J Incl Phenom Macrocycl Chem (2011) 70:389–396
DOI 10.1007/s10847-010-9857-6

ORIGINAL ARTICLE

Improvement of pharmaceutical potential of all-*trans* retinoic acid with hydroxypropyl-*β*-cyclodextrin

Keiko Takahashi · Sayo Morimoto · Hiroto Nakamura · Toshiaki Narusawa · Taizo Seki · Masahiko Ooe · Keigo Aoi · Akiyoshi Takada

Received: 14 June 2010 / Accepted: 2 September 2010 / Published online: 18 September 2010

Abstract 2-Hydroxypropyl-*β*-cyclodextrin (HP-*β*-CyD) includes all-*trans* retinoic acid (RA), covering the double-bond area of RA with substituted hydroxypropyl groups on CyD ring, as proved by the nuclear Overhauser effect (NOE) between methylene protons on the hydroxypropyl groups and the proton on RA. The formation of an inclusion complex results in hydrophilicity and stability. The effect of RA/HP-*β*-CyD and that of RA without HP-*β*-CyD on wrinkle scores and skin elasticity during skin treatment were identical, and the cutaneous stimulus was reduced comparing with RA. The results indicated that the RA/HP-*β*-CyD complex should help to realize new approaches in skin rejuvenation therapy.

15th International Cyclodextrin Symposium.

K. Takahashi (✉) · S. Morimoto · H. Nakamura · T. Narusawa
Department of Life Science and Sustainable Chemistry, Faculty of Engineering, The Center for Nanoscience and Technology, Tokyo Polytechnic University, 1583 Iiyama, Atsugi Kanagawa 243-0297, Japan
e-mail: takahasi@chem.t-kougei.ac.jp

T. Seki · K. Aoi
Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan

M. Ooe · A. Takada
Department of Cosmetic Medicine, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka Suita, Osaka 565-0871, Japan

T. Seki · M. Ooe
Development Laboratory, Tokiwa Pharmaceutical Co., Ltd, 6-13-1 Minatojima Nakamachi, Chuou-ku, Kobe Hyougo 650-8521, Japan

Keywords Tretinoin · Hydroxypropyl-*β*-cyclodextrin · Skin rejuvenation therapy · Inhibition of side effects · NMR

Introduction

All-*trans* retinoic acid (RA, tretinoin; Fig. 1) causes a wide range of biological effects and is used for treating acne and for hyperkeratinization [1–3]. It has been reported that topical tretinoin decreases the cohesiveness of follicular epithelial cells as well as microcomedo formation; further, it has been reported that topical tretinoin stimulates mitotic activity and increases the turnover of follicular epithelial cells that cause extraction of the comedones. Tretinoin also exhibits therapeutic activity in the treatment of photo-aging, e.g., treatment of wrinkles, dyspigmentations and tactile roughness [4]. However, sensitive patients may experience inflammation, redness, scaling, itching, and burning [5, 6]. The technological disadvantages of RA are its lack of solubility in water and its sensitivity to environmental factors such as light, heat, and air. Solutions of RA in organic solvents are reasonably stable when stored in the dark, but RA deteriorates rapidly in aqueous solution [7]. Cyclodextrins (CyDs), which are cyclic oligosaccharides consisting of six to eight glucose moieties linked through α-1,4 glycosidic bonds, form inclusion complexes with various drug molecules in aqueous solution and in the solid state; they have been successfully utilized to improve certain properties such as the solubility, stability, and bioavailability of drugs [8–12]. 2-Hydroxypropyl-*β*-CyD (HP-*β*-CyD), which has been known as a poly(hydroxypropyl)ether of *β*-CyD with random substitutions at the 2, 3, and 6 positions of the glucose ring, has been produced on an industrial scale. In commercial HP-*β*-CyDs, the

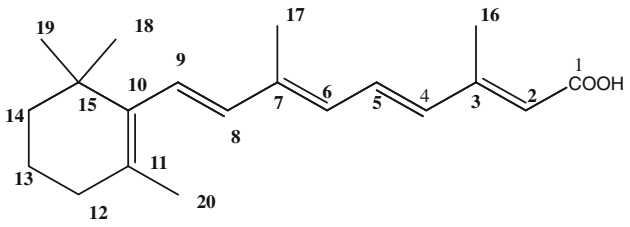

Fig. 1 Structure of all *trans*-retinoic acid (tretinoin; RA)

positions and the number of substitutions of CyD isomers vary, because it has been difficult to regulate hydroxypropylation with precision. Even though it suffers from these disadvantages, HP-β-CyD not only possesses properties of the native CyD but also has greater water solubility than the native CyD; HP-β-CyD is used in various products and techniques [13–15]. In order to improve the bioavailability of RA, inclusion complexes of RA with β-CyD, heptakis(2,6-di-*O*-methyl)-β-CyD, or HP-β-CyD) have already been developed [16–23]. The photostability, water solubility, and diffusion rate through the membrane have been improved, and RA-induced skin irritation has been reduced by complexation with CyDs. We prepared the HP-β-CyD/RA inclusion complex and mixed it in a moisturizing base for skin treatment. In this paper, we indicate the activities of RA/HP-β-CyD on the skin during the treatment of photo-aging and present NMR spectral data as evidence of the formation of an inclusion complex of RA and HP-β-CyD.

Experimental

Materials

All-*trans* retinoic acid (RA, tretinoin) was purchased from Sigma Co. Ltd. HP-β-CyD (DS = 6.3, 4.2) was supplied by Ensuiko Sugar Co. HP-β-CyD (DS = 4.2) was supplied by Nihon Shokuhin Kako Co., Ltd. Mono-2-*O*-hydroxypropyl-β-CyD (MHP-β-CyD; molecular weight: 1193) was obtained from Cyclo Lab. Co. Other chemicals and solvents were of analytical reagent grade.

Apparatus

^{1}H NMR and ^{13}C NMR spectroscopy was performed using a JEOL NM Lambda 500 spectrometer. NMR spectra of pure components and inclusion complexes, except for pure RA, were obtained in D_2O. The NMR spectra of RA were obtained in DMSO-d_6 at r.t. The concentrations of the components were 1.17 mg/mL (3.33 mM) for RA and 7.75 mg/mL (3.91 mM), which are 1 molar equivalent for HP-β-CyD. A small amount of acetone was added to all samples as a reference. Chemical shifts were reported in units of ppm, and were referenced to the acetone signal (2.100 ppm).

Molecular drawing

Molecular structures were optimized and drawn using the program WinMOPAC [24]; this program included MOPAC2002 as a program for semi-empirical molecular orbital calculation [25] and an exclusive program for three-dimensional visualization. The analysis method used was AM1 [26]. A Fujitsu CE/C90N (Quad 2.33 GHz CPU, 4 GB RAM) computer was used.

The clinical trial involving photo-aging therapy with topical CD tretinoin

Preparation of RA/moisturizing base

The test samples were prepared using RA (0.05 wt%), HP-β-CyD (DS = 6.3), and a moisturizing base. RA and excess HP-β-CyD were suspended in cool water, and the solution was vigorously stirred. After filtration, the filtrate was freeze-dried. One gram of the powdered RA/HP-β-CyD complex (RA: 5 wt%) and 99 g of the moisturizing base were kneaded. The formations of the moisturizing base are listed in Table 1.

Treatment protocol and evaluation of results

The anti-wrinkle functions were evaluated according to the guidelines reported in the literature [28]. Twelve female patients (average age: 58 years) applied a formulated RA complex containing HP-β-CyD tretinoin in the moisturizing base at the lateral angle of the eye on one side of the face and applied conventional RA in the same base on the opposite side in a single-blind manner. Wrinkling, inflammation, and skin elasticity were clinically evaluated prior to the study and 8 weeks after the beginning of the trial. The effect of the RA/HP-β-CD complex on human

Table 1 The formations of the moisturizing base

Ingredient	
Water	Glyceryl stearate
Phenyltrimethycone	Glyceryl isostearate PeG-60
1,3-Butylene glycol (BG)	Citric acid
Tetrahexyldecyl ascorbate	Hydroxyethylcellulose
Glycerin	Sodium hydroxide
Palm oil	Methyparabens
Batyl alcohol	
Behenyl alcohol	
Cetyl palmitate	

skin was investigated by evaluating the transepidermal water loss (TEWL). The TEWL, which is expressed in grams per square meter per hour, was measured in order to study the water barrier function of human skin. The statistical difference was determined by performing the two-sided paired *t* test.

Results and discussion

Action of tretinoin on skin

Preparation of RA/moisturizing base

A dispersion of RA with HP-β-CD in water was smoother than that in which there was no HP-β-CD. The RA/HP-β-CD moisturizing base was stable. No separation of RA in the RA/HP-β-CD moisturizing base occurred after mixing.

Evaluation of anti-wrinkle function

The RA/HP-β-CD complex was applied at the lateral angle of the eye, and the appropriate amount of the applied complex was 0.13 g, or about 1/4 fingertip units (FTU; 1 FTU = 0.5 g). The statistical difference was determined by the two-sided paired *t* test. A difference with $p < 0.05$ was considered significant. The dependence of the wrinkle area (expressed as a percentage) on time, as determined by replica analysis, is presented in Fig. 2 and Table 2. The wrinkle areas decreased by over 20% on both sides, namely, the side treated with RA containing HP-β-CD ($p = 0.046$) and that treated with RA not containing HP-β-CD ($p = 0.048$). The change was estimated as a decrease of two in the wrinkle score, which is a significant improvement. The skin elasticity was also improved to the same degree by RA ($p = 0.006$) and RA/HP-β-CyD ($p = 0.003$). Nine patients (75% of the patients) asserted that the dermal irritation caused by the RA moisturizer with HP-β-CyD was lower than that caused by the RA moisturizer without HP-β-CyD.

Table 2 Dependence of wrinkle area on time (replica analysis)

No.	Age	Wrinkle area (%)			
		RA[a]		RA/HP-β-CyD[b]	
		1st Day	8 Weeks	1st Day	8 Weeks
1	52	100.0	82.9	100.0	66.9
2	54	100.0	55.3	100.0	85.3
3	56	100.0	80.8	100.0	79.6
4	63	100.0	76.0	100.0	82.2
5	56	100.0	98.8	100.0	102.9
6	62	100.0	59.5	100.0	76.8
7	51	100.0	62.8	100.0	50.4
8	62	100.0	57.8	100.0	50.1
9	72	100.0	94.5	100.0	76.6
10	62	100.0	111.0	100.0	73.9
11	53	100.0	67.0	100.0	80.1
12	57	100.0	77.3	100.0	64.5
Average	58.3	100.0	77.0	100.0	74.1
SD			17.7		14.7
p value[c]			0.0009		0.00008

[a] RA: all-*trans* retinoic acid (tretinoin)

[b] HP-β-CyD: hydroxypropyl-β-cyclodextrin

[c] Paired *t* test

Cutaneous stimulus of RA

The effect of the HP-β-CyD inclusion complex in the RA moisturizing base on the upper arm skin was examined for 8 weeks. The concentrations of RA in moisturizing base were adjusted to 0.1 wt%. More than 10 days after the examination began, the erythema, dryness and desquamation of skin

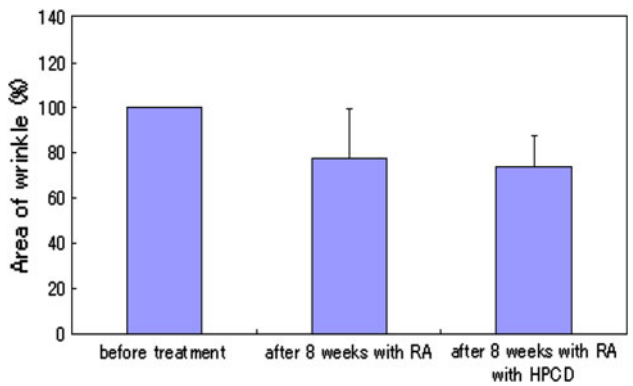

Fig. 2 Dependence of wrinkle area on time (replica analysis)

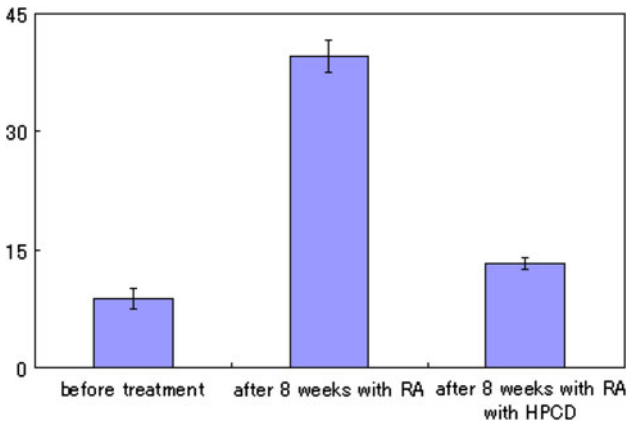

Fig. 3 Transepidermal water loss from upper arm skin within 8 weeks, the treatment using RA/HP-β-CD, the treatment using RA, and for the control

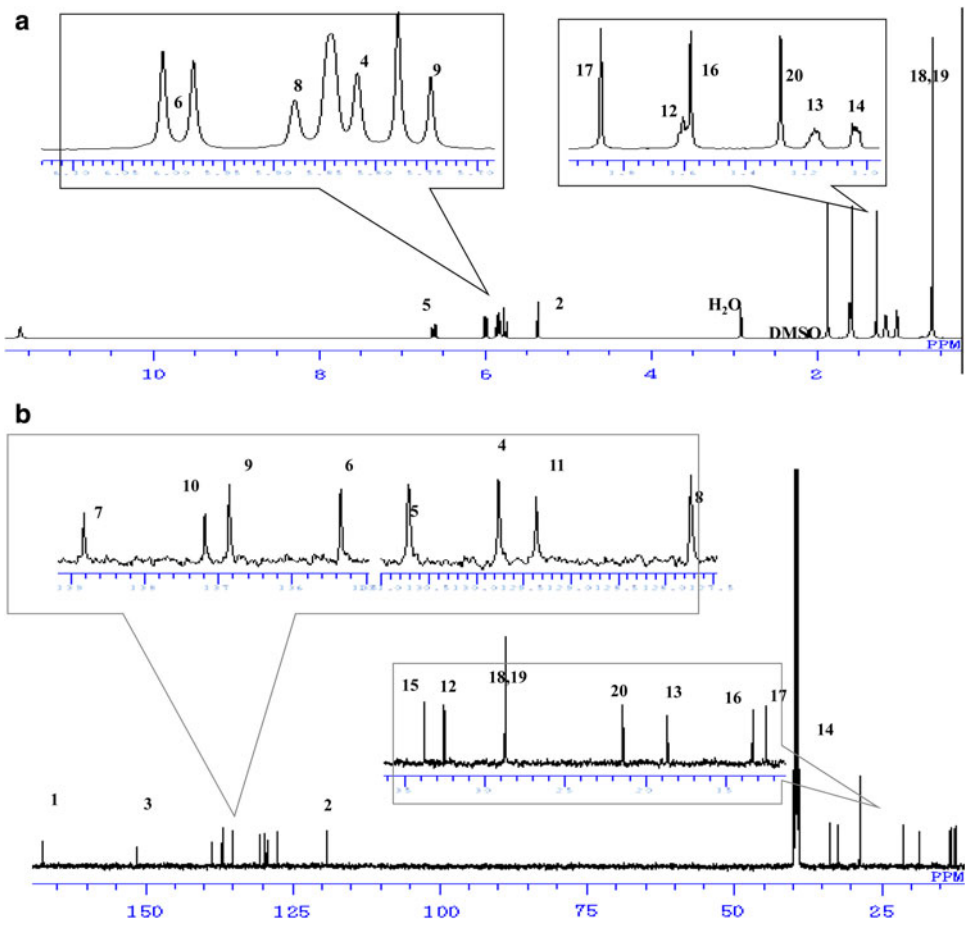

Fig. 4 ^{1}H (**a**) and ^{13}C NMR (**b**) spectra and assignment of RA signals in DMSO-d_6

treated using the RA/HP-β-CD complex were observed to be less than those of skin treated using RA. The TEWL at the site treated with conventional RA was higher than that at the site treated with the RA/HP-β-CD complex and that at the control site (Fig. 3). The above results show that the RA/HP-β-CD inclusion complex in a moisturizing base is suitable for skin treatment. The effect of RA/HP-β-CyD and that of RA without HP-β-CyD on the wrinkle scores and skin elasticity during treatment were identical; further, the cutaneous stimulus in the case of RA/HP-β-CD was lower than that in the case of conventional RA.

Structure of RA/HP-β-CyD complex

Characteristics of NMR spectra of RA and HP-β-CyD before complexation

The unambiguous assignment of as many signals in NMR spectra as possible is a prerequisite for successfully performing structural and conformational analysis. The water solubility of RA is too low for observing either H or C chemical shifts in D_2O. The RA protons and carbons in DMSO-d_6 were assigned using H–H and H–C COSY spectral data (Fig. 4). Figure 5 shows the ^{1}H NMR spectra of HP-β-CyD (DS = 4.2 (a) and 6.3 (b)) in D_2O. The signals due to the CyD moiety were broadened because the sample was a mixture. It was possible to assign only the signals around 5 ppm that correspond to the glycosidic anomeric protons. In order to assign the chemical shifts of HP-β-CyD, the ^{1}H NMR spectrum of mono{2-*O*-(2′-hydroxypropyl)} β-CyD(MHP-β-CyD) in D_2O was obtained, as shown in Fig. 6. Well splitted signals due to the methyl and methylene groups are observed at 1.04 and 3.76 ppm, respectively. The signals due to the CyD moiety also had reasonable area ratios. Using H–H COSY methods, the weak signals at 5.1, 3.9, 3.8, 3.6, and 3.4 ppm were assigned to the anomeric H1′, H3′, H6′, H5′, and H2′ protons of the modified glucose unit of the CyD ring. The H4′ proton was observed at 3.5 ppm only as a cross peak between H3′ and H5′. The glycosidic proton on hydroxypropyl-substituted glucose at secondary sites shifted to low fields. The ratio of the areas of the peaks at 5.1 and 4.96 ppm was 1:6. From these results, it was concluded that the signals of the methyl and methylene groups on HP-β-CyD were located at 1.05 and 3.78 ppm together, respectively. Glycosidic protons were observed at 5.13 and 4.97 ppm. The values that are lower and higher than the

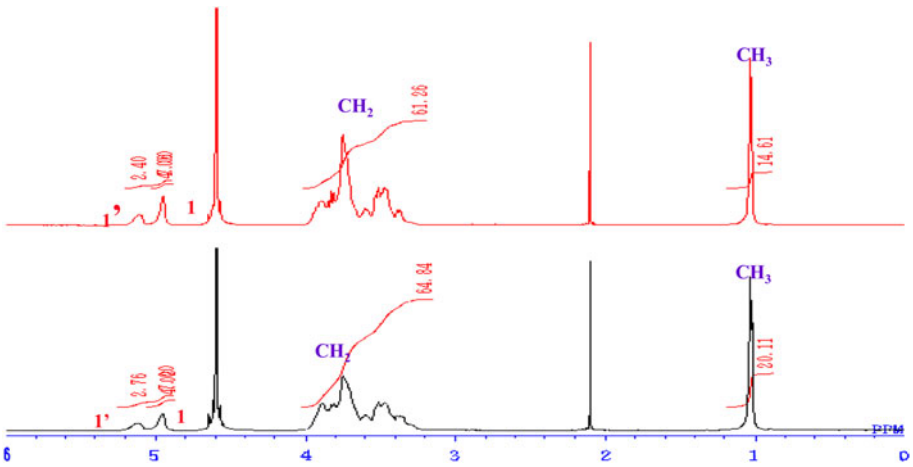

Fig. 5 ^{1}H spectra and assignment of HP-β-CyD (**a** DS = 4.2, **b** DS = 6.3) in D_2O

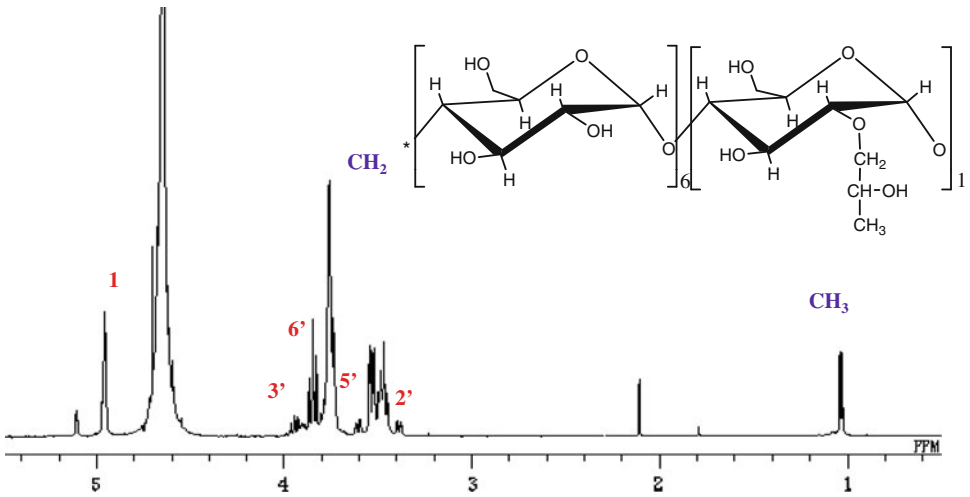

Fig. 6 ^{1}H NMR spectra and assignment of mono{2-*O*-(2′-hydroxypropyl)} β-CyD(MHP-β-CyD) signals in D_2O

chemical shifts of MHP-β-CyD are attributed to anomeric protons on the glycosyl group at the secondary site modified by a hydroxypropyl group and to anomeric protons on the unmodified secondary site, respectively. The MS, which is defined as the average number of substitutions per glucose ring and is one-seventh of DS, can be obtained by calculating the ratio of the signal due to the methyl group of the hydroxypropyl group at 1 ppm divided by three times the area of the glycosidic hydrogen signal at 5 ppm [27]:

$$MS = DS/7 = A_1/3A_2$$

where A_1 is the area of the signal due to the three protons of the methyl groups that are part of the hydroxypropyl groups, and A_2 is the area of the signal due to the glycosidic protons. The sum of the two signals is used as the value of A_2.

$$A_2 = A_{22} + A_{20}$$

where A_{22} is the area under the peak due to the glycosidic protons in the presence of hydroxypropyl groups on the secondary hydroxyl groups, while A_{20} is the area under the peak due to glycosidic protons in the absence of a hydroxypropyl group. When A_2 is 7, A_{22} is the average number of the glucose that is substituted on the secondary position. In Fig. 5a, A_1 = 14.61, A_{22} = 2.60, and A_{20} = 4.40, and in Fig. 5b, A_1 = 20.11, A_{22} = 2.85, and A_{20} = 4.15. The MS values of HP-β-CyD were 0.70 and 0.95; the DS values were 4.9 and 6.6 close to 4.2 and 6.3, respectively. Where are the other hydroxypropyl groups substituted? The substitution pattern of HP-CyD has been studied in detail [28, 29]. For low average molar substitution (DS < 5) the substitution occurs almost exclusively on the CyD ring, while an increase in DS results in an increase in the probability that the (2-hydroxy)-propylating agent will react with the hydroxyl groups of the HP substituents of the CyD ring, and this will result in the formation of (oligo)propylene glycol chains instead of the (2-hydroxy)-propyl groups. Contrary to common belief, the (2-hydroxy)-propyl substituents for lower DS values are located mainly on the *O*-2 positions and not on the *O*-6 positions [30]. It is suggested that the HP-β-CyD used in

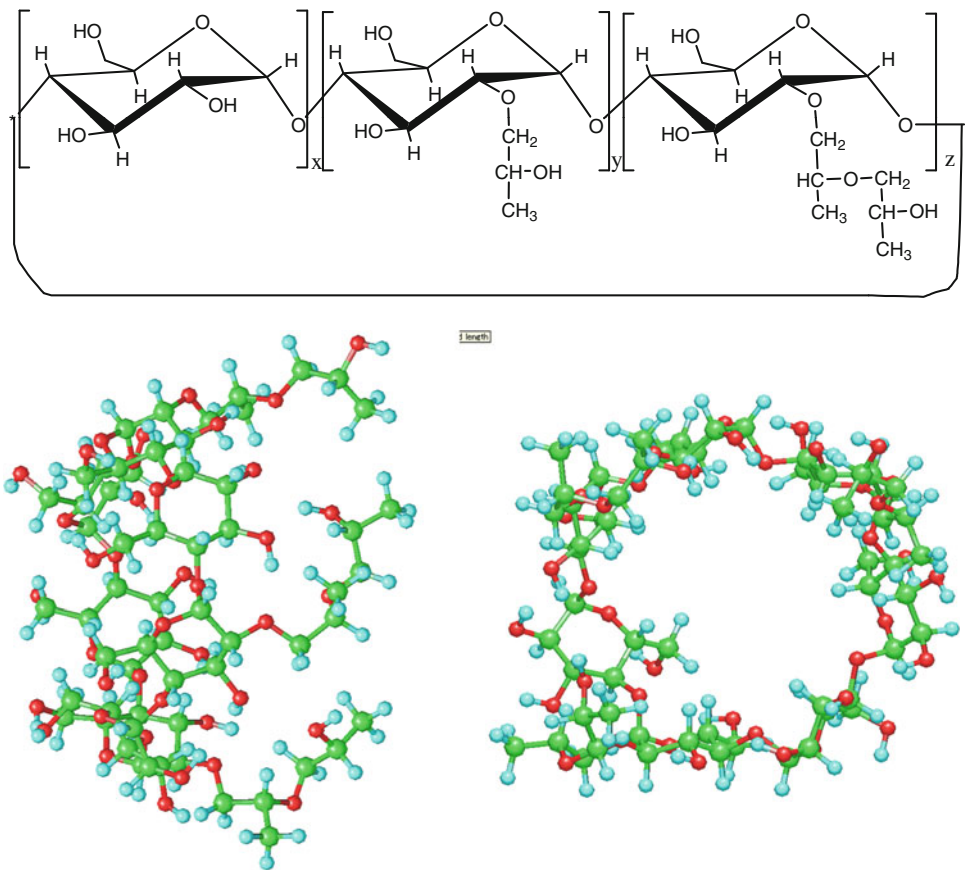

Fig. 7 Possible HP-β-CyD structure; top view (*right*) and side view (*left*)

this investigation has three di- and/or tripropylene glycol chains on the secondary site of CyD (Fig. 7).

The evidence for inclusion and the structure of RA/HP-β-CyD

When an inclusion complex is formed, the upfield shift of the proton of the guest molecule with CyD, a change in the chemical shifts of the proton of the H3 and H5 of the CyD with the included guest molecular or NOE cross peaks of H3 and H5 protons of CyD is observed; however, both observations cannot be made in the case of the RA/HP-β-CyD system. It was possible to observe the chemical shifts of RA in D_2O, because of getting water-solubility. The ^1H chemical shifts of RA with 1 and 2 molar equivalents of HP-β-CyD (DS = 6.3) in D_2O indicated that the resonances due to protons at positions 2, 4, 5, 6, 3, 11, and 12 were shifted upfield, those due to protons at positions 1 and 13 were shifted slightly downfield, and those due to protons at positions 7, 8, and 9 on the cyclic structure experienced no shift in the absence of CyD in DMSO-d_6. The chemical shift pattern did not depend on the concentration of HP-β-CyD. Unfortunately, the chemical shift due to methylene groups at positions 14 and 15 overlapped with that due to the hydroxypropyl group on HP-β-CyD, and hence information on the interaction between both ends of the molecule could not be obtained. ^1H homonuclear NOE measurement is an effective technique for obtaining information about through-space interactions among proton nuclei within 0.4 nm of each other. NOE magnitudes depend on the effective correlation times characterizing overall molecular tumbling and intermolecular motion. Since the NOE in the rotating frame (ROE) is always positive, the ROESY spectrum is usually chosen in cyclodextrin studies [31–32]. Whether the ROESY method or NOESY method is used should depend on the molecular motion. The resonance due to H3 and H5 proton of HP-β-CyD was broadened. The resonance peak due to the methyl group of the hydroxypropyl group overlapped with that due to methyl group of RA. The resonances due to the methylene protons bound to the second carbon occurred simultaneously at 3.78 ppm. The cross peaks between the protons of methylene on the hydroxypropyl group and RA, rather than the cross peaks between H3 or H5 protons and RA were observed in the NOESY spectrum of the RA/HP-β-CyD complex, and this suggests that RA remained inside CyD. In other word, the cross peak between the protons of methylene on the hydroxypropyl group and RA protons is the only direct evidence for inclusion. The cross peaks appeared with the 16, 17 and 13, 13′ protons, not with the

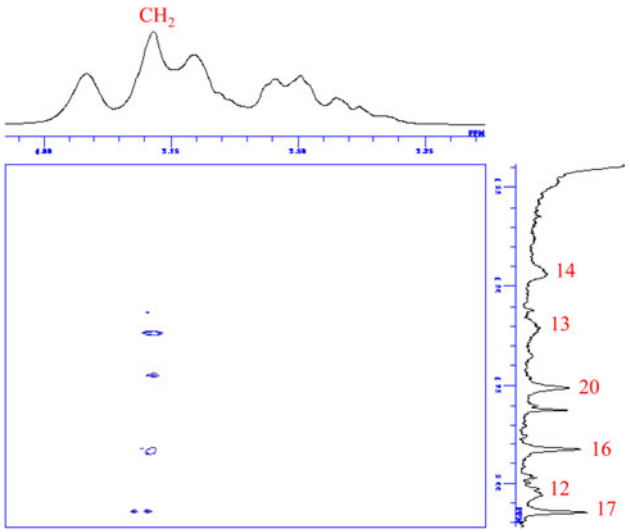

Fig. 8 NOESY spectra of RA/HP-β-CyD in D_2O

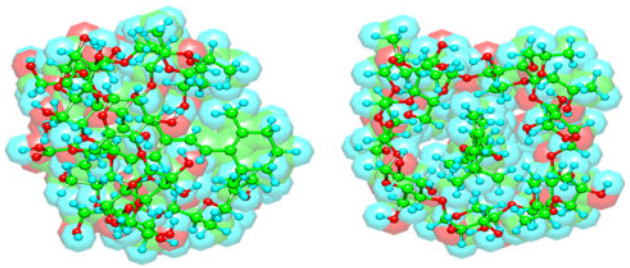

Fig. 9 Possible RA/HP-β-CyD structure; top view (*right*) and side view (*left*)

4, 6, 8, 9, 12, and 12′ protons (Fig. 8). The experimental results helped to determine the average structure of the inclusion complex. Figure 9 shows examples of the complex structure, which was drawn using MOPAC. It was found that in the average structure of HP-β-CyD, three dipropylene glycol chains are introduced at the secondary site. Methylene groups exist at two different depths of CyD. As a result, in the case of RA, the cross peaks correspond to different positions. The inner diameter of the HP-β-CyD cavity is equal to that of the native β-CyD cavity; however, the depths of the cavities are different. The hydroxypropyl arms fit the RA molecule; this explains why the second CyD could not get closer to the RA molecule.

Conclusions

In clinical trials of the RA/HP-β-CyD complex (0.05 wt%), the wrinkle areas observed in 12 women decreased by over 20% within 8 weeks, and no skin irritation was reported. HP-β-CyD with oligopropylene glycol at the secondary site includes RA, and covers the double bond chain of RA, as evidenced by the NOE between methylene protons on the hydroxypropyl chain and RA. The above results suggest that the inclusion of the RA/HP-β-CyD complex in a moisturizing base will help to realize new perspectives in skin rejuvenation therapy.

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