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Characterization of inclusion complexes of betamethasone-related steroids with cyclodextrins using high-performance liquid chromatography

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Abstract

HPLC was used to study the inclusion complexes formed between various β- and γ-cyclodextrins and a series of corticosteroids related to betamethasone. Apparent association constants were measured in acetonitrile–water for a set of 13 steroids. An increase in the stability of the steroid–cyclodextrin complex is observed at lower concentrations of acetonitrile. The effects of the nature of the halide at the 9-position, the location of a double bond within the C-ring, substitution at the 9- and 11-positions, and modification of the D-ring of the steroid backbone were studied. The 11- and 17-positions were found to be critically involved in the inclusion process. Larger apparent association constants were obtained with γ-cyclodextrin (γ-CD) than with β-cyclodextrin (β-CD) due to the increased diameter of the γ-CD cavity. Van't Hoff plots were constructed to examine the thermodynamic properties of the inclusion process. Plots constructed using retention factors were found to be nonlinear when γ-CD was present in the mobile phase. This is due to an increase in the strength of the inclusion complex as temperature decreases. Plots constructed using apparent association constants were linear, indicating that the mechanism of inclusion does not change over the range of temperatures studied (10 to 80°C). Enthalpy–entropy compensation was observed for 11 of the 13 steroids studied. The usefulness of cyclodextrins to achieve the separation of steroids in HPLC is discussed and a practical application for the analysis of a steroid and three potential impurities is described.

Keywords: Inclusion complexation; Betamethasone; Steroids; Cyclodextrins

1. Introduction

Cyclodextrins are cyclic oligo-saccharides consisting of six or more α-1,4-linked D-glucopyranose units. They exhibit the ability to form highly selective inclusion complexes with a variety of guest molecules [1,2]. This characteristic has led to the development of commercial stationary phases for both liquid and gas chromatography. A wide variety of native and derivatized cyclodextrins are available

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for use as mobile phase additives in HPLC and as run buffer additives for capillary electrophoresis (CE). While the majority of the published research using cyclodextrins in chromatography focuses on the separation of enantiomers, cyclodextrins demonstrate exceptional utility for separating closely related compounds including geometrical structural isomers [3–6].

Betamethasone (**1**) is a synthetic adrenocorticosteroid used in pharmaceutical products for its anti-inflammatory, anti-pruritic, and vasoconstrictive actions. Numerous researchers have investigated the interactions of **1** and other related steroids with various cyclodextrins. Cyclodextrins have been shown to improve bioavailability of steroids in topical formulations [7] and in oral formulations [8], to enhance the solubility [9,10] and dissolution rates [11,12] of steroids, and to increase the stability of formulations for some steroids [13–16]. Cyclodextrins have also been used as mobile phase modifiers in HPLC applications for the analysis of steroids [17–20]. Highly selective β-cylcodextrin-based molecular imprinted polymers have been synthesized using steroids as a template [21,22]. A modified cyclodextrin has been used to control the hydroxylation of an androstanediol derivative through inclusion [23]. The first effort to study the interactions of steroids with various cyclodextrins in a comprehensive fashion was made by Uekama et al. [24]. Solid-state complexes of 18 steroids with various cyclodextrins were characterized and apparent association constants (K_f) were determined for each steroid using phase solubility techniques. The investigators concluded that stronger complexes were obtained for steroids that were more hydrophobic, with inclusion occurring primarily at the A–B ring of the steroid. Shimada et al. later found that the stability of inclusion could be related in part to the substitution at the 3-position of the steroid [25]. K_f was found to increase in the order 3-β-OH>3-α-OH>3-oxo. This data conflicts with the earlier findings of Uekama since the addition of hydroxyl groups causes a steroid to be less hydrophobic. Work by Liu et al. describes the formation of a complex having two cyclodextrins per steroid molecule [26]. This can occur when the concentration of cyclodextrin is high relative to the concentration of a steroid present in solution. They propose that the C–D ring of a steroid is the first to be included into β-CD and then the A–B ring is included into a second β-CD. Others report that this is not the case and that inclusion occurs primarily at the A–B rings as described by Uekama [10,27].

Other research groups have attempted to relate structural features of various steroids to the strength of the complexes those steroids form with cyclodextrins. Lam and Malikin found that the orientation of the 5-position hydrogen affects the strength of complexation with cyclodextrins [28]. Additionally, Djedaini and Perly found that substitution at the 11-position affects complex stability and stoichiometry [29]. Mathematical models describing particular aspects of steroids such as hydrophobicity (determined from migration in thin-layer chromatography), steric parameters, and partition coefficients have been used to examine the inclusion process of steroids into cyclodextrins with little success [30–32].

The goal of our research is to probe the nature of the steroid–cyclodextrin inclusion complex using HPLC to measure K_f. HPLC has been shown to be a powerful tool for studying molecular interactions [33]. Inclusion complexes of steroids with cyclodextrins have been studied using HPLC, with two different methods being used to determine K_f [25,27,34,35]. The Hummel–Dreyer method [36], adapted to study inclusion, employs a mobile phase containing the guest molecule. A solution of cyclodextrin is injected. Two peaks are observed: an unretained positive peak and a negative peak at a retention time equal to that of the guest injected into the system can be quantified to determine the K_f. An alternate method described by Fujimura et al. [37] calculates K_f from the change in retention factor obtained using mobile phases containing varying concentrations of cyclodextrin, assuming a 1:1 stoichiometry is observed for the complex. Armstrong et al. expanded this model to include higher complex stoichiometry [38]. Others have described similar methods for determining K_f values from the change in retention factors as the concentration of cyclodextrin in the mobile phase is changed [39,40]. Fujimura's method was chosen for this work because K_f values can be determined for multiple steroids in one injection. If the Hummel–Dreyer method was used then a separate mobile phase would need to be

prepared for each steroid being studied. This was not practical given the limited availability of several of the steroids. Three test mixtures were used to analyze a series of structurally similar steroids chosen to determine key regions of interaction with various cyclodextrins. The thermodynamics of the inclusion phenomenon were investigated using the Van't Hoff method [41].

2. Experimental

2.1. Apparatus

The HPLC systems used were 1050 Series HPLCs (Agilent Technologies, Palo Alto, CA, USA) with in-line degassers. One system was equipped with a variable-wavelength detector while the other had a diode-array detector. Symmetry C_{18} columns, 150 mm×4.6 mm I.D., 5 μm particle size (Waters Corporation, Milford, MA, USA) were used. A refrigerated circulating bath (Neslab Instruments, Portsmouth, NH, USA) was used to control column temperature. A YMC J'Sphere H-80 column, 150 mm×4.6 mm I.D., 4 μm particle size (Waters Corporation) was used for the separation of a steroid from several impurities. Depending on the organic composition of the mobile phase, flow-rates of 1.0 or 1.8 mL/min were used to maintain reasonable run times. The steroids studied possess a UV absorption maximum at a wavelength of 240 nm, so this wavelength was used for detection. Turbochrom 4.0 (Perkin-Elmer, Norwalk, CT, USA) was used to acquire and process the chromatographic data.

2.2. Chemicals

2.2.1. Mobile phase preparation

HPLC-grade acetonitrile, methanol, and water (Fisher Scientific, Springfield, NJ, USA) were used without further purification. β-CD, hydroxyethyl-β-cyclodextrin (HE-β-CD; average degree of substitution 5), hydroxypropyl-β-cyclodextrin (HP-β-CD; average degree of substitution 5.9), and γ-CD were purchased from Cerestar USA, Inc. (Hammond, IN, USA). Hydroxypropyl-γ-cyclodextrin (HP-γ-CD; average degree of substitution 4.6) was purchased from Fluka (Milwaukee, WI, USA). Mobile phases were prepared by mixing the appropriate amount of acetonitrile and water and then dissolving cyclodextrin in the resulting solution with the aid of a sonic bath (Fisher Scientific). Mobile phases containing cyclodextrin were filtered through two Metrigard glass fiber filters (Gelman Sciences, Ann Arbor, MI, USA).

2.2.2. Steroid solutions

The Schering-Plough Research Institute (Kenilworth, NJ, USA) generously provided the 13 corticosteroids studied.

Stock solutions containing approximately 2 m*M* of each steroid were made in acetonitrile except for beclomethasone (**3**), the 9-bromo analogue (**4**), and the 9,11-epoxide alcohol compound (**5**) (see Fig. 1) which were prepared in methanol. Test mixtures were prepared by transferring 1.0 mL of the stock solutions to 50 mL volumetric flasks and diluting to volume with acetonitrile–water (35:65, v/v). Three test mixtures containing different steroids were prepared in this way. Betamethasone (**1**), the 9-bromo analogue (**4**), the $\Delta^{11,12}$ compound (**6**), betamethasone 21-acetate (**8**), the 9,11-epoxide, 21-acetate compound (**10**), and betamethasone 21-monopropionate (**11**) were added to one flask. Beclomethasone (**3**), the 9,11-epoxide alcohol compound (**5**), betamethasone 17-monopropionate (**12**), and betamethasone 11,21-diacetate (**13**) were added to a second flask. Dexamethasone (**2**), the $\Delta^{9,11}$ compound (**7**), and dexamethasone 21-acetate (**9**) were added to a third flask. The identity of each peak was confirmed by injecting solutions containing the individual steroid. The 9-bromo analogue (**4**) was found to irreversibly convert to the 9,11-epoxide alcohol compound (**5**) in solution over a period of about 24 h at room temperature. Betamethasone 17-monopropionate (**12**) converts more slowly to betamethasone 21-monopropionate (**11**) in solution. The change in concentration of these steroids in the test mixtures was not observed to affect the retention factor for either compound in the presence or absence of cyclodextrin in the mobile phase.

3. Results and discussion

Fig. 1 shows the chemical structures of the

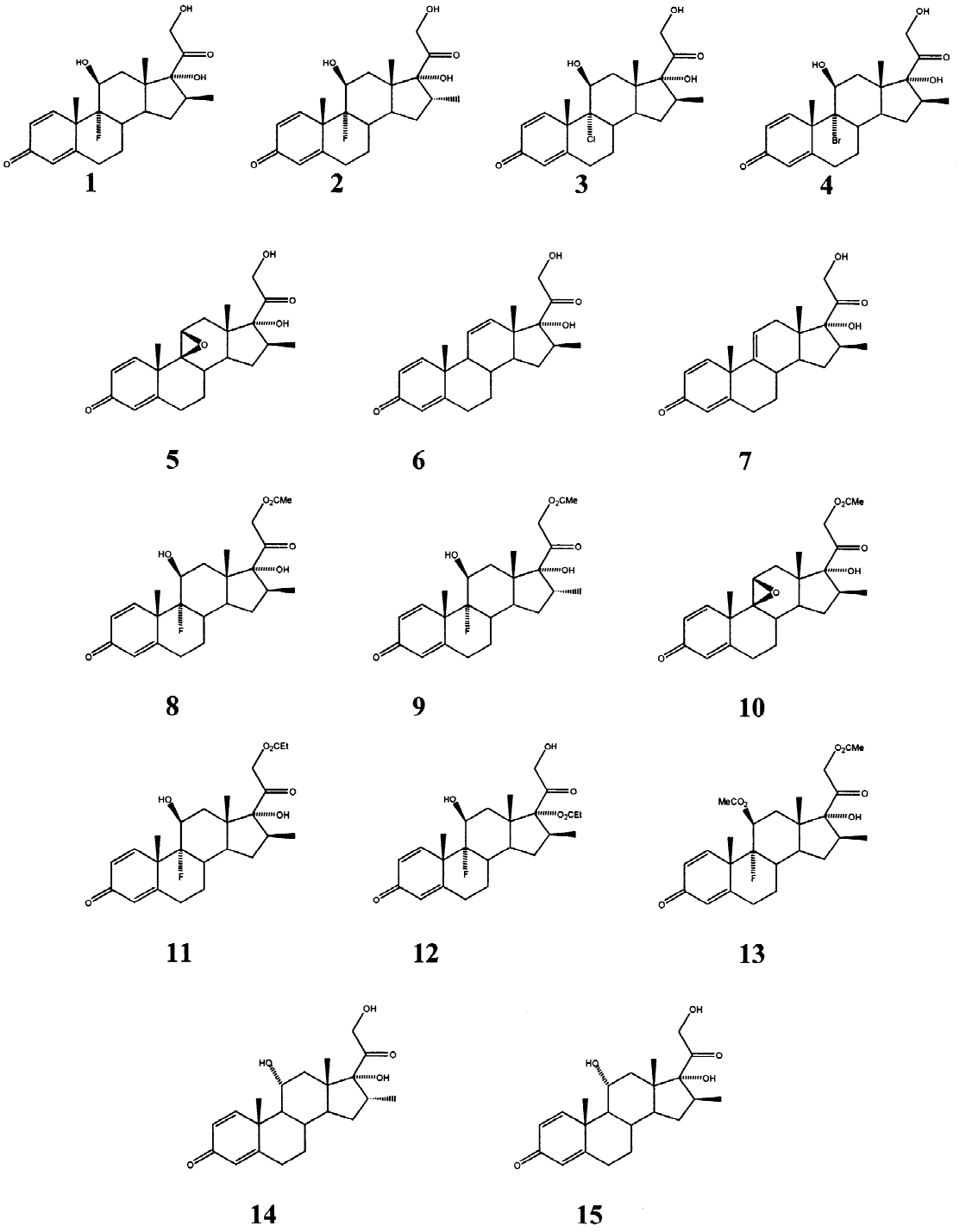

Fig. 1. Chemical structures of steroids analyzed: betamethasone (**1**); dexamethasone (**2**); beclomethasone (**3**); 9-bromo analogue (**4**); 9,11-epoxide alcohol (**5**); $\Delta^{11,12}$ compound (**6**); $\Delta^{9,11}$ compound (**7**); betamethasone 21-acetate (**8**); dexamethasone 21-acetate (**9**); 9,11-epoxide, 21-acetate (**10**); betamethasone 21-monopropionate (**11**); betamethasone 17-monopropionate (**12**); betamethasone 11,21-diacetate (**13**); epimer 1 (**14**); epimer 2 (**15**).

steroids selected for this study. The series was developed to illustrate the effect of changing various regions of a steroid on complex formation with cyclodextrin. The effect of the halide in the 9-position was investigated using betamethasone (**1**), beclomethasone (**3**), and the 9-bromo analogue (**4**). These compounds contain an α-fluorine atom, an α-chlorine atom, and an α-bromine atom in the 9-position, respectively. Dexamethasone (**2**), an epimer of betamethasone (**1**), was also investigated. Dexamethasone (**2**) differs from betamethasone (**1**) only by the orientation of the 16-methyl group. It was chosen to determine if such a small change on the D-ring of the steroid structure would impact the strength of the inclusion complex formed with cyclodextrin. Betamethasone 17-monopropionate (**12**), betamethasone 21-monopropionate (**11**), and betamethasone 21-acetate (**8**) are alkyl ester derivatives of betamethasone (**1**). They were included in this study to determine the significance of the 17- and 21-positions in the inclusion process. If inclusion occurs at the A–B rings of the steroid, then the structure of the C-ring and the nature of the groups located at the 11-position should have a dramatic impact on the inclusion process because this region should closely interact with the hydroxyl rim of the cyclodextrin. To determine if this is the case, the 9,11-epoxide alcohol compound (**5**) and the 9,11-epoxide, 21-acetate compound (**10**) were used for comparison to betamethasone (**1**) and betamethasone 21-acetate (**8**), respectively. Betamethasone 11,21-diacetate (**13**) was also investigated. The $\Delta^{9,11}$ compound (**7**) and the $\Delta^{11,12}$ compound (**6**) possess unsaturated C-rings. Dexamethasone 21-acetate (**9**) was also investigated.

Retention factors decrease when cyclodextrin is added to a mobile phase. This is due to the formation of inclusion complexes between cyclodextrin and the analytes being chromatographed. Complexation increases the solubility of the analyte in the mobile phase and thus decreases its residency time in the HPLC column. K_f values for the steroids described above were determined by HPLC with a variety of β- and γ-CDs using the method described by Fujimura et al. [37]. Changes in retention factors for each steroid were monitored as the concentration of cyclodextrin in the mobile phase was varied. The following expression is true when the inclusion complex has no retention in the LC system:

$$\frac{1}{k} = \frac{[(CD)_T]K_f}{k_0} + \frac{1}{k_0} \quad (1)$$

where k is the retention factor at a particular concentration of cyclodextrin, $(CD)_T$, and k_0 is the retention factor in the absence of cyclodextrin. For a compound with a 1:1 stoichiometry with cyclodextrin, a plot of $1/k$ vs. $[(CD)_T]$ yields a straight line which has a slope equal to K_f/k_0. Since retention factors are used, the exact void volume of the HPLC system and column being used need to be known for accurate results to be achieved. For example, a 10% variation in the void volume results in a 25% variation in the K_f obtained for the 9-bromo analogue (**4**) due to its low capacity in the system, especially at high concentrations of cyclodextrin. Comparisons can still be made between K_f values obtained using the same system if inaccuracies exist in the measurement of the void volume and the effects of structural features of a set of compounds can still be evaluated. However, if comparisons are to be made between different techniques of determining K_f (such as phase solubility, spectroscopic methods, etc.) or between results generated by different labs for a similar set of compounds then an error in void volume will confound any conclusions that might be made. For this reason, the minor disturbance method described by Kazakevich and McNair was used to determine the exact void volume of the HPLC column used [42]. The void volume for the entire HPLC system was found to be 1.58 mL using this method. The void volume determined from the baseline perturbation after injection of a dilute solution of acetone was 1.64 mL.

K_f values obtained using γ-CD are shown in Table 1. Relative standard deviations (RSD) ranged from 1.0 to 7.0% with an average RSD of 2.8% for all 13 steroids. This data was obtained on five different days over a 3-month period using two different HPLC systems and three different columns. The steroids were analyzed in three different solutions to minimize peak overlap as the cyclodextrin concentration was increased. Fig. 2 shows chromatograms obtained for a solution containing all 13 steroids being studied using mobile phases prepared in the presence and absence of γ-CD. The elution order of several peaks changes as cyclodextrin is added to the mobile phase. The 9-bromo analogue (**4**) and beclomethasone (**3**) elute after betamethasone (**1**) when

Table 1
K_f values measured for γ-CD complexes in acetonitrile–water (35:65, v/v)

Component	Set 1	Set 2	Set 3	Set 4	Set 5	Average	SD[a]
9-Bromo analogue	977	937	970	895	970	950	34.4
Beclomethasone	621	604	617	586	634	612	18.2
Betamethasone	213	208	211	206	221	212	5.8
Dexamethasone	216	213	214	209	223	215	5.1
9,11-Epoxide alcohol	142	144	144	141	150	144	3.5
$\Delta^{9,11}$ Compound	151	150	151	148	154	151	2.2
$\Delta^{11,12}$ Compound	93	101	103	101	107	101	5.1
Betamethasone 21-acetate	148	147	149	146	151	148	1.9
Dexamethasone 21-acetate	146	147	148	147	150	148	1.5
9,11-Epoxide, 21-acetate	178	178	179	178	182	179	1.7
Betamethasone 17-monopropionate	100	102	102	101	106	102	2.3
Betamethasone 21-monopropionate	118	127	128	126	130	126	4.6
Betamethasone 11,21-diacetate	42	48	47	48	51	47	3.3

[a] SD, standard deviation.

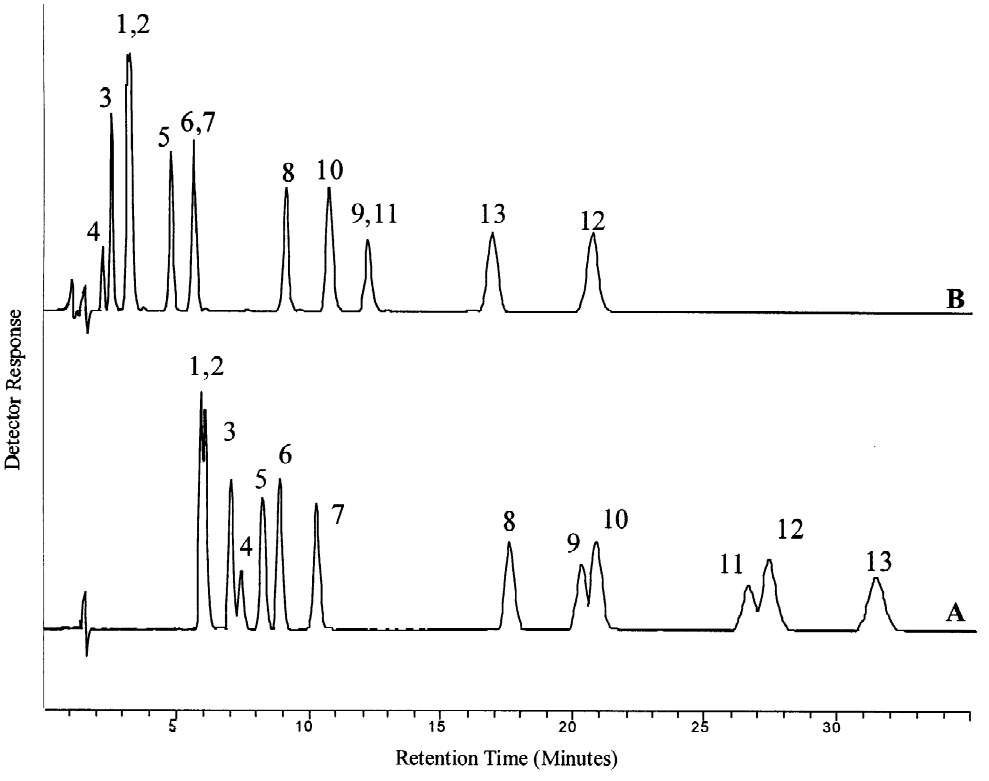

Fig. 2. Chromatograms of 13 steroids without cyclodextrin in mobile phase (A) and with 7.5 m*M* γ-CD in mobile phase (B). Peaks: 1=betamethasone, 2=dexamethasone, 3=beclomethasone, 4=9-bromo analogue, 5=9,11-epoxide alcohol, 6=$\Delta^{9,11}$ compound, 7=$\Delta^{11,12}$ compound, 8=betamethasone 21-acetate, 9=betamethasone 17-monopropionate, 10=dexamethasone 21-acetate, 11=9,11-epoxide, 21-acetate, 12=betamethasone 11,21-diacetate, 13=betamethasone 21-monopropionate.

no cyclodextrin is present, but elute before betamethasone (**1**) when γ-CD is added to the mobile phase. It is this phenomenon that makes cyclodextrins such useful mobile phase additives for the development of practical HPLC methods. The change in elution order is due to differences in K_f values for each component being analyzed. Large K_f values translate to a greater mobile phase residency and thus a shorter retention on the HPLC column. The largest K_f values for all of steroids studied were observed with γ-CD, except for betamethasone 21-monopropionate (**11**), which yielded a higher value of K_f with HP-γ-CD.

Table 2 lists K_f values obtained for each of the steroids studied with β-CD, HE-β-CD, HP-β-CD, γ-CD, and HP-γ-CD. The low values obtained for β-CD and its derivatives indicate that the size of the cavity is too small to allow a strong interaction with the steroid. The poor fit of steroids into the β-CD cavity has been reported by other researchers [24,35]. The length of a typical steroid is about 12 Å, whereas the diameter of the secondary hydroxyl rim of β-CD and γ-CD is 6.5 and 8.3 Å, respectively [35]. This size restriction prevents the entire steroid from entering the cyclodextrin cavity.

The K_f values calculated for the cyclodextrins and steroids studied provide information about the nature of the inclusion complex formed. Relating structure to the strength of inclusion can suggest possible inclusion geometry. However, spectroscopic data is required for the absolute determination of the structure of the inclusion complex. The primary objective was to determine which region of the steroid is included in the cyclodextrin cavity for betamethasone-related steroids. Complexes could be proposed with either the A–B rings or the C–D rings contained within the cyclodextrin cavity. No 1:2 complexes were observed for the 13 steroids investigated at the cyclodextrin concentrations and acetonitrile concentration which were used for this work.

The halide in the 9-position of the steroid has a tremendous impact on the strength of the inclusion complex. Betamethasone (**1**) has a fluorine atom present in the 9-position and is observed to have the lowest K_f of the halide series studied. The 9-bromo analogue (**4**) was found to have the highest K_f. For the halide in the 9-position, the order of decreasing K_f was found to be Br>Cl>F. Other researchers have observed the preferential inclusion of bromine-containing compounds into the cyclodextrin cavity [43]. Connors and Pendergast propose that this occurs due to the polarizability of the bromine atom [43]. Bromine is more polarizable than chlorine and thus it is more able to disperse its electrons over a larger area. This allows for a stronger association with the glycosidic bonds that make up the interior of the cyclodextrin cavity. This evidence supports the inclusion of the A–B rings of the steroids into the cyclodextrin cavity. The trend observed for the halide in the 9-position would indicate that the halide is located within the cyclodextrin cavity. The opposite trend would be observed if the C–D rings were included into the cavity. In this case, the halide would be interacting with the hydroxyl rim of the

Table 2
K_f values obtained using five different cyclodextrins in acetonitrile–water (35:65, v/v)

Compound	β-CD	HE-β-CD	HP-β-CD	γ-CD	HP-γ-CD
9-Bromo analogue	28	26	28	950	667
Beclomethasone	39	32	34	612	454
Betamethasone	27	21	19	212	180
Dexamethasone	22	17	17	215	173
9,11-Epoxide alcohol	14	7	9	144	119
$\Delta^{9,11}$ Compound	39	20	20	151	145
$\Delta^{11,12}$ Compound	28	20	23	106	84
Betamethasone 21-acetate	16	14	18	148	142
Dexamethasone 21-acetate	15	13	16	148	132
9,11-Epoxide, 21-acetate	9	6	11	179	172
Betamethasone 17-monopropionate	1	2	6	102	70
Betamethasone 21-monopropionate	16	15	19	126	128
Betamethasone 11,21-diacetate	−2	2	5	47	35

cyclodextrin. It might then be expected that a very electronegative halide such as fluorine would stabilize the complex due to hydrogen bonding with the secondary hydroxyl groups of cyclodextrin. A less electronegative halide such as bromine would be expected to have a lower K_f because it could not participate in strong hydrogen bonding. The data show that this is the opposite of what is observed; therefore, inclusion must be occurring primarily at the A–B ring. While electrostatic forces are critical in determine the strength of inclusion, steric factors are also very important. It is obvious from the low K_f values generated for β-CD that its cavity is too small to properly fit the steroids screened. The cavity of β-CD is too constricting to allow the bromine group of the 9-bromine analogue (**4**) to enter. This explains the trend Cl>Br~F for the halide in the 9-position with β-CD and its derivatives. No significant differences were observed between the K_f values for betamethasone (**1**) and dexamethasone (**2**) for any of the cyclodextrins studied.

The results obtained using HP-γ-CD provide additional insight as to how the steroid molecule resides in the γ-CD cavity. Hydroxypropyl groups are added to the primary alcohol groups located at the narrower end of the cyclodextrin cavity; therefore, the depth of the cyclodextrin cavity is effectively increased. A decrease in K_f of about one-third is observed for most steroids when HP-γ-CD is used as the host when compared to K_f values when γ-CD is used. It is interesting to note that the compounds which do not show this decrease in K_f all have alkyl ester groups at the 21-position. Assuming that the A–B rings are included in the cyclodextrin cavity, it seems likely that the 21-OH of the steroids studied here is interacting with the secondary hydroxyl groups along the wider rim of the cyclodextrin cavity resulting in an increase in the strength of the inclusion complex. When the steroids penetrate further into the cyclodextrin cavity, as with HP-γ-CD, this interaction may be disrupted resulting in a weaker complex.

Another steroid series that demonstrates the effect of sterics is betamethasone (**1**), betamethasone 21-acetate (**8**), and betamethasone 21-monopropionate (**11**). A decrease of about 60 M^{-1} in K_f is observed when the alcohol in betamethasone (**1**) is replaced with a methyl ester. The addition of another methyl group to generate an ethyl ester results in a further decrease of about 20 M^{-1}. The larger increase observed for the replacement of 21-OH for 21-acetate relative to the replacement of 21-acetate with 21-propionate could be due to the additional loss of hydrogen bonding with the secondary hydroxyl groups of cyclodextrin when the 21-OH group is lost. A more dramatic decrease in K_f is observed when this bulk is placed in the 17-position. The replacement of the alcohol in the 17-position of betamethasone (**1**) with an ethyl ester results in a drop of approximately 110 M^{-1}. These data suggest that the 17-position is in closer proximity to the cyclodextrin molecule than the 21-position.

The steroid C-ring is also intimately involved with the cyclodextrin. A comparison of betamethasone (**1**) and the 9,11-epoxide alcohol compound (**5**) shows that a 68 M^{-1} decrease in K_f occurs when the proton in the 11-position is not available for hydrogen bonding. Comparing betamethasone 21-acetate (**8**) to betamethasone 11,21-diacetate (**13**) shows that the presence of an acetate group in the 11-position results in a loss of 101 M^{-1} in K_f. This data suggests that the 11-position is interacting with the secondary hydroxyl groups along the rim of the cyclodextrin. This supports the results of Djedaini and Perly who found that the presence of an alcohol at the 11-position of prednisolone promoted a strong 1:1 complex with β-CD while an oxo group at the 11-position of prednisone resulted in the formation of a weaker 1:1 complex [29]. These authors also observed 1:2 complexes for steroids with no hydrophilic groups on the D-rings. These results point to a complex where the A-ring and B-ring of the steroids studied fit into the cyclodextrin cavity leaving the C-ring and D-ring to interact with the rim of the cyclodextrin. Spencer and Purdy propose a similar model for inclusion of a series of steroids related to estrone [27]. They observed significant changes in K_f when a hydroxy group was added to the 3- or 5-position of the A-ring.

Interpreting the results obtained for the $\Delta^{9,11}$ compound (**7**) and the $\Delta^{11,12}$ compound (**6**) poses an interesting challenge. The presence of a double bond in the 9,11-position disrupts the *cis*-configuration of the B–C ring junction. Molecular modeling shows that this results in a bulkier steroid backbone compared to the $\Delta^{11,12}$ compound, as shown in Fig. 3.

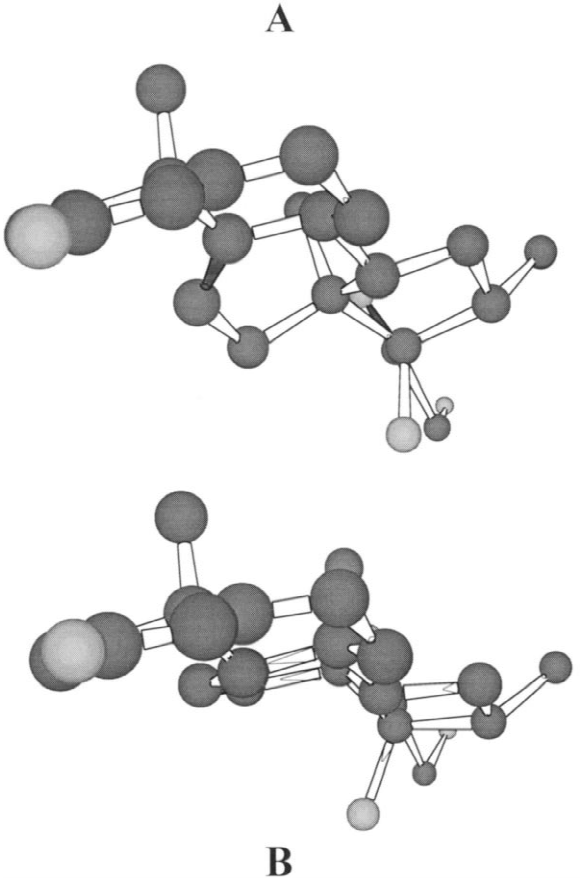

Fig. 3. Energy minimized structures for the $\Delta^{9,11}$ compound (A) and the $\Delta^{11,12}$ compound (B).

These structures were minimized using the MM2 feature of Chem3D Pro (Cambridge Software, Cambridge, MA, USA). The energy minimized structures for these two steroids are viewed with the 3-ketone group of the A-ring in the foreground. The molecular model shows that the C-ring and D-ring are out of plane for the $\Delta^{9,11}$ compound (**7**), resulting in a less compact structure. If size were the only factor involved in complex formation, then it would be expected that the $\Delta^{9,11}$compound (**7**) would have a lower K_f than the $\Delta^{11,12}$compound (**6**). This is not the case, however, with values of 151 M^{-1} for the $\Delta^{9,11}$compound (**7**) and 106 M^{-1} for the $\Delta^{11,12}$compound (**6**). Having demonstrated that the 9- and 11-positions are intimately involved in the inclusion process indicates that the presence of π-electrons in this region may promote interaction with hydrogen atoms present in the secondary alcohol groups of γ-CD.

The dependency of K_f on the amount of acetonitrile present in the mobile phase was investigated. A plot of the natural logarithm of K_f for γ-CD with the 9-bromo analogue (**4**), beclomethasone (**3**), dexamethasone (**2**), the $\Delta^{9,11}$compound (**7**), and the $\Delta^{11,12}$compound (**6**) versus the percent acetonitrile contained in the mobile phase can be found in Fig. 4. These plots are representative of those generated for the other steroids investigated. The average correlation coefficient for the set of 13 steroids was 0.9918. The linear nature of the plots indicates that the inclusion process follows a similar trend of linear solvent strength as seen in reversed-phase chromatography. As the amount of acetonitrile increases, the solubility of the steroid in solution increases. Much of the driving force for inclusion is removed when the guest is highly soluble in solution containing the cyclodextrin and the guest. This data indicates that no change in the mechanism of inclusion is apparent as the acetonitrile content is varied from 20 to 45%. Lower concentrations of acetonitrile were not evaluated using the method described by Fujimura because the retention times became prohibitively long when no cyclodextrins were present in the mobile phase. A less retentive column packed with a stationary phase such as C_4 or C_8 derivatized silica gel or a size exclusion polymer could be used to study the steroid set at a lower concentration of acetonitrile. The mechanism of inclusion may change at very low organic compositions, perhaps resulting in the formation of a 1:2 complex between the steroid and cyclodextrin.

The thermodynamics of the inclusion process were investigated by determining the K_f for each steroid using mobile phases with varying concentrations of γ-CD at temperatures ranging from 10 to 80°C. Fig. 5A shows the chromatogram obtained for one of the test mixtures used in this study at various temperatures in the absence of cyclodextrin. The retention times of the peaks present in this test mixture increase as the temperature decreases, as expected for a typical reversed-phase system. When cyclodextrin is present in the mobile phase this trend of decreasing retention is observed at higher temperatures, but a decrease in retention is observed at lower

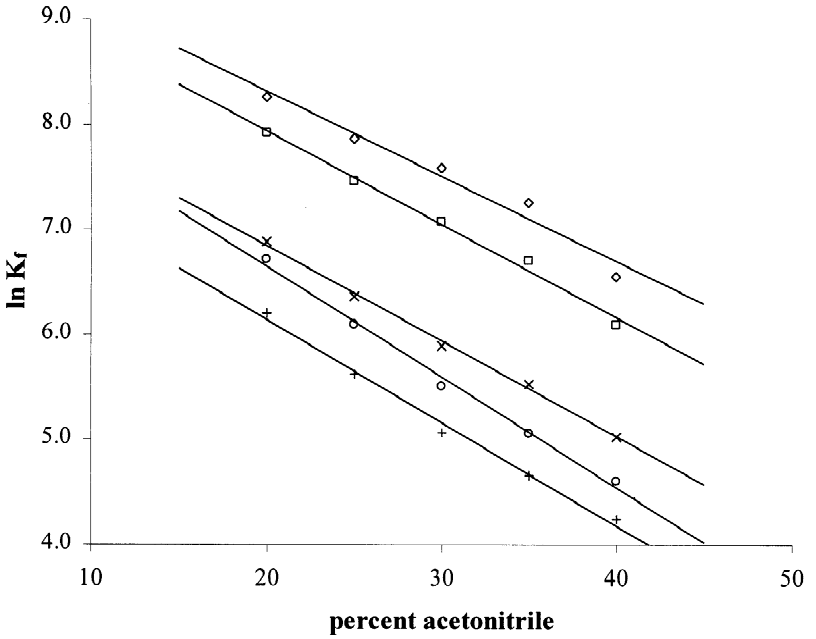

Fig. 4. Dependency of K_f on percent acetonitrile for γ-CD inclusion complexes of the 9-bromo analogue (◊), beclomethasone (□), dexamethasone (×), the $\Delta^{9,11}$ compound (○), and the $\Delta^{11,12}$ compound (+).

temperatures due to the increased association of the steroids with cyclodextrins. This data is shown in Fig. 5B. The peak area for the 9-bromo analogue (**4**) decreased as the column temperature was increased due to the conversion of this steroid into the 9,11-epoxide alcohol (**5**). The peak disappears almost completely at a column temperature of 80°C. However, the decrease in the peak area did not affect the peak's retention factor.

Van't Hoff plots [44] were generated by plotting the natural logarithm of chromatographic retention factors obtained for each mobile phase concentration of γ-CD against the reciprocal of absolute temperature and are described by the following equation:

$$\ln k = -\Delta H^\circ/RT + \Delta S^\circ/R + \ln \phi \quad (2)$$

where k is the retention factor at a particular absolute temperature, ΔH° and ΔS° are the standard enthalpy and entropy changes associated with the chromatographic process, R is the gas constant, and ϕ is the phase ratio of the column. Typical Van't Hoff plots generated for the steroids investigated using mobile phases containing 0, 2.5, 5.0 and 7.5 mM γ-CD are shown in Fig. 6A and B. Non-linear Van't Hoff plots are observed when a process occurs which changes the enthalpy or entropy of the separation system [44–46]. Numerous researchers have observed a deviation from linearity in Van't Hoff plots when cyclodextrins are present in the mobile phase [47–49]. This is due to the competing retention processes at work when cyclodextrin is in the mobile phase. As the column temperature is lowered, the strength of inclusion increases due to the increase in the strength of hydrogen bonding between the guest and the hydroxyl groups of the cyclodextrin. This results in an increase in the residency time of the guest in the mobile phase and thus a decrease in the retention factor measured for that guest. Opposing this process is the increase in retention as temperature decreases due to the change in the equilibrium between the stationary phase and the mobile phase. The natural logarithm of the retention factor is expected to decrease in a linear fashion for a reversed-phase HPLC system when cyclodextrins are absent from the mobile phase and the processes controlling retention are not changing. A slight deviation from linearity is observed at a temperature of about 25°C

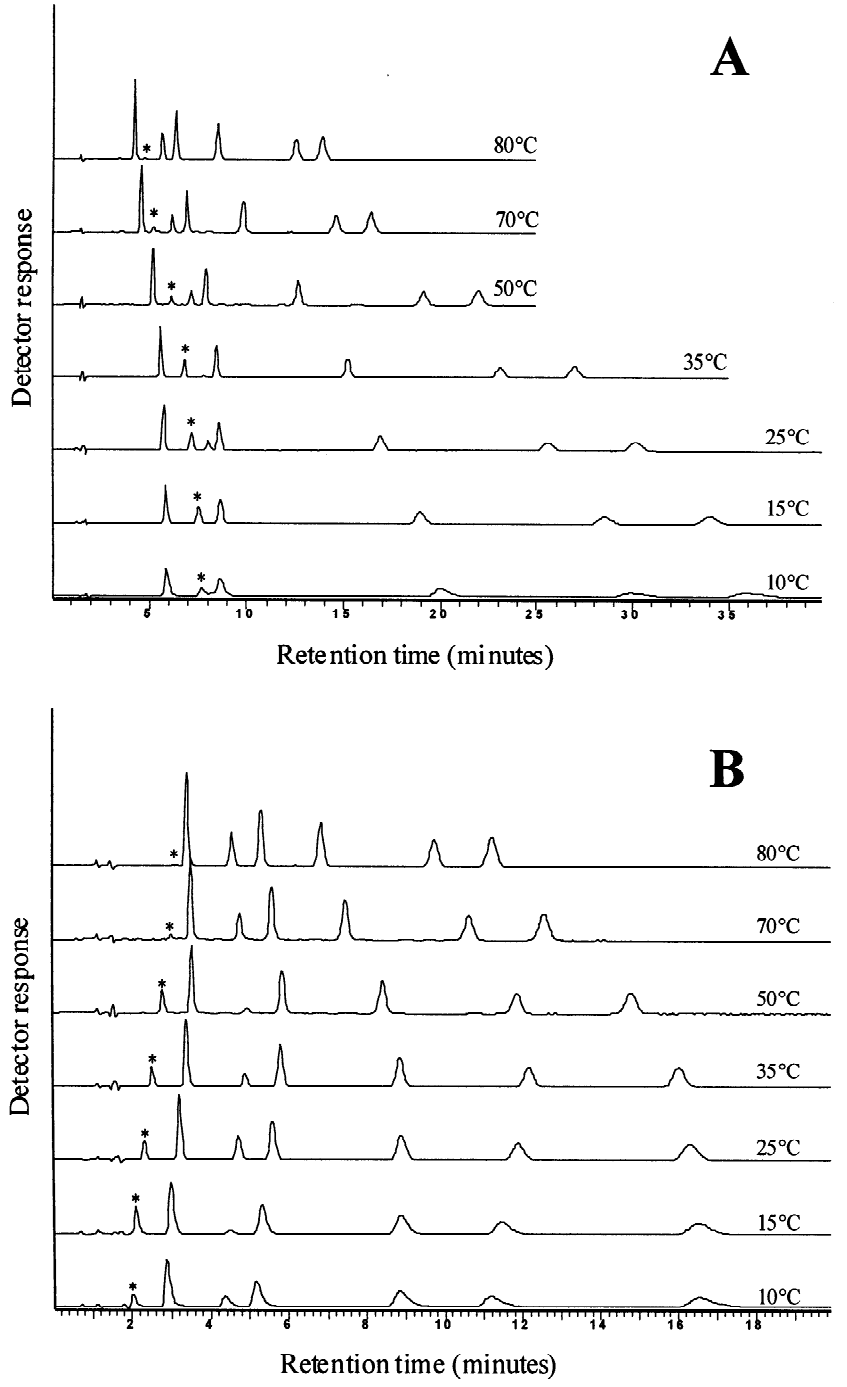

Fig. 5. Overlaid chromatograms for a steroid test mixture with no γ-CD in the mobile phase (A) and with 7.5 m*M* γ-CD in the mobile phase (B) at the column temperature indicated. *The 9-bromo analogue peak.

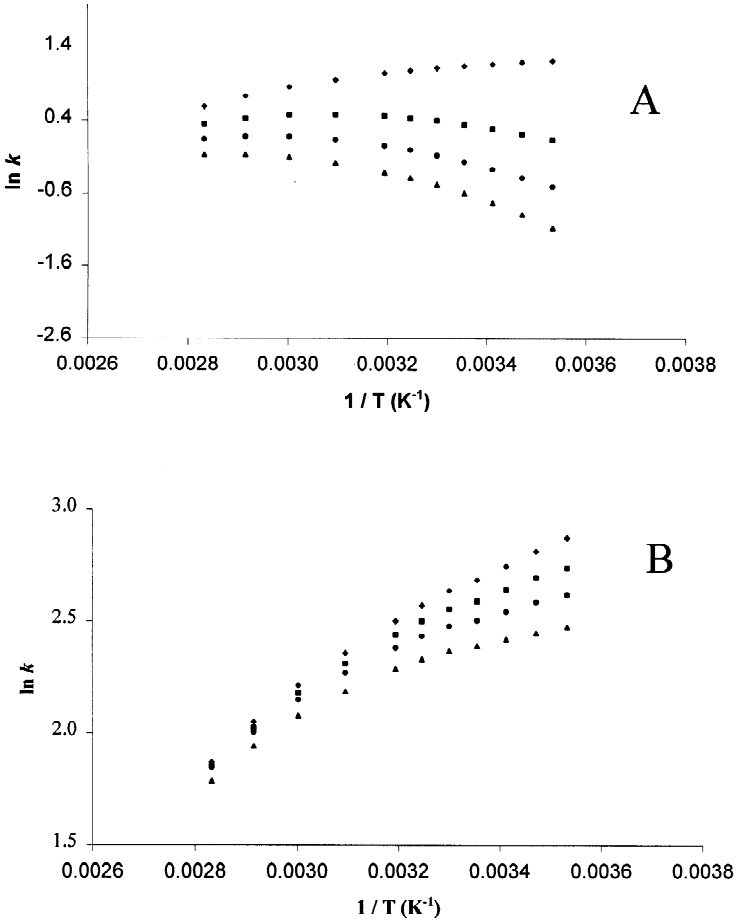

Fig. 6. Van't Hoff plots generated using retention factors of beclomethasone (A) and betamethasone 11,21-diacetate (B) with no γ-CD (♦), 2.5 m*M* γ-CD (■), 5.0 m*M* γ-CD (●), and 7.5 m*M* γ-CD (▲) in the mobile phase.

in the Van't Hoff plot generated in the absence of γ-CD in the mobile phase. This phenomenon has been observed by other research groups and has been attributed to a change in the configuration of the stationary phase, which effects the separation process [50–52]. Differential scanning calorimetry (DSC) has been used to study the stationary phase used for this work to demonstrate that a change in the conformation of the stationary phase is the cause of the deviation from linearity observed in the Van't Hoff plot when no γ-CD is present. A transition was observed at a temperature of 28°C. Similar results were observed by Dorsey and Cole [50] for a reversed-phase column with similar bonding density as the one used in this research. They found transition temperatures of 26.7 and 35.7°C for stationary phases with bonding densities of 2.84 and 4.07 $\mu mol/m^2$, respectively [50]. The bonding density of the Symmetry C_{18} column on which DSC was performed was 3.18 $\mu mol/m^2$.

For mobile phases containing γ-CD, a more interesting though somewhat expected result is the inflection observed in Van't Hoff plots. The inflection becomes more pronounced as the concentration of γ-CD in the mobile phase increases. The change in slope observed in the Van't Hoff plots is related to K_t. Fig. 6A shows a large deviation in the slope obtained for beclomethasone (**3**) as temperature decreases. Fig. 6B shows a much less severe departure from linearity in the Van't Hoff plot for

betamethasone 11,21-diacetate (**13**). The K_f values obtained with γ-CD at 25°C are 612 M^{-1} for beclomethasone (**3**) and 47 M^{-1} for betamethasone 11,21-diacetate (**13**).

At first glance, it appears that a very complicated change in retention processes is occurring at lower temperatures. Further analysis of the data reveals that this is not the case. K_f values can be calculated at each temperature studied since several concentrations of γ-CD were used. Additional Van't Hoff plots were generated by plotting the natural logarithm of K_f values (instead of retention factors) against the inverse of absolute temperature. By relating $\Delta G°$ directly to an equilibrium constant and not to a retention factor, the value of ϕ is no longer needed to explicitly calculate $\Delta S°$ from the *y*-intercept of the Van't Hoff plot. The thermodynamic parameters extracted from Van't Hoff plots generated in this way relate only to the process of inclusion. Van't Hoff plots generated using retention factors are more difficult to interpret due to the complex nature of reversed-phase retention.

Fig. 7 shows the plots of the natural logarithm of K_f against the inverse of absolute temperature for five of the steroids studied here. These plots are representative of the rest of the steroids analyzed. Values for $\Delta H°$ and $\Delta S°$ for the inclusion process extracted from the slope and intercept of the Van't Hoff plots using the natural logarithm of K_f are listed in Table 3. The overall process of inclusion for betamethasone and the related steroids investigated here is enthalpy driven. Similar results were obtained by Sadleg-Sosnowska for four steroids not used in this study [53]. K_f values reported by Sadleg-Sosnowska were obtained using the Hummel–Dreyer method [53]. A plot of $\Delta S°$ against $\Delta H°$ for the 13 steroids studied is shown in Fig. 8. A linear relationship between $\Delta S°$ and $\Delta H°$ for all but two of the steroids exists ($r^2 = 0.983$). As the magnitude of the enthalpy associated with inclusion increases an offsetting decrease in the entropy occurs. A relationship of this type is referred to as enthalpy–entropy compensation [54]. This data exhibits a compensatory enthalpy–entropy relationship for all of the steroids examined except betamethasone 11,21-diacetate (**13**) and betamethasone 17-monopropion-

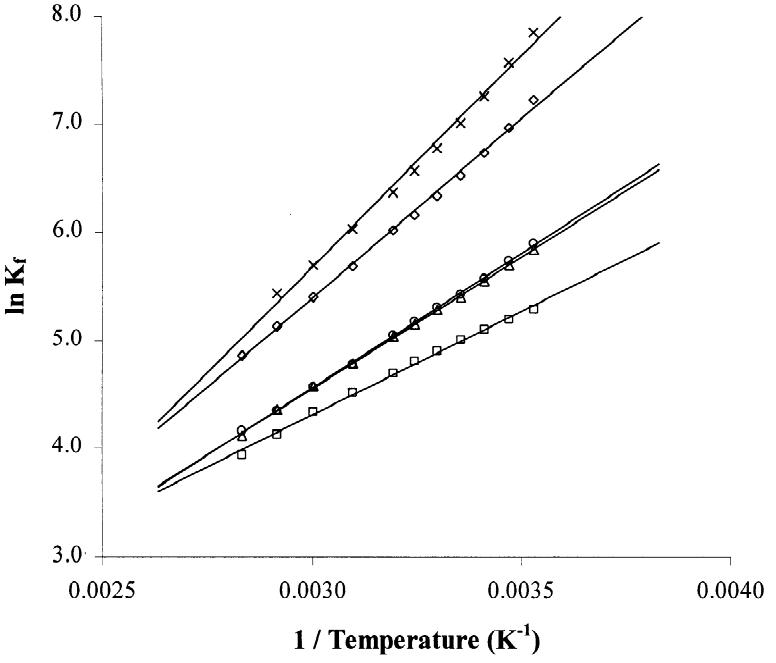

Fig. 7. Van't Hoff plots generated using K_f values for γ-CD complexes of the 9-bromo analogue (×), beclomethasone (◊), betamethasone (△), dexamethasone (○), and 9,11-epoxide alcohol (□).

Table 3
Thermodynamic properties of complex formation with γ-CD in acetonitrile–water (35:65, v/v)

Component	$-\Delta H^\circ$ (kJ/mol)	$-\Delta S^\circ$ (kJ/mol K)
9-Bromo analogue	32.4	0.05010
Beclomethasone	27.4	0.03740
Dexamethasone	20.6	0.02389
Betamethasone	20.2	0.02271
9,11-Epoxide, 21-acetate	20.2	0.02479
Betamethasone 17-monopropionate	20.1	0.02892
Betamethasone 11,21-diacetate	19.2	0.03217
Dexamethasone 21-acetate	19.1	0.02265
Betamethasone 21-acetate	18.4	0.02011
Betamethasone 21-monopropionate	17.6	0.01891
9,11-Epoxide alcohol	16.0	0.01214
$\Delta^{9,11}$ Compound	15.0	0.00839
$\Delta^{11,12}$ Compound	14.6	0.01041

ate (**12**). Enthalpy–entropy compensation is frequently observed when a series of related compounds undergo a similar process [54–56]. Due to the offsetting variance in the entropy, the change in free energy of the process is always lower than the change in entropy of the process. This is represented by a linear dependence of the plot shown in Fig. 8. Only betamethasone 11,21-diacetate (**13**) and betamethasone 17-monopropionate (**12**) deviate significantly from this line. This data indicates that these steroids are not forming a complex with γ-CD in exactly the same way as the other betamethasone-related steroids studied. Further evidence that these steroids undergo a slightly different mechanism of inclusion arises from plots of ΔG° versus the inverse of absolute temperature. One of the requirements for enthalpy–entropy compensation outlined by Krug [57] is that plots of ΔG° versus the inverse of absolute temperature must intersect at a single temperature for all of the compounds undergoing the same process in the same manner. This intersection occurs at a temperature of about 450°C for all of the steroids tested except betamethasone 11,21-diacetate (**13**) and betamethasone 17-monopropionate (**12**). From this data, it is clear that the 11-position and the 17-position are critically involved in the inclusion process, as the analysis of K_f values has indicated. Significant changes to the chemical structure at these

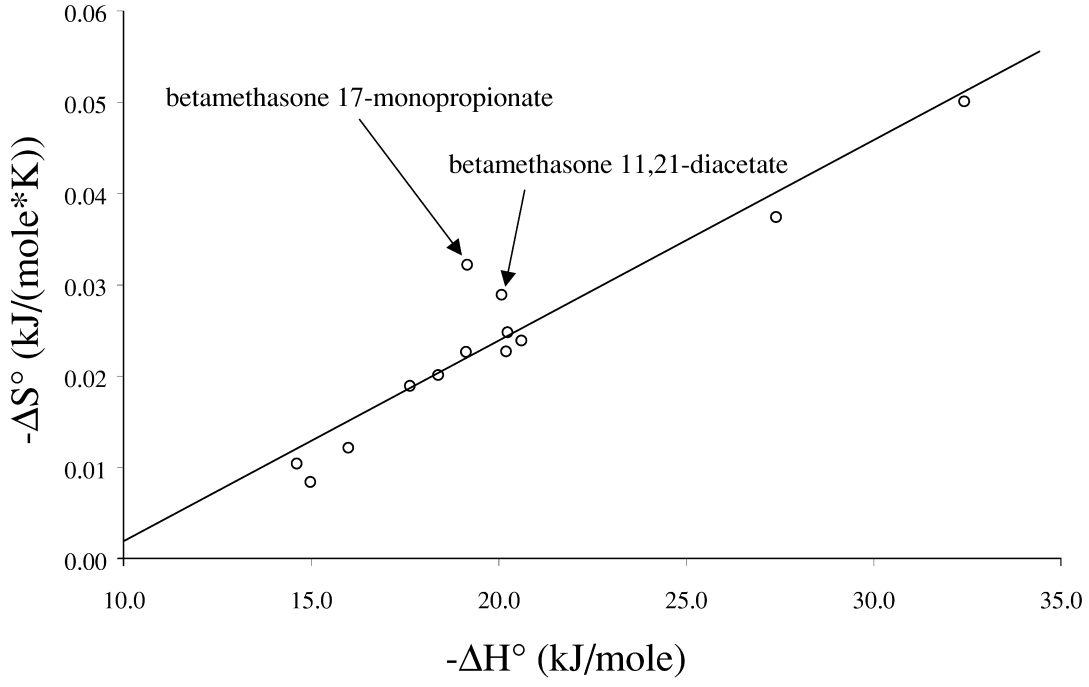

Fig. 8. Enthalpy–entropy plot for 13 steroids.

positions result in a change in the stability of the inclusion complex, as well as a change in the mechanism of the inclusion process.

A useful consequence of the complex nature of inclusion with cyclodextrins is the ability of cyclodextrins to resolve closely related compounds when added to an HPLC mobile phase. Small differences in K_f values for two closely eluting peaks will yield significant changes in the resolution between these peaks. A practical example of this is shown in Fig. 9. Separate solutions containing compounds **14** and **15** were analyzed using mobile phases containing no γ-CD and 3.2 m*M* γ-CD. In the pharmaceutical industry, it is necessary to separate and correctly identify each component present in a material. The synthetic chemists working with these materials need to be aware of the presence of significant amounts of an unknown compound so that they can modify their reactions or purification methods. In the absence of γ-CD, an impurity related to compound **14** coelutes with compound **15**. Upon adding γ-CD to the mobile phase it becomes apparent that compound **15** is one of two impurities present at significant levels in compound **14**. The K_f values calculated for **14**, **15**, impurity one, and impurity two are 112, 148, 212 and 93 M^{-1}; respectively. A difference of 50 M^{-1} in the value of K_f facilitates the separation of two peaks that coelute in the absence of γ-CD. Impurity one is the 11-β-OH epimer of compound **15**. Here is another example of the significance of the 11-position on the inclusion complex formed with γ-CD. An additional benefit of using cyclodextrins to modify a mobile phase is a decrease in retention time and thus improved sensitivity. It is possible to increase resolution and shorten run time by adding cyclodextrins to the HPLC mobile phase. Since K_f increases as organic content decreases it is possible to achieve rapid separations using a minimal amount of organic modifier. Typically, increasing the organic content of mobile phase decreases retention in a reversed-phase

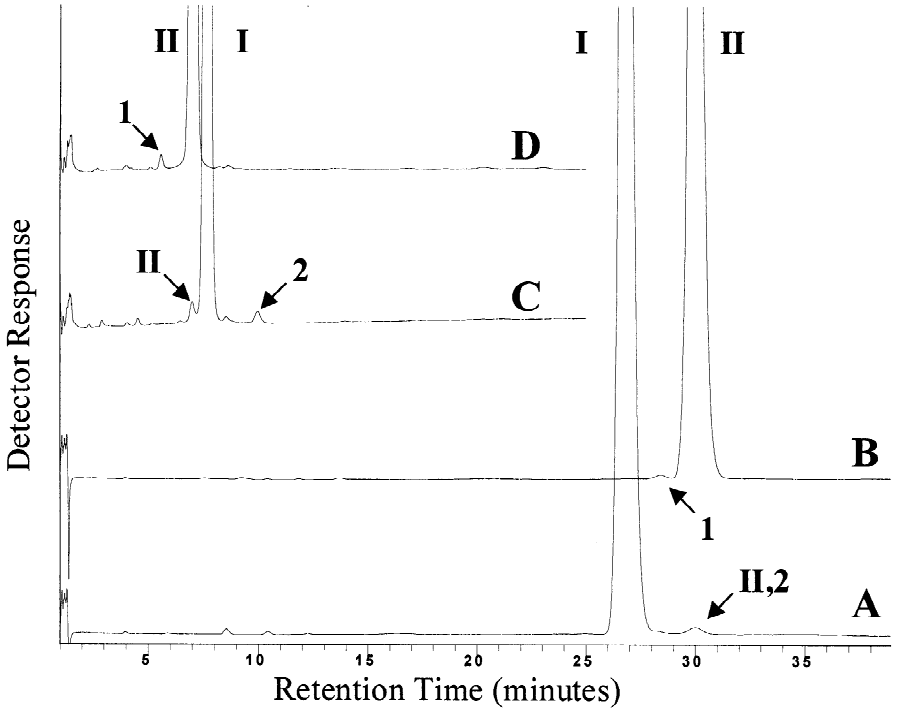

Fig. 9. Overlaid chromatograms obtained for compound **15** (A) and compound **14** (B) with no γ-CD in mobile phase and for compound **15** (C) and compound **14** (D) with 3.2 m*M* γ-CD in mobile phase. Peaks: I=compound **15**, II=compound **14**, 1=impurity one, 2=impurity two. Conditions: acetonitrile–water (20:80, v/v) at 1.0 mL/min, YMC J'Sphere ODS-H80, 150×4.6 mm, 4 μm particle size, UV detection at 240 nm.

HPLC system. However, this is usually done at the expense of resolution. The inclusion process is much more sensitive to small changes in the structure of a compound than the retention process using an alkyl derivatized stationary phase. Large changes in selectivity can be achieved using small amounts of cyclodextrin in the mobile phase. It is not possible to relate K_f to the retention time of a compound when no cyclodextrins are present in the mobile phase. The overall hydrophobicity of a compound determines its retention time but it is the location of regions of hydrophobicity and key structural features of a compound that will determine how strongly it associates with cyclodextrin.

4. Conclusions

The inclusion complexes of 13 steroids with several cyclodextrins were investigated using the HPLC method described by Fujimura. It was found that precise K_f values can only be determined if the void volume of the HPLC column is known exactly. Stronger inclusion complexes were obtained with γ-CD and HP-γ-CD than with β-CD and its derivatives. A combination of steric factors and electronic factors were found to control the inclusion process. A decrease in K_f and a change in the inclusion mechanism were observed when the hydroxy group in the 9- or 17-position is replaced with an alkyl ester. A decrease in K_f is observed when the bulkiness of the substituent at the 11-position, 17-position, or 21-position is increased. The mechanism of inclusion was found to remain constant using mobile phases with acetonitrile content between 20 and 40%. No change was observed in the inclusion mechanism from 10 to 80°C, based on the linear results of the Van't Hoff plots generated using the natural logarithm of K_f. A practical application for separating several structurally similar impurities from a steroid demonstrates the usefulness of cyclodextrins as HPLC mobile phase modifiers.

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