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Research Paper

NMR Studies of the Inclusion Complex of Cloprostenol Sodium Salt with β-cyclodextrin in Aqueous Solution

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Purpose. Cloprostenol sodium salt (referred as cloprostenol) may be used for the synchronization of estrous cycles in farm animal species. Cyclodextrins (CDs) have potential as drug delivery systems through the formation of inclusion complexes between CDs and drugs. This is the first study of the inclusion complex of cloprostenol with β-cyclodextrin (β-CD) in aqueous solution using NMR and 3D molecular dynamics simulations.
Methods. 1D proton NMR spectra of β-CD, a complex of cloprostenol with β-CD, and cloprostenol in D_2O were assigned and confirmed. The cross relaxation interactions from ROESY were used as constraints for 3D molecular modeling studies.
Results. In the 2D ROESY of the complex, cross-peaks were observed between the aromatic protons of cloprostenol and protons of the β-CD as well as between aliphatic protons and protons of the β-CD. The stoichiometry of the complex was found that β-CD forms a 1:1 inclusion complex with cloprostenol. The association constant K was 968±120 M^{-1} at 298 K.
Conclusions. Aromatic side and/or aliphatic side chains of the cloprostenol is included in the β-CD while aliphatic side and/or aromatic side chains wraps around β-CD, respectively. The molecular modeling also confirms that β-CD forms a 1:1 inclusion complex with cloprostenol.

KEY WORDS: cloprostenol sodium salt; cyclodextrins; inclusion complexes; molecular modeling; nuclear magnetic resonance.

INTRODUCTION

Cyclodextrins (CDs) are oligosaccharides containing 6 (α-CD), 7 (β-CD) or 8 (γ-CD) α-1,4-linked glucose units produced from degradation of starch by naturally occurring protein enzymes in certain bacteria. CDs have a truncated conical shape with a hollow interior. Although the depth of the CD cavities is the same (7.8 Å), the size of the cavity depends upon the number of glucose units in the cyclodextrin ring. The diameters of the α-, β-, and γ-CD cavities are ~5.7, 7.8, and 9.5 Å, respectively (Fig. 1). CDs have polar, hydrophilic exteriors and hydrophobic interiors, making them soluble in water and able to form non-covalent inclusion complexes (ICs) with a large variety of guest molecules by including them in their interiors (1).

CDs have the potential for drug delivery through the formation of inclusion complexes (ICs) between CDs and drugs. Drug-CD-ICs may enhance the bioavailability of poorly soluble drugs by reducing their hydrophobicity when complexed with CDs (2). On the other hand, they may also be useful to modify the release rate of drugs. For example, hydrophobic cyclodextrin derivatives such as ethylated and acylated β-CD can be used as prolonged release type carriers for water soluble drugs such as diltiazem hydrochloride, buserelin acetate, and molsidomine (3).

Prostaglandins are a group of compounds formed in animals and humans that are derived from unsaturated hydroxyl fatty acid derivatives. They are endogeneous cell hormones, which are active in minute amounts, and are divided into types E, F, A, B, C, and D based on the functional groups in their cyclopentane rings (See Fig. 2). The majority of these compounds are unstable and insoluble in water, although they exhibit strong biological activities. There are several reports of using cyclodextrins for the solubilization and stabilization of prostaglandins, such as prostaglandin E_1 (PGE_1) and prostaglandin E_2 (PGE_2), through formation of their inclusion complexes (4–8). Cloprostenol is a synthetic analog of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and may be used for the synchronization of estrous cycles in farm animal species. Cloprostenol sodium salt is freely soluble in water and is the crystalline form of cloprostenol. For species like the pig it is desirable to develop a delayed release formulations of cloprostenol (referred to a cloprostenol instead of cloproste-

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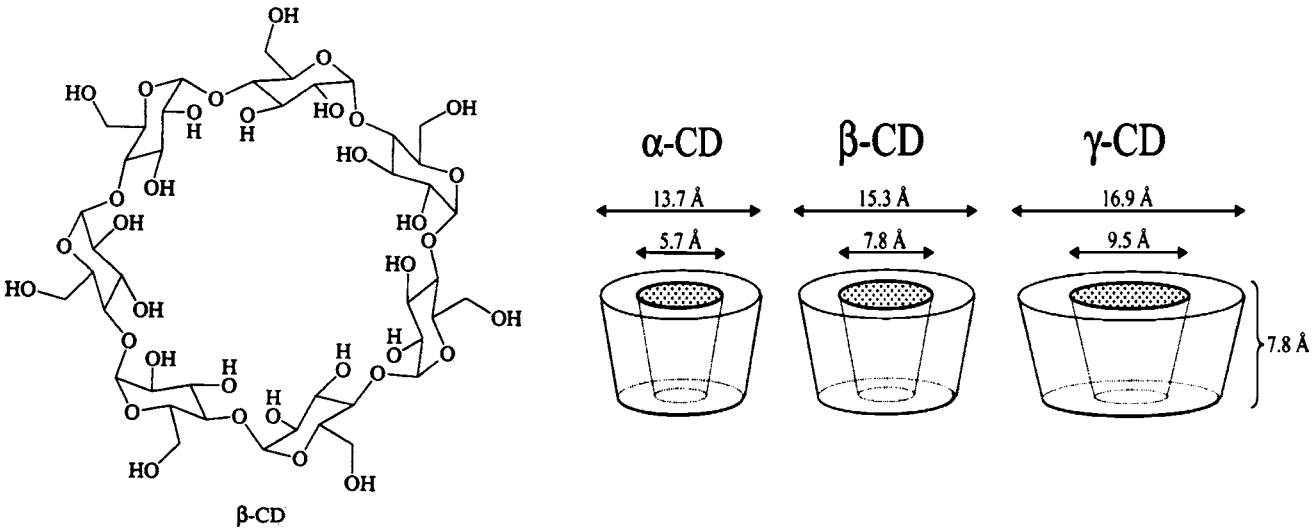

Fig. 1. Cyclodextrin structures.

nol sodium salt from now on), using inclusion complexes with CDs.

Nuclear Magnetic Resonance (NMR) spectroscopy is one of the most efficient techniques for the structural elucidation of inclusion complexes between prostaglandins and CDs. The structures of prostaglandins-CD complexes have been studied by NMR (4,5,9–14), however, there are no reported studies investigating the inclusion complexation of cloprostenol with CDs. Although nuclear overhauser effect (NOE) or rotating-frame overhauser effect (ROE) measurements have been successfully applied for determining the two and three dimensional characteristics of peptides and proteins in solutions (15,16), relatively little work has been conducted for elucidating the three dimensional characteristics of inclusion complexes involving prostaglandins. For the first time, we investigate an inclusion complexation of cloprostenol with β-CD using NMR spectroscopy and molecular modeling. In addition, the cross relaxation interactions from ROESY were used as constraints for molecular modeling studies of the inclusion complexation of cloprostenol by β-CD.

MATERIALS AND METHODS

Materials

Cloprostenol, β-CD, and D_2O (99.9 atom %D) were purchased from Pharmatech International, Inc., Cerestar Company, and Aldrich, respectively, and were used as supplied.

NMR Methods

All of the pulsed-field gradient NMR experiments were performed on a Bruker Avance 500 MHz Spectrometer equipped with an Oxford narrow bore magnet, REdHat Linux host workstation, and XwinNMR software (version; 3.6). A 5 mm ID inverse ^{1}H/BB (broad band) (^{109}Ag–^{31}P) triple-axis gradient probe (ID500-5EB, Nalorac Cryogenic Corp.) has been used for all measurements. The NMR probe was tuned to the ^{13}C frequency, which is 125.75 MHz in the 500 MHz spectrometer (^{1}H frequency of 500.13 MHz).

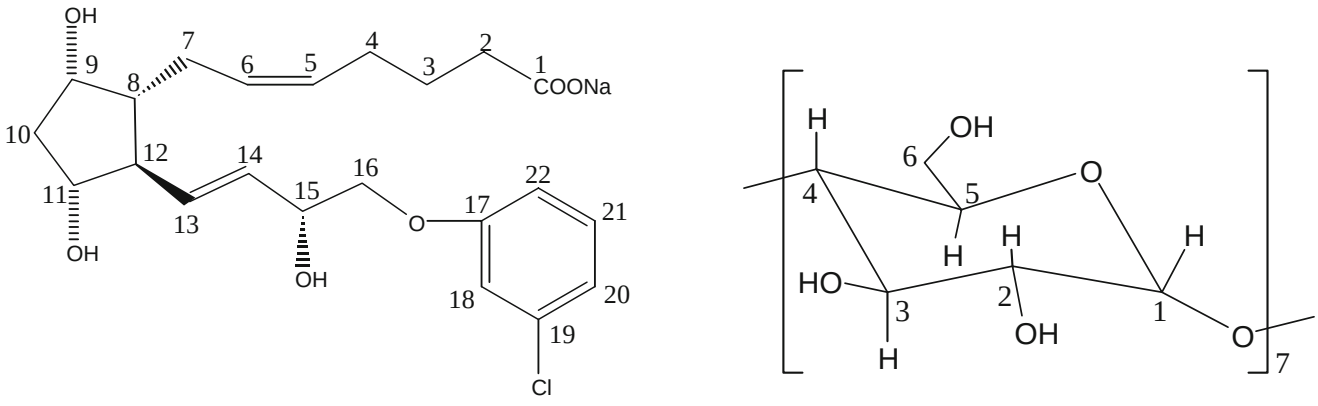

Fig. 2. Atom numbering of cloprostenol (**a**) and β-cyclodextrin (**b**).

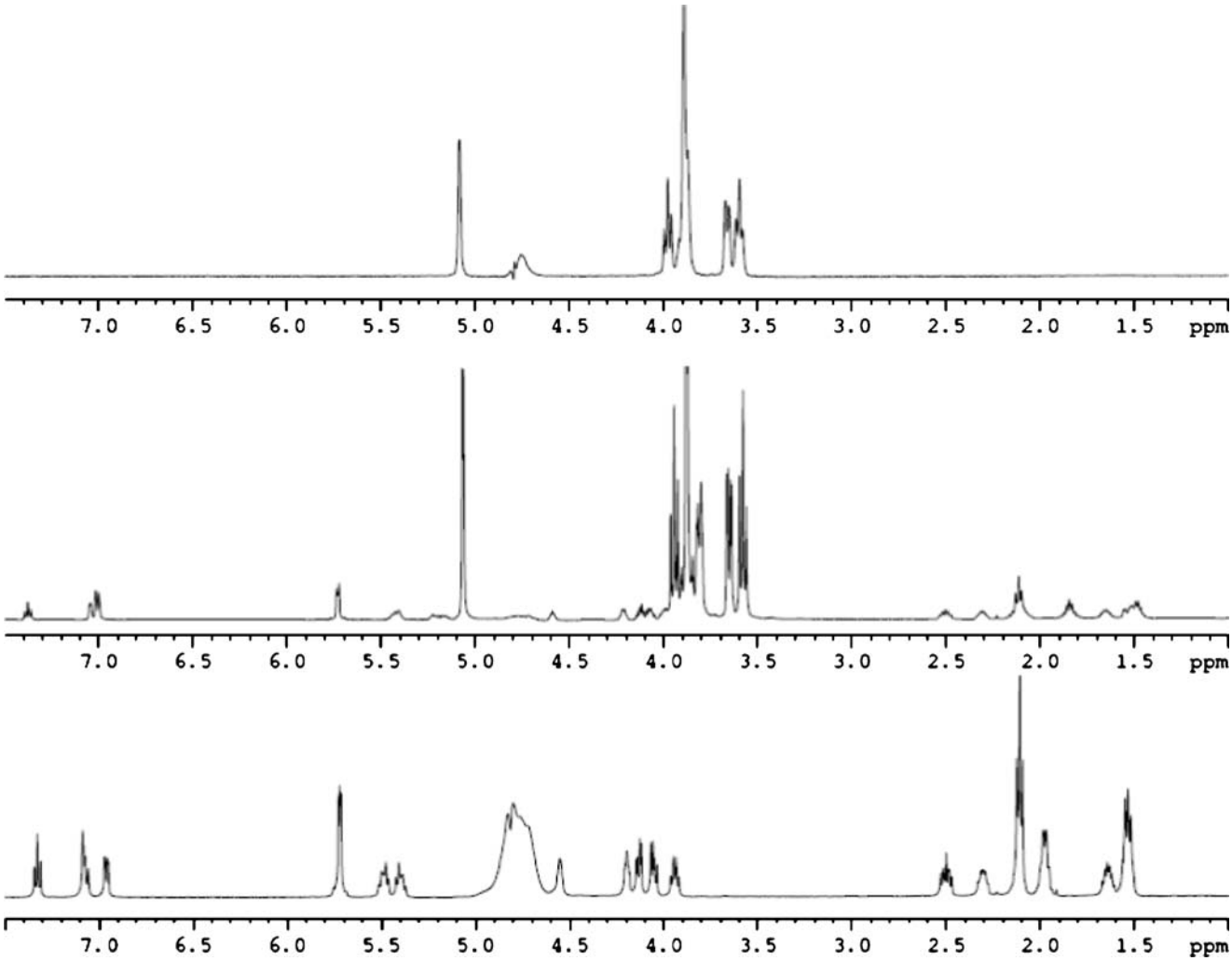

Fig. 3. One-dimensional (1D) ^{1}H-NMR solution spectra of β-CD solution (*top*), complex of cloprostenol with β-CD (*middle*), and cloprostenol in D_2O (*bottom*).

The spectra of β-CD, cloprostenol, and the complex of cloprostenol with β-CD in aqueous solution (D_2O) were assigned and confirmed using two-dimensional (2D) Homonuclear Correlation [2D COSY (^{1}H–^{1}H)], 2D heteronuclear multiple quantum coherence (HMQC) (2D ^{1}H–^{13}C one bond) and 2D heteronuclear multiple bond correlation (HMBC) (2D ^{1}H–^{13}C long range) NMR experiments. The standard instrumental parameters for acquisition of the 2D COSY, 2D HMQC, and 2D HMBC spectra were used. All data were processed with Bruker software XWINMR 3.6.

Two dimensional (2D) ROESY spectra with water presaturation were acquired using a phase sensitive pulse sequence with TPPI (time proportional phase incrementation), with two mixing times of 50 ms, 350 ms and recycle delay of 3 s. Data sets with 2048 complex points in t_2 and 512 complex points in t_1 were acquired with a 6,000 Hz sweep width in both dimensions and 128 scans per slice. All spectra were processed with a combination of exponential weighting and sine–skewed functions and zero-filled to 2k × 2k data points.

Determination of the Stoichiometry of the Complex

The stoichiometry of complexes formed between β-CD and cloprostenol were determined using the continuous variation method (17). Two stock solutions of cloprostenol (5 mM) and β-CD (5 mM) in D_2O were prepared. The total concentration of the cloprostenol ([A]) and β-CD ([B]) was kept constant ($[A]_{total}+[B]_{total}=5$ mM), while the ratio $r=[A]_{total}/([A]_{total}+[B]_{total})$ was varied from 0 to 1. A series of cloprostenol and β-CD mixtures were obtained by preparing equimolar solutions of cloprostenol and β-CD and mixing them to constant volume to the desired ratio (r=0, 0.25, 0.5, 0.75, and 1), and their proton NMR spectra were acquired immediately.

Modeling of Closprostenol

Semi-quantitative distance constraints between the non-exchangeable protons were estimated from the intensity of the cross-peaks in the 2D ROESY spectra observed in the mixing time study (18,19). Using the invariable covalent

Table I. Effects of β-cyclodextrin on the Proton Chemical Shifts of Cloprostenol

Proton #	Cloprostenol	In the Presence of -CD
1	–	–
2	2.014	1.950
3	1.437	1.317
4	1.879	1.658
5	5.308	5.006
6	5.390	5.254
7	2.006	1.917
8	1.542	1.500
9	4.103	4.055
10 a and b	2.410/1.437	2.345/1.369
11	3.840	3.826
12	2.216	2.142
13	5.622	5.565
14	5.622	5.565
15	4.462	4.435
16 a and b	4.035/3.953	3.960/3.910
17	–	–
18	6.972	6.881
19	–	–
20	6.950	6.849
21	7.214	7.221
22	6.848	6.849

H21–H22 distance (2.4 Å) of the benzene ring of cloprostenol (Fig. 2) as reference, the ROESY cross peak intensities were classified as strong, medium, weak or very weak. Therefore, the corresponding assigned proton pairs yielded bound distance constraints of 2.4 (±0.6), 3.0 (±1), 4.0 (±1) and 5.0 (±1) Å, respectively.

The distances involving the unresolved protons, i.e. methylene protons, were assigned using pseudo-atom notation to make use of the pseudo-atom correction automatically computed by the Crystallography and NMR System (CNS) (20). Before proceeding to the NMR structure determination, the atomic coordinates of cloprostenol were obtained by drawing its two dimensional (2D) structure with the JME Molecular Editor (Peter Ertl, Novartis Institutes for BioMedical Research, Basel, Switzerland). The above coordinates were then transferred to the Dundee PRODRG2 server (21) and converted into the Protein Data Bank (PDB) format. The topology and parameter files compatible with CNS 1.1 (20), which are required for structure calculation, were generated with XPLOR-2D software (Gerard J. Kleywegt, Dept. of Cell and Molecular Biology, Uppsala University, Biomedical Centre). Partial charges for individual atoms of cloprostenol were determined using Antechamber (22), with the AM1-BCC charge model (23). The NMR structure determination of closprostenol was achieved by using the CNS 1.1 software according to published protocols with minor modifications (20, 24). The molecular visualization software PyMOL (DeLano Scientific LLC, San Carlos, California, USA) was used to display and analyze the above resulting structures.

Docking Model of the Complexation of Cloprostenol with β-CD

The PDB coordinates of β-CD were taken from HIC-UP (Gerard J. Kleywegt, Department of Cell and Molecular Biology, Uppsala University, Biomedical Centre). The atomic charges for individual atoms of β-CD were calculated as described above. The structural geometry of β-CD was optimized by minimizing its energy using HyperChem with the Amber force field (25). Then, the β-CD model was subjected to 60 ps of MD simulation at 300 K in vacuo followed by energy minimization. The aromatic ring of the energy minimized structural model of closprostenol was placed randomly inside the cavity of β-CD. The complex of cloprostenol with β-CD was minimized by using the Polak–Ribiere algorithm (conjugate gradient method) and the NMR derived distance constraints found in the case of the complex formation. Following geometry optimization, the complex (docking model) was subjected to a MD simulation of 1 ns at constant temperature (300 K). Snapshots of representative models were recorded every 100 ps.

RESULTS AND DISCUSSION

A series of NMR experiments in aqueous solution were immediately carried out to assign peaks and to study the structure of cloprostenol, β-CD, and the complex of cloprostenol with β-CD. Figure 2 shows the atomic numbering schemes of cloprostenol and β-CD used for ^{1}H spectral assignments.

Figure 3 shows one-dimensional (1D) ^{1}H-NMR spectra of β-CD, a complex of cloprostenol with β-CD, and cloprostenol in D_2O. The 1D proton spectra of cloprostenol and β-CD revealed the absence of impurities, which could interfere with the accuracy of the NMR spectral analysis. The aromatic protons H18, H20, H21 and H22 of cloprostenol were readily identified due to their characteristic low field resonance peaks between 6.5 – 7.5 ppm. The peaks corresponding to the H5, H6, H13 and H14 protons of the ethylene methine carbons were identified between 5 – 6 ppm. As expected, the methylene and methine protons attached to C2–C4, C7, C8, C10 and C12 were observed between 1 – 2.5 ppm. Due to the proximity of the oxygen atom between C16 and C17, and the OH groups, the resonance peaks corresponding to H16a/b, H9, H11, H15 were deshielded and identified between 3.5 – 4.5 ppm. These identifications were further confirmed by analyzing the 2D COSY ^{1}H–^{1}H, 2D ^{1}H–^{13}C one bond HMQC, and ^{1}H–^{13}C 2D long bond HMBC NMR experiments (See Figures S1-A and S1-B in the supporting information). The assignment of the β-CD protons was obtained using ^{1}H–^{1}H, 2D COSY and 2D ^{1}H–^{13}C HMQC experiments, and were further confirmed (26).

Table II. Effects of Cloprostenol on the Proton Chemical Shifts of β-cyclodextrin

Proton #	β-CD	In the Presence of Cloprostenol
1	4.965	4.902
2	3.55	3.491
3	3.86	3.782
4	3.483	3.421
5	3.76	3.645
6	3.77	3.716

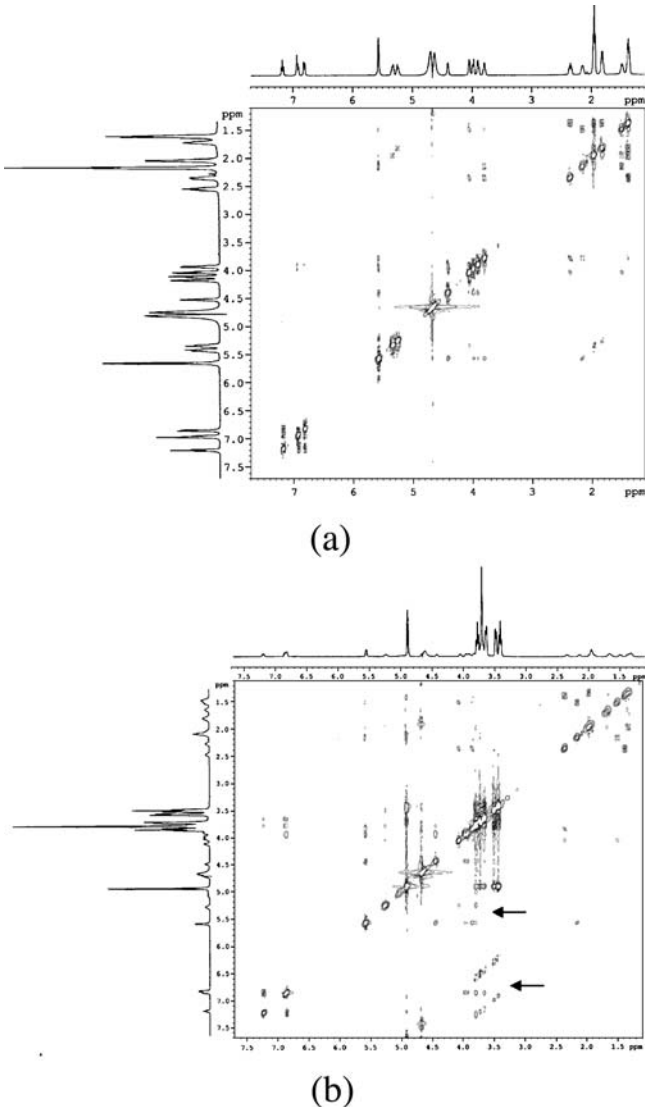

Fig. 4. Two-dimensional ROESY spectra of cloprostenol in D_2O in the absence of β-CD (**a**) and the complex of cloprostenol with β-CD (**b**). *Arrows* indicate cross-peaks.

The proton chemical shifts for cloprostenol and β-CD in the presence and absence of each other are summarized in Tables I and II, respectively. As shown in Table I, the protons H3, H4, H5, H6, H20 of the cloprostenol in the presence of β-CD show considerable chemical shift differences *(Δδ=0.3–0.13 ppm),* while other individual protons show only minimal chemical shift differences. In particular, the H5 proton shows the greatest proton chemical shift difference of *(Δδ=0.3 ppm)*. From Table II, the proton H5 of β-CD exhibits the greatest chemical shift difference *((Δδ=0.12 ppm)* compared with the other protons of β-CD in the presence of cloprostenol. There are potentially several different reasons for this effect; for example, changes in the molecular geometry, intermolecular interactions by association, or formation of an inclusion complex.

Figure 4 presents the 2D ROESY spectra for cloprostenol in the absence (a) and in the presence (b) of β-CD. The results of the 2D ROESY spectra were used to monitor the proximity between protons through space (^1H–^1H). In terms of

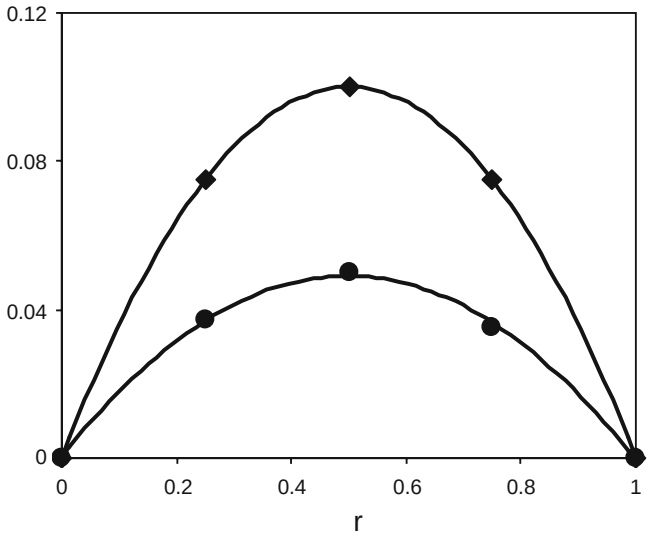

Fig. 5. Plot of the observed $\Delta\delta_{obs}$ $[cloprostenol]_{total}$ (*filled diamond* proton H21 of cloprostenol) or $\Delta\delta_{obs}$ $[\beta\text{-CD}]_{total}$ (*filled circle* proton H2 of β-CD) as a function of ratio *r*.

cloprostenol in the absence of β-CD, the two side chains of cloprostenol appeared to be close in space, as indicated by the cross-peaks observed between protons H13, H14 and protons H2, H3. However, these close distances through space were interrupted when β-CD was added to the cloprostenol in aqueous solution. Also, cross-peaks between protons H5 and H12, between H16 and H18, and between H16 and H22 of cloprostenol disappeared. New cross-peaks between H7 and H2, between H10 and H4 of cloprostenol appeared in the ROESY spectrum. The above appearance and disappearance of cross-peaks are apparently caused by the interaction between cloprostenol and β-CD. For the analysis of the ROESY spectrum corresponding to the complex of cloprostenol with β-CD in Fig. 4b, weak cross-peaks between the aliphatic protons (H4, H5, H6, H13, H14 and H15) and H3 of the β-CD were observed. Strong cross-peaks were observed between the aromatic H18–H22 protons and the H3, H4, H5 and H6 protons of the β-CD. Due to the nature of the observed ROESY signals upon complex formation, one could conclude that the aromatic and aliphatic protons of the cloprostenol were closely interacting with the protons

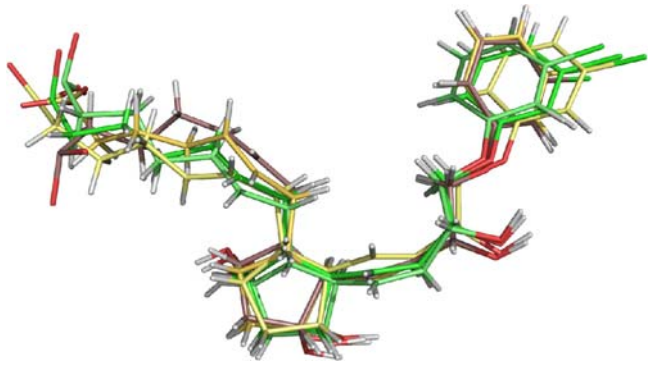

Fig. 6. Ensemble of solution structures of free cloprostenol determined by NMR.

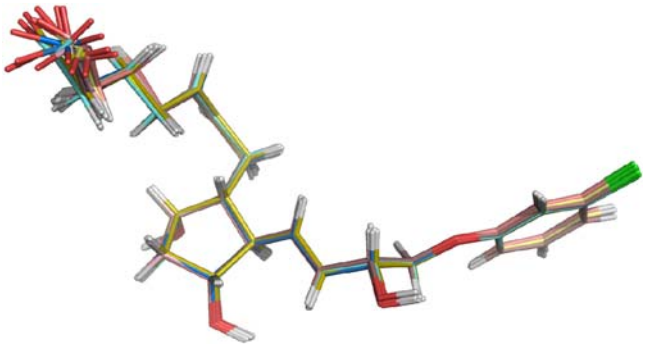

Fig. 7. Reduction of the dynamics of the aliphatic and aromatic sections of the cloprostenol molecule upon complex formation (21).

oriented toward the cavity and located outside the cavity in the β-CD. These results clearly demonstrated that two different modes of complex between cloprotenol and β-CD are formed simultaneously. First, the aromatic side chain is included in the cavity of the β-CD, and the aliphatic side chain interacts with the H4, H6 protons of the β-CD situated outside the cavity. Second, the aliphatic side chain of the cloprostenol is inserted in the β-CD cavity, and the aromatic side chain is engaged in interactions with H6 and H4 protons of the β-CD.

The stoichiometry of the complex between cloprostenol and β-CD was determined using the continuous variation method. The quantity $\Delta\delta_{obs}$ $[cloprestenol]_{total}$ and $\Delta\delta_{obs}$ $[\beta\text{-CD}]_{total}$ represent the chemical shift difference between free cloprostenol in the absence of β-CD and the value observed for a given ratio of *r* and the chemical shift difference between free β-CD in the absence of cloprostenol and the value observed for a given ratio of r, respectively. Plots of the observed $\Delta\delta_{obs}$ $[cloprostenol]_{total}$ and $\Delta\delta_{obs}$ $[\beta\text{-CD}]_{total}$ as a function of r are presented in Fig. 5. Only a few protons of cloprostenol and β-CD have been selected in the host and guest molecules. The plots shows maxima at r=0.5 and have highly symmetrical shapes. This indicates that β-CD forms a 1:1 inclusion complex with cloprostenol in aqueous solution. In addition, the association constant K obtained from the curve fitting analysis is 968±120 M^{-1} at 298 K (27).

Following the sequence specific assignment of the ROESY cross-peaks, 39 distance constraints were generated. The solution structure of apo-cloprostenol (without the

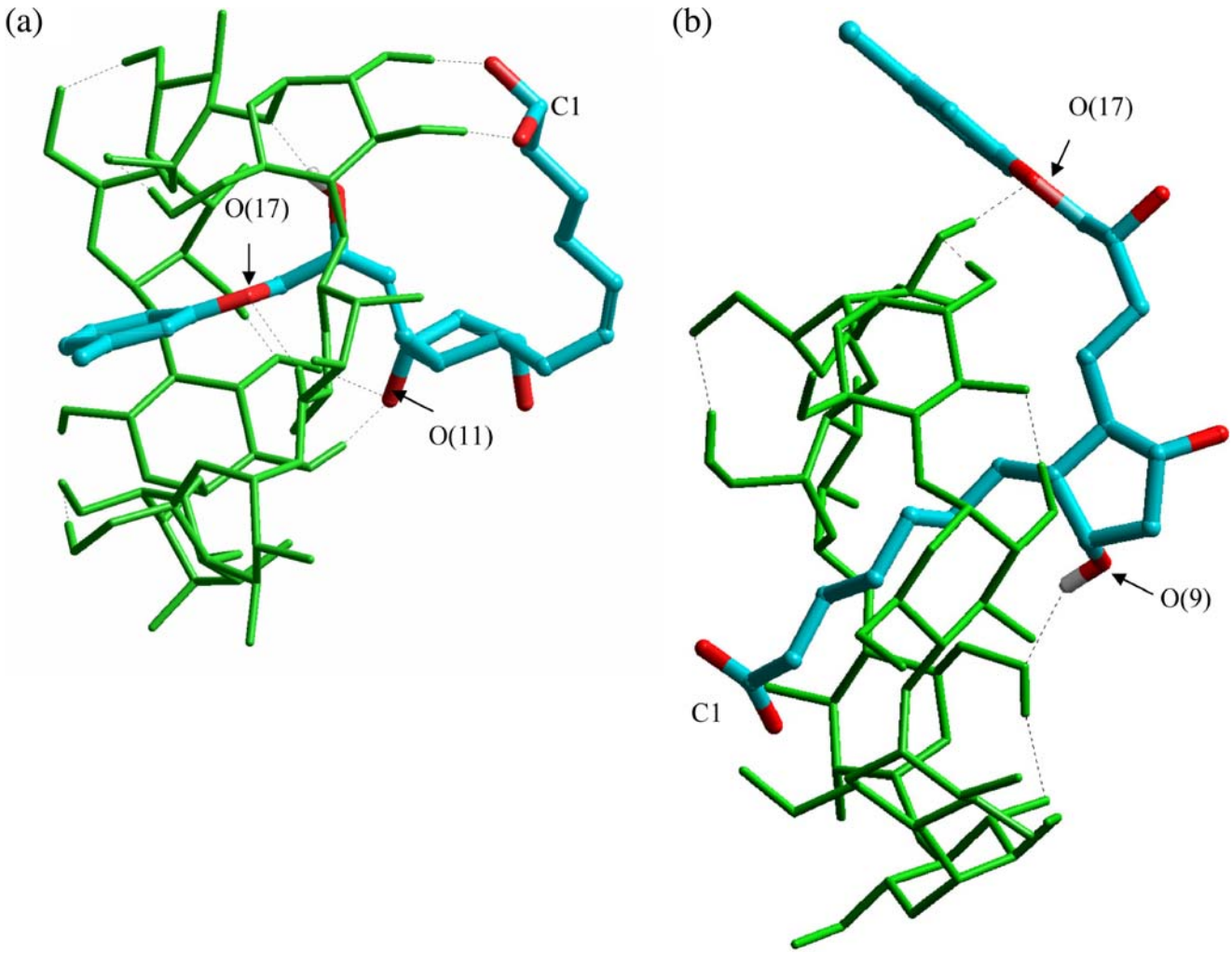

Fig. 8. **a** Cloprostenol interacting with β-CD by intercalation of its aromatic side chain within the cavity of β-CD. **b**. Cloprostenol interacting with β-CD by intercalation of its aliphatic side chain within the cavity of β-CD. The *dashed lines* represent H-bonds.

presence of β-CD) was determined by torsion angle molecular dynamics simulation (21). Twenty-five (25) structures contained no distance violation greater than 0.5 Å and dihedral angle violation were less than 5°. In addition, structures were rejected when the root mean standard deviation (RMSD) of bonds was higher than 0.02 Å or if the RMSD of angles was above 2.0°. Among the above twenty-five (25) generated structures, ten (10) were found to have the lowest energy target function and no constraint violations. These structures are in agreement with the NMR data and were chosen to represent the final ensemble as shown in Fig. 6. Analyses of these structures showed that an extended strand conformation was adopted by the apo-cloprostenol in aqueous solution. The aliphatic arm of the cloprostenol was found to be highly dynamic, due to the rotations around the bonds terminated by C7, C4 and C2 of the cloprostenol. The cyclopentane ring adopted the envelope conformation, which is thermodynamically the most stable one. The dynamics of the aromatic ring was less pronounced compared to that of the aliphatic strand. This is due to slight rotation around the bonds terminated by C16. Therefore, the orientation of the substituted aromatic ring seems to be dictated by the Cl substituent at C19 of the cloprostenol.

However, a noticeable decrease in dynamics of the aliphatic and aromatic sections of cloprostenol were revealed upon complex formation with β-CD, as shown in Fig. 7. A single bend conformation was adopted by the cloprostenol when complexed with β-CD. Thus, a change of conformation was induced during the interaction between cloprostenol and β-CD.

In addition to the NMR data, the molecular model of the complex of cloprostenol with β-CD highlighted a number of key interactions between the two molecules, as shown in Fig. 8a,b. The cloprostenol interacted with the β-CD by intercalation of its aromatic ring or aliphatic side chain within the cavity of β-CD. In the case where the aromatic ring was inside the cavity, inter-hydrogen bond formation was observed between the O (11) and O (17) atoms of the cloprostenol and the exterior OH groups of the β-CD. Inter-hydrogen bonds were also observed between the oxygen of the carbonyl group of cloprostenol and the exterior OH of the β-CD. When the aliphatic side chain was inside cavity of the β-CD, hydrogen bond interactions were observed between the O (17) and O (9) atoms and of the cloprostenol and the exterior OH groups of the β-CD. Finally, the molecular modeling also confirmed that in aqueous solution β-CD forms a 1:1 inclusion complex with cloprostenol.

CONCLUSIONS

The H5 proton of cloprostenol in the presence of β-CD shows the greatest chemical shift difference of *(Δδ=0.3 ppm)*. On the other hand, the H5 proton of β-CD exhibits the greatest chemical shift difference *(Δδ=0.12 ppm)* in the presence of cloprostenol. This may be due to some reasons such as changes in the molecular geometry, intermolecular interactions by association, or formation of an inclusion complex. The 2D ROESY spectra indicate that the two side chains of cloprostenol in the absence of β-CD appear to be close in space. However, their vicinity through space distances was interrupted when β-CD was added to the cloprostenol in aqueous solution. In the ROESY spectrum corresponding to the complex of cloprostenol with β-CD, strong cross-peaks were observed between the aromatic H18–H22 protons and the H3, H4, H5 and H6 protons of the β-CD, suggesting that the aromatic and aliphatic protons of the cloprostenol were closely interacting with the protons in the β-CD cavity. The stoichiometry of the complex determined by the continuous variation method showed that β-CD forms a 1:1 inclusion complex with cloprostenol in aqueous solution. The NMR-derived distance constraints were generated and used to determine the 3D structure of the complex of cloprostenol with β-CD by molecular modeling. The NMR data were supported by the molecular model of the complex of cloprostenol with β-CD, which shed light on possible interactions between the two molecules. Our results demonstrated that the structure of apo-closprostenol experienced a high level of dynamics, compared to the motions when complexed with β-CD. It is also clear that two different modes of complex between cloprotenol and β-CD are formed simultaneously. Aromatic side chain and/or aliphatic side chain of the cloprostenol is included in the β-CD while aliphatic side chain and/or aromatic side chain wraps around β-CD, respectively. In addition, inter-hydrogen bonds were observed to form between other portions of cloprostenol and the exterior hydroxyl groups of β-CD. The molecular modeling also confirmed that in aqueous solution β-CD forms a 1:1 inclusion complex with cloprostenol.

ACKNOWLEDGEMENTS

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