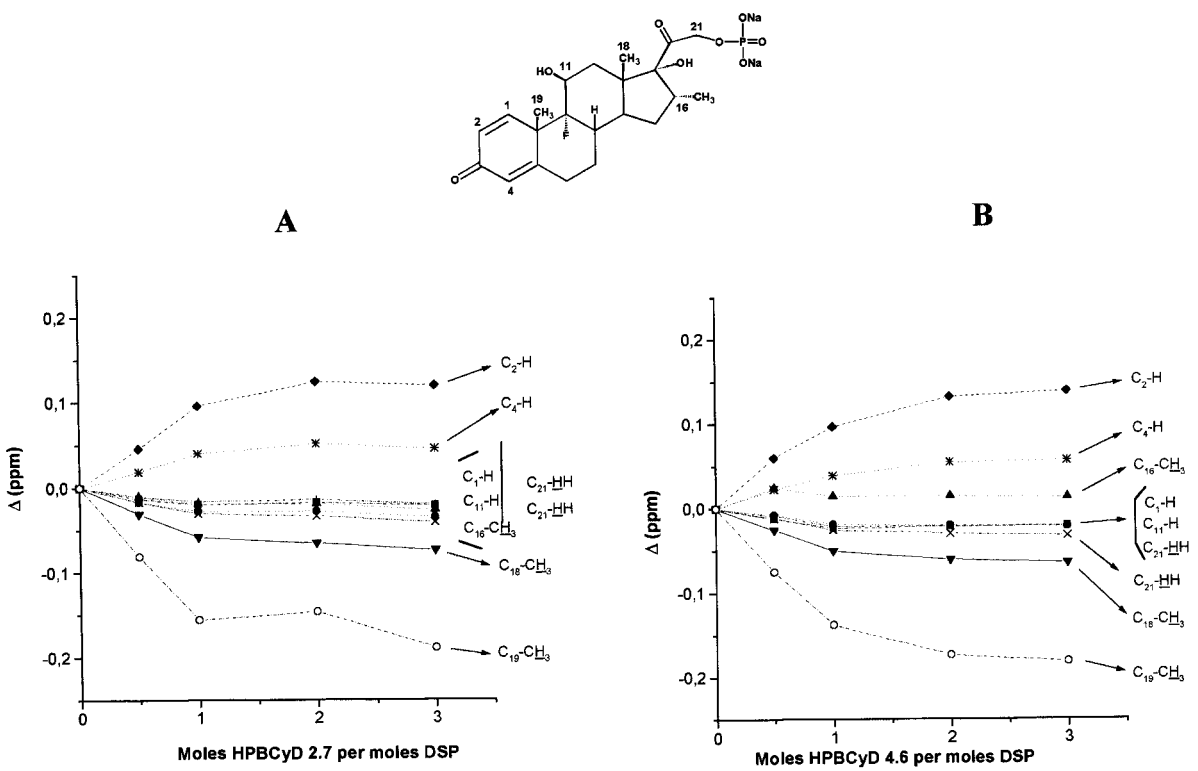

Figure 2. Chemical shifts of DSP protons in the presence of (A) HPβCyD 2.7 and (B) HPβCyD 4.6.

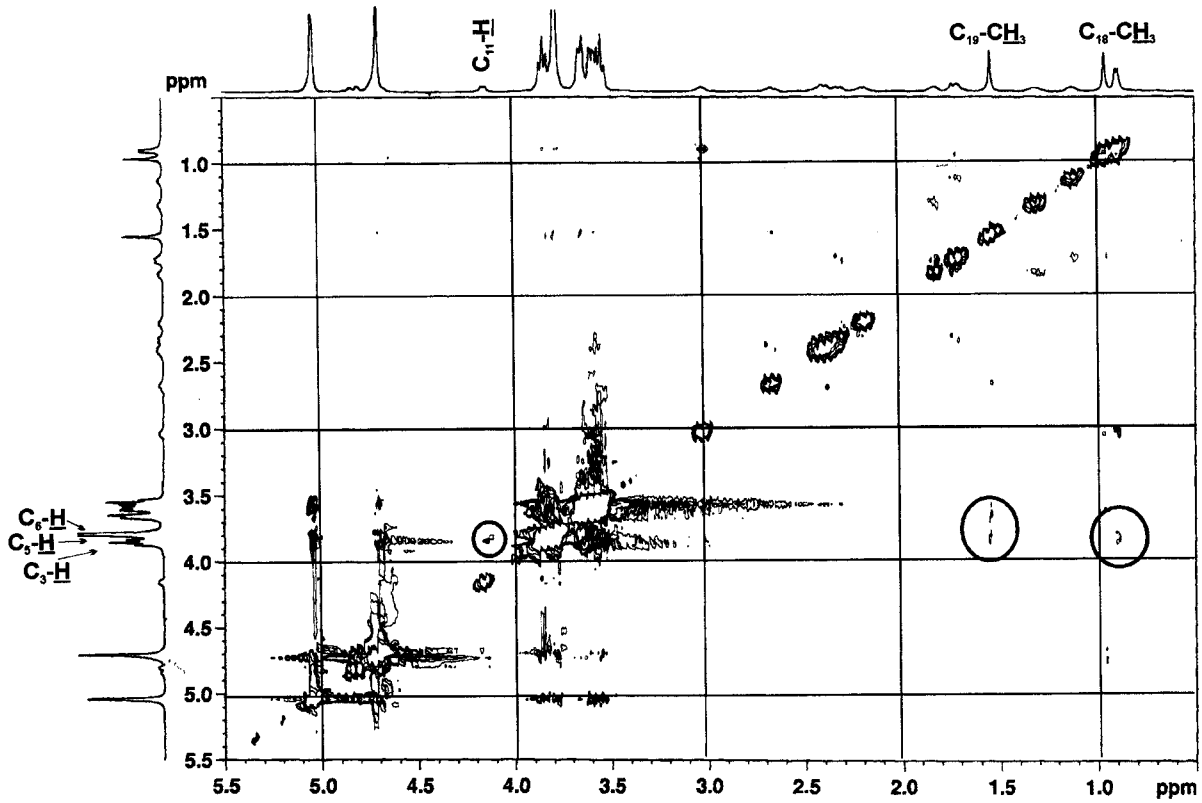

Figure 3. NMR spectrum corresponding to the 2D ROESY experiments carried out on the DSP–γ-CyD complex in solution.

peaks are similar to those found for γ-CyD but they have higher intensity between $C_{19}-C\underline{H}_3$ (1.65 ppm) and H_3, H_5, and H_6 and between both $C_2-\underline{H}$ (6.23 ppm) and $C_4-\underline{H}$ (6.11 ppm) and H_2, H_3, and H_4 (Figure 4). The difference in the intensity could be explained on the basis of the highest depth of the DIMEB.

Therefore, according to these shifts, the A ring protons may interact with the edge of the CyDs, resulting in an upfield shift, but the A ring protons are not located inside the CyD cavity. These results do not agree with the data reported by Viana et al.,[6] for dexamethasone acetate with β-CyD, HPβCyD, and γ-CyD, or with the results of Uekama et al.,[5] for dexamethasone base with α-, β-, and γ-CyD. These authors claim that the "A" ring of the molecule was included in the cavity of the CyDs. These discrepancies allow us to conclude that the complexation of a drug into the CyD cavity depends not only on the CyD used but also on the drug derivative considered.

The results of Job's method of continuous variations for some of the protons of DSP, located in ring A, and for methyl groups are shown in Figures 5 and 6. The results indicate a different drug–CyD ratio depending on the CyD derivative considered: 1:1 mol:mol for DSP–HPβ-CyD, 1:2 mol:mol for DSP–DIMEB, and 5:2 mol:mol for DSP–γ-CyD. This 5:2 drug–CyD relationship has not been described for any complex but, because the shape of the plots is not symmetrical, more than one complex may be present in the solution.[26] Indeed, it is know that DSP self-associates to form micelles. The measurement of surface tension of DSP dissolution (figure not shown) indicates that the cmc of DSP is in the range 0.01–0.02 M, which is lower than the concentration used in the Job diagrams (from 4×10^{-3} M to 4×10^{-2} M versus 2×10^{-2} M). The Job's plots provided same preliminary information on the stoichiometry of the complexes but did not seem to be absolutely reliable, perhaps because of the self-association of the drug molecules and the formation of multiple type complexes.[27]

Molecular Modeling

Molecular modeling has become a useful technique for studying host–guest complexes, especially as a complement to structural studies on the

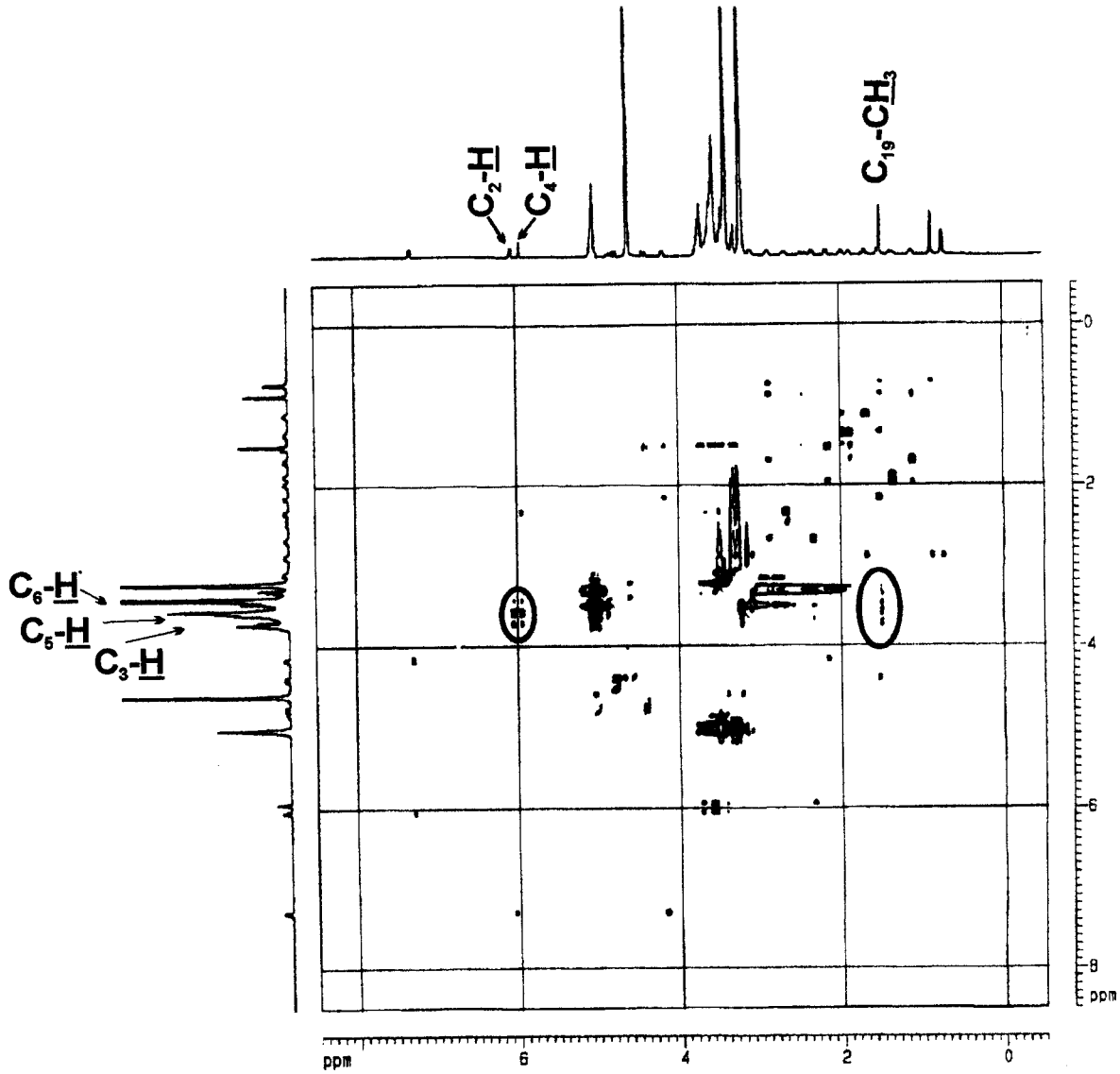

Figure 4. NMR spectrum corresponding to the 2D ROESY experiments carried out on the DSP–DIMEB-CyD complex in solution.

orientation of the host in guest cavities.[28–30] In this sense, we have carried out molecular mechanics calculations toward the determination of the geometrical conformation of DSP in the cavities of γ-CyD and DIMEB. Structural models of HPβCyD have been reported in the literature. However, the existence of HPβCyD as a complex mixture of different structures makes it impossible to conduct modeling experiments on its interactions with DSP.

DSP can be included into the CyD cavities (γ-CyD and DIMEB) in six different orientations, as illustrated in Figure 7. According to the results obtained by MM2 calculations in vacuum, DSP penetrates with the oxo group by the broad side of the CyDs in an orientation keeping both central rings of DSP (B and C) inside the hydrophobic core of CyDs (see Figure 7 (2)). However, there are small energy differences between the complexes in which the host penetrates the cavity, with the oxo group by the wider side and that in which it comes from by the narrow side. These energy differences are < 1 kcal/mol, which indicates that in vacuum, both orientations have similar probabilities of occurrence. Very large energy gaps are obtained from the inclusion of any of the extreme groups of DSP into the CyD cavities (see Figure 7 (1), (3), (4), and (6)). These groups

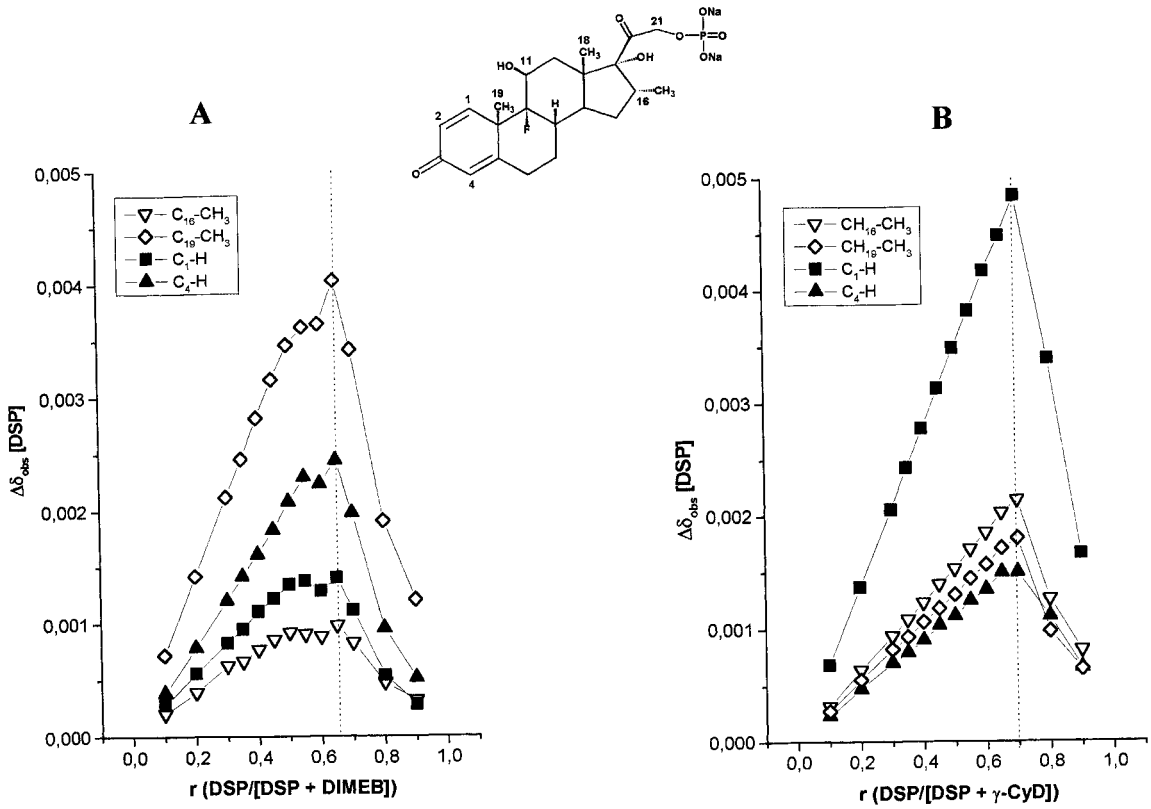

Figure 5. Job diagrams for the (A) DSP–DIMEB complex and (B) DSP–γ-CyD complex plots data corresponding to protons $C_1-\underline{H}$, $C_4-\underline{H}$,$C_{16}-C\underline{H}_3$, and $C_{19}-C\underline{H}_3$ of DSP. The values on the x-axis correspond to guest/(host+guest) ratio (*r*).

(oxo or phosphate) are polar groups that are not welcomed in the hydrophobic cavity of CyDs. These energy differences are increased when the study is performed in water solvent as a consequence of the preferred interaction of these groups with water. In this case, two effects contribute to increase the energy differences; they are, the preference of hydrophobic groups for a hydrophobic core and the interactions of polar groups with water.

If we consider only the structures in which rings B and C are inside the CyD cavity, the following results are obtained. In this case, for DIMEB, there are no significant energy differences for orientations of the DSP in which oxo group comes from the wider or narrow sides. However, a significant increment of energy difference appears in the case of γ-CyD for which the penetration of oxo group for the narrow side (see Figure 7 (2)) is favored by 17.2 kcal/mol (see Table 1) respect to its penetration from the wider side (see Figure 7 (4)). From these results we can conclude that the penetration of DSP with the oxo group coming from the narrow side leaves the phosphate group more free to interact with water molecules than in the case when the penetration occurs from the wider side. This effect is not observed in the case of DIMEB because of the interactions with the methyl groups that are at both sides of the CyD cavity and bury more of the structure of DSP, thereby preventing its interaction with water. The results obtained here on the orientation of DSP in CyD cavities, by molecular modeling techniques, agree with those derived experimentally from NMR studies and discussed previously (Figure 8).

Effect of Polymyxin B, Trimethoprim, and Benzalkonium Chloride on DSP–CyD Complexation

The displacement of a drug from the CyD cavity usually modifies the effect of complexation.[13–15] Therefore, it is important to investigate the interaction of the molecules present in the formulation with the CyD to avoid undesirable outcomes. The approach used in this paper to

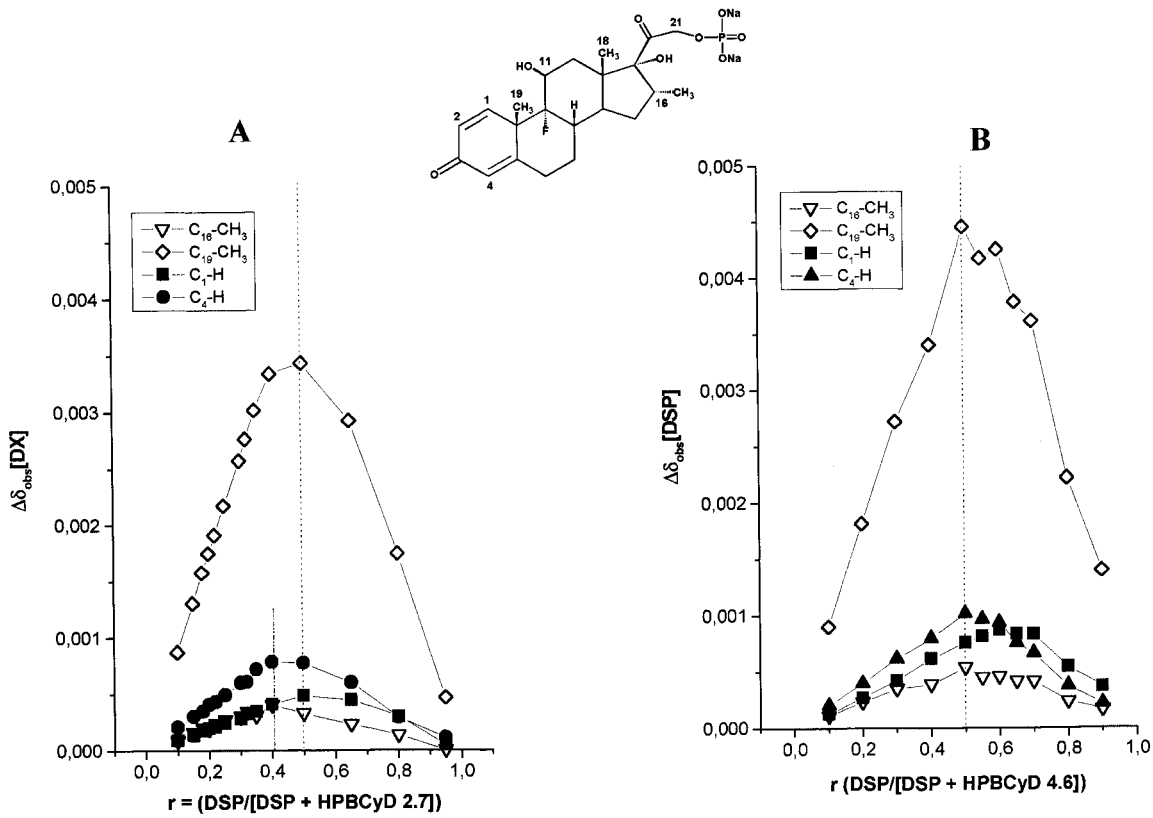

Figure 6. Job diagram for (A) the DSP–HPβCyD2.7 complex, and (B) the DSP–HPβCyD4.6 complex corresponding to protons $C_1-\underline{H}$, $C_4-\underline{H}$, $C_{16}-C\underline{H}_3$, and $C_{19}-C\underline{H}_3$ of DSP. The values on the x-axis correspond to guest/(host + guest) ratio (r).

analyze the potential displacement of DSP by polymyxin B, trimethoprim, and benzalkonium chloride form the cavity of the CyDs was to study the ^{1}H NMR chemical shifts of dexamethasone protons in the presence of those compounds. The main problem of this strategy may be the overlapping of the signals of DSP by those of the molecules. When this overlapping occurs, it is not possible to see the variation of the chemical shifts of DSP protons. This problem is common in the case of benzalkonium chloride and, in many cases, it has not been possible to clearly determine the chemical shifts variations for DSP protons.

The effect of these drugs on the chemical shifts of DSP protons $C_2-\underline{H}$, $C_4-\underline{H}$, and $C_{18}-C\underline{H}_3$ and $C_{19}-C\underline{H}_3$ with HPβCyD 4.6 are shown in Figure 9. The protons of DSP show variations in the same direction—either downfield or upfield—in the presence of the three molecules, and the differences in the intensity of those changes can be considered not significant on the NMR scale. Similar results where found with the rest of the CyD derivatives used (figures not shown). Therefore, from these results it can be concluded that the presence of polymyxin B, trimethoprim, and benzalkonium chloride do not interfere in the complexation of DSP with the four CyD derivatives used.

In conclusion, the structures of the inclusion complexes in solution of DSP with DIMEB, γ-CyD, and two HPβCyD derivatives have been characterized by NMR techniques. The results show no experimental evidence of inclusion of A ring in the cavity. This ring may interact with the edge of the CyDs, and the B and C rings remain inside. These results are supported by the molecular mechanic calculations for DIMEB and γ-CyD. NMR experiments were successfully used in the determination of a possible interference of different drug molecules presents in a pharmaceutical preparation on the complexation of DSP with the CyDs used.

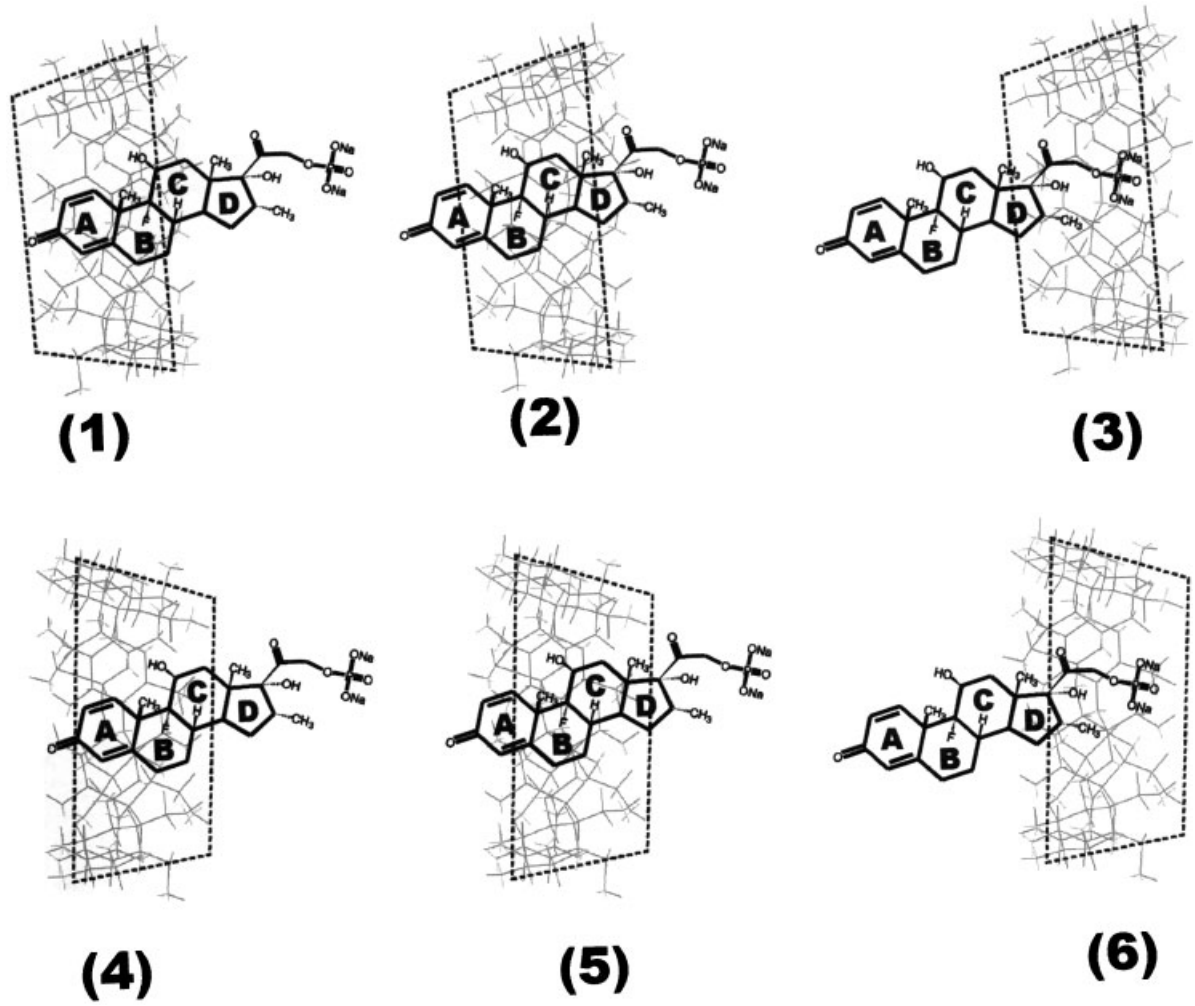

Figure 7. Possible orientations of DSP in the CyD cavity.

Table 1. Relative Energy of the Possible DSP–DMEB and DSP–γ -CyD Complexes[a]

Conformer	DIMEB in Vacuum	DIMEB with Aqueous Solvent	γ-CyD in Vacuum	γ-CyD with Aqueous Solvent
"A" ring inside the cavity in the broad end	4.30	17.16	4.46	7.09
"A" ring inside the cavity in the la narrow end	8.74	9.58	3.48	14.62
"C" and "D" rings inside the cavity in the broad end	1.04	0.00	0.46	17.24
"C" and "D" rings inside the cavity in the narrow end	0.00	0.54	0.00	0.00
Phosphate group inside the cavity in the broad end	5.54	14.85	5.95	25.00
Phosphate group inside the cavity in the narrow end	7.91	11.10	11.38	26.01

[a]Determined in a vacuum or in aqueous solvent by MM2 (Kcal/mol).

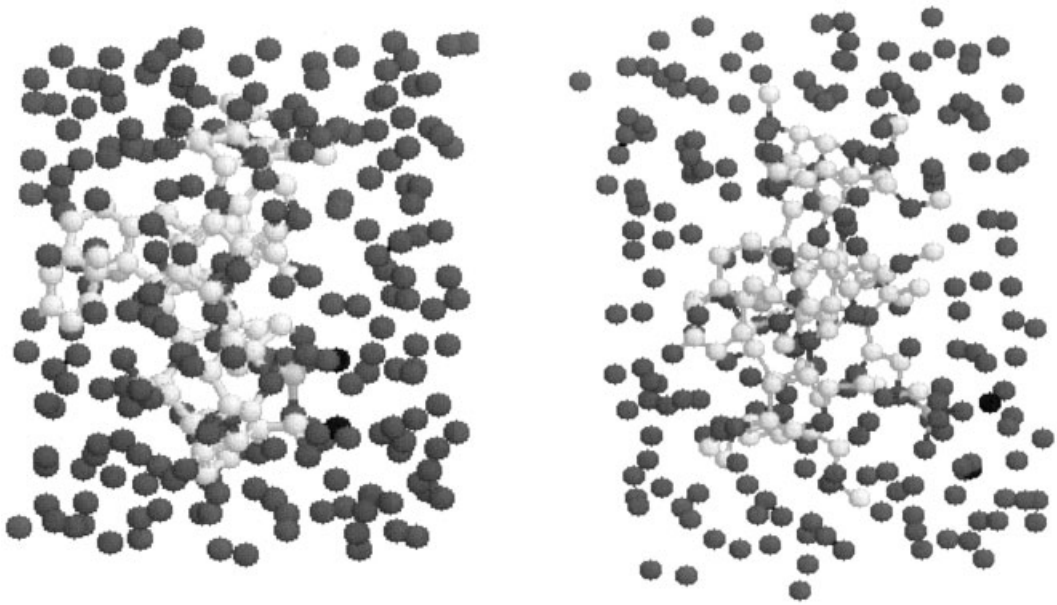

Figure 8. Energy-minimized (MM2) structures of DSP–DIMEB and DSP–γ-CyD complexes in aqueous solvent.

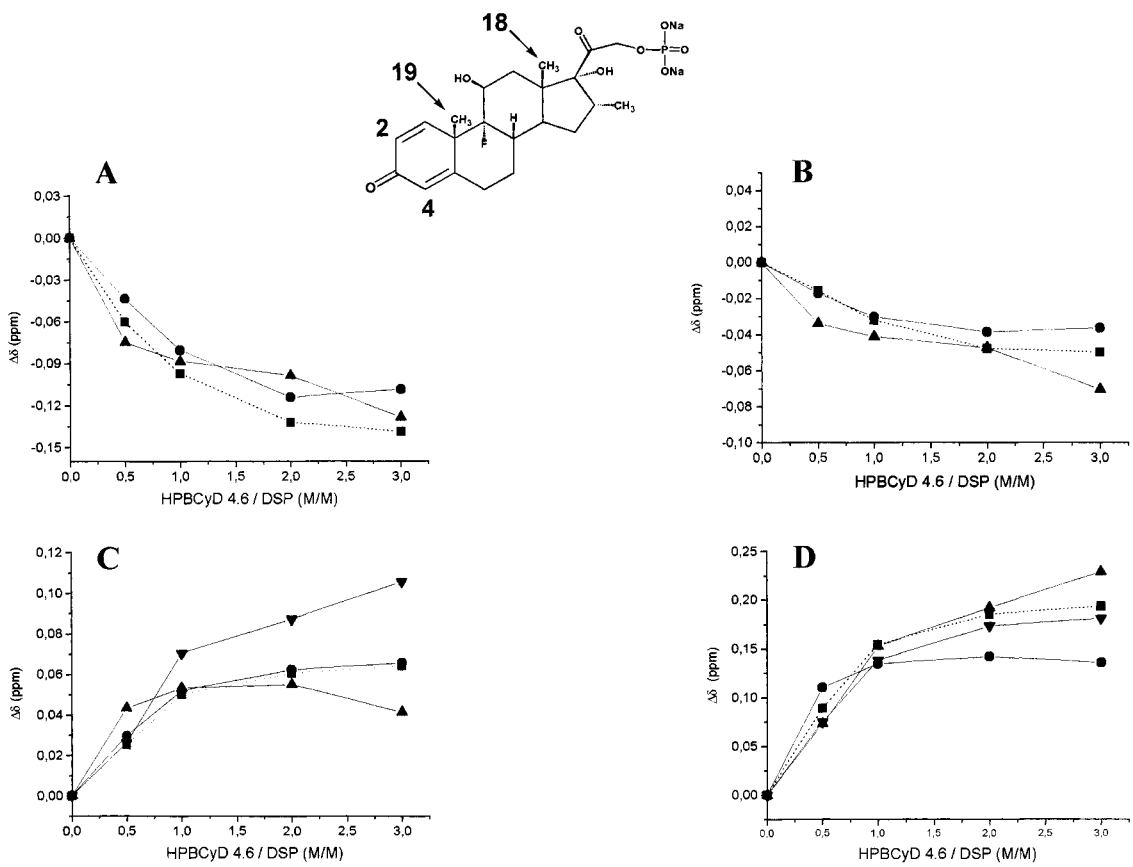

Figure 9. Chemical shifts of proton (A) $C_{18}-C\underline{H}_3$, (B) $C_{19}-C\underline{H}_3$, (C) $C_2-\underline{H}$, and (D) $C_4-\underline{H}$ of DSP in the presence of benzalkonium chloride, polymyxin B, and trimethoprim with HPβCD 4.6. Key: (■) DSP; (●) DSP + trimethoprim; (▲) DSP + polymyxin B; (▼) DSP + benzalkonium chloride.

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