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Semi-preparative separation of polyhydroxylated sterols using a β-cyclodextrin high-performance liquid chromatography column

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ABSTRACT

A series of closely related polyoxygenated sterols from the sponge *Dysidea etheria* was resolved by high-performance liquid chromatography on a β-cyclodextrin column, a stationary phase apparently not used previously for the preparative separation of natural products. Analysis of the chromatographic behavior of the various sterols on the β-cyclodextrin column indicated that formation of an inclusion complex was not the separation mechanism involved in this case. Rather, the comparative analysis suggested that hydrogen bonding between the solute molecules and the hydroxyl groups on the rim of the β-cyclodextrin cavities best explained the results observed.

INTRODUCTION

Cyclodextrins are D(+)-glucopyranose units connected by α-(1,4) bonds to form cyclic oligosaccharides [1]. An interesting high-performance liquid chromatography (HPLC) column stationary phase has been produced by covalently bonding these molecules to silica gel via a seven to nine atom spacer [2]. β-Cyclodextrin is a non-ionic, cyclic, chiral carbohydrate that has the shape of a hollow truncated cone in which the side of the torus with the greater circumference has fourteen secondary hydroxyl groups, while that of the smaller side has seven primary hydroxyl groups [3]. Due primarily to favorable hydrophobic (interior of cavity) and/or hydrogen bonding interactions (hydroxyl containing faces), guest molecules can bind to and form inclusion complexes with β-cyclodextrin. This host–guest spatial interaction can result in appreciably different complexes from a pair of guests which differ only slightly in structure.

β-Cyclodextrin HPLC stationary phases have probably found their most important utility as chiral stationary phases (CSPs) [4–6]. The chiral recognition has been rationalized in term of several possible interactions between the β-cyclodextrin

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host and guest racemates [7]. Guest solutes can interact via Van der Waals–London dispersion forces with the hydrophobic cavity. In order for this to be effective, however, the molecule must fit tightly within the cavity. In most cases, the cylindrical binding characteristics to a racemic guest molecule are too symmetrical to induce large enantioselectivities. Consequently, there must be other points of interaction in order to achieve chiral recogition. The fourteen chiral secondary alcohols situated around the mouth of the cavity, as well as the seven primary hydroxyl groups situated around the opposite rim provide a number of these possible stereospecific interactions.

Numerous other types of closely related compounds that are not enantiomers, but which are also poorly resolved on more traditional columns, can be readily separated on this stationary phase. A variety of structural isomers has been resolved, including positional and geometrical (*cis–trans*) isomers of prostaglandins [8,9], polycyclic aromatic hydrocarbons, and steroid epimers [10]. These results encouraged us to apply the β-cyclodextrin column to the separation of the closely related polyhydroxylated sterols from the sponge *Dysidea etheria*. To the best of our knowledge, this stationary phase had not been used previously for the preparative separation of natural products.

EXPERIMENTAL

Instrumentation

HPLC separations were performed on a Perkin–Elmer Series 3B liquid chromatograph using a Knauer differential refractometer for detection. The flow-rate was either 1.0 or 3.0 ml/min as noted; the injection volume was 40 μl.

Column

A Hamilton PRP-1 column (300 mm × 7 mm I.D.) was used for the reversed-phase separations. An ASTEC β-cyclodextrin column (250 mm × 4.6 mm I.D.) was used for the β-cyclodextrin separations.

Samples

All sterols were naturally occurring sterols isolated from the sponge *Dysidea etheria*. Their structures were solved by spectroscopic methods [11,12]. Injection concentrations were approximately 10 mg/ml for all sterols; 0.5–1.0 mg of sterol mixtures were injected per run.

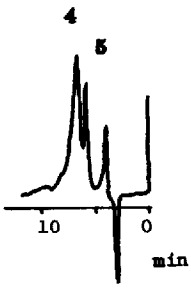
RESULTS AND DISCUSSION

The extremly polar polyhydroxylated sterols which we encountered in extracts of *D. etheria* were not amenable to normal-phase chromatography. While some of the sterols could be purified on octadecylsilyl bonded phase columns, some mixtures remained unresolved. The β-cyclodextrin column, however, using acetonitrile–water (1:1) as the mobile phase, gave nearly bascline separation of sterols **1** and **3** (Fig. 1). This initial success was followed by the separations of **4** and **5** (Fig. 2), **2** and **6** (Fig. 3), and **7** and **8** (Fig. 4) using the β-cyclodextrin column with various ratios of acetonitrile–water. What these separations had in common was that the nuclear framework and functional group substitution patterns on the sterol nuclei were the

Fig. 1. β-Cyclodextrin separation of **1** and **3**. Acetonitrile–water (1:1); 1.0 ml/min.

same for both members of the separated pairs, while the differences resided only in the side chains of each pair (Table I).

Once the sterols were purified by these β-cyclodextrin chromatographies and their structures determined, it became obvious that we had not separated epimeric pairs as we had originally assumed, but rather analogues in which the side chains of each member of a pair differed only slightly from one another. The sterols separated in the chromatographies shown in Figs. 1, 3, and 4 differed in that one member of each set contained a carbon–carbon double bond at C-22 and an extra methyl group at C-24 in the C_8 sterol side chain. The fact that these congeners co-eluted by reversed phase chromatography in all three cases (non-acetylated, mono-acetylated, and di-acetylated) with sterols possessing a normal C_8H_{17} side chain indicated that the apparent polarity of these two very different side chains must be similar. It was interesting to note that whereas the reversed-phase column could separate sterols differing only by



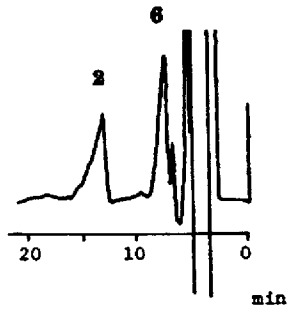


Fig. 2. β-Cyclodextrin separation of **4** and **5**. Acetonitrile–water (9:11); 1.0 ml/min.

Fig. 3. β-Cyclodextrin separation of **2** and **6**. Acetonitrile–water (4:1); 1.0 ml/min.

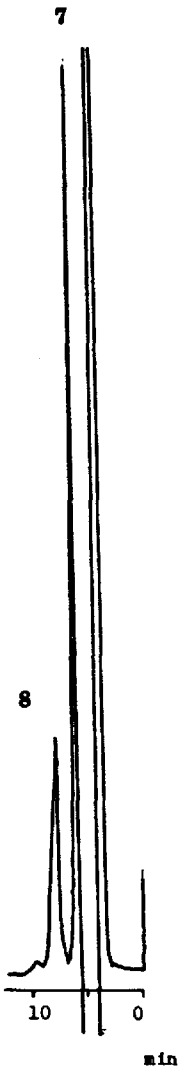
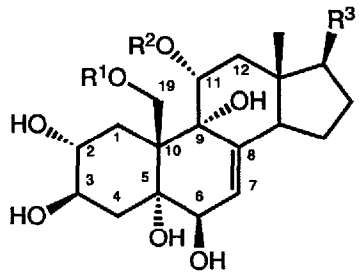


Fig. 4. β-Cyclodextrin separation of **7** and **8**. Acetonitrile–water (5:1); 1.0 ml/min.

Δ^{22} double bond from those having a normal, saturated side chain, the addition of a methyl group at C-24, along with the unsaturation, posed a very difficult problem for a reversed phase separation.

The remaining β-cyclodextrin separation, which is shown in Fig. 2, was interesting in that it represented a separation between two much more closely related sterols than the other three separations. Sterols **4** and **5** were positional isomers of one another, since **4** possessed a normal C-26, 27 isopropyl group, whereas **5** was missing the C-27 methyl, but had a methyl branch at C-24 instead. Djerassi and co-workers [13,14] have reported the separation of sterols differing by these same side chains situated on



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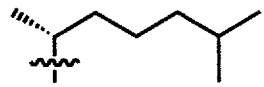
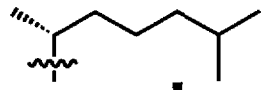
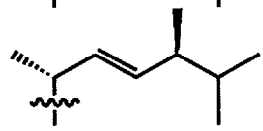
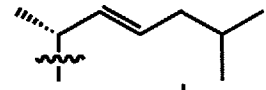
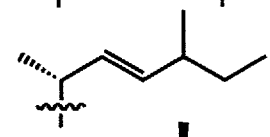
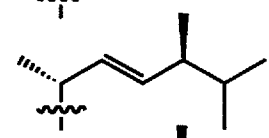
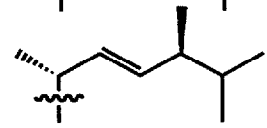
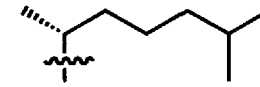
1	Ac	Ac	
2	H	H	
3	Ac	Ac	
4	Ac	Ac	
5	Ac	Ac	
6	H	H	
7	H	Ac	
8	H	Ac	

identical sterol nuclei. Only relative retention time (RRT) data were given in both instances, which makes it difficult to determine how well resolved the two components were. In both cases, though, the RRT values were close enough (0.72 to 0.76, and 0.70 to 0.75) to indicate that these closely related compounds were not as well resolved using reversed-phase chromatography as **4** and **5** were on the β-cyclodextrin column.

TABLE I

EXAMPLES OF β-CYCLODEXTRIN SEPARATIONS OF 5α-CHOLESTA-7-ENE-2α, 3α, 5α, 6β, 9α, 1α, 19-HEPTOL STEROLS

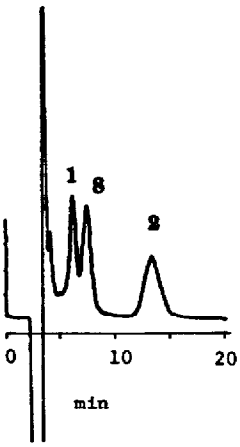
t_R = Retention time; k' = capacity factor; R_s = resolution.

Eluent: acetonitrile–water	Sterol	No. of acetates	Side chain substitutions	t_R (min)	k'	R_s
1:1	1[a]	11,19-Diacetate	(Normal) C_8H_{17}	8.6	3.3	0.93
	3[a]	11,19-Diacetate	Δ^{22}, 24-Methyl	6.5	2.3	
9:11	4[b]	11,19-Diacetate	Δ^{22}	6.4	1.3	0.71
	5[b]	11,19-Diacetate	Δ^{22}, 27-Nor, 24-methyl	5.6	1.0	
4:1	2[c]		(Normal) C_8H_{17}	13.5	3.8	3.0
	6[c]		Δ^{22}, 24-Methyl	7.5	1.7	
5:1	7[d]	11-Acetate	Δ^{22}, 24-Methyl	6.1	0.9	1.05
	8[d]	11-Acetate	(Normal) C_8H_{17}	7.9	1.4	

There seems to be general agreement that when aqueous–organic mobile phases are used, the mechanism of separation on the β-cyclodextrin column is the formation of inclusion complexes with solute molecules [1–3,15–17]. Armstrong *et al.* [18] have suggested that in order for chiral recognition to be effective a compound needs at least one aromatic ring, preferably two, and that the chiral center must be in close proximity to the ring moieties. The formation of an inclusion complex, however, has also been invoked to explain the separation mechanism of diastereomers and geometric isomers which lack aromatic rings such as steroid epimers [3,19] and prostaglandins [8,9]. In the separation of *cis–trans* isomers of prostaglandins [9], for example, it was assumed that an incluson complex would form in such a way that the polar functional groups would stay away from the hydrophobic center and/or allow hydrogen bonding with the hydroxyl groups on the edge of the cavity. The elution order of *cis* isomer, followed by *trans,* was explained by the more compact conformation of the *cis* isomer, which allowed for a tighter complex.

It appeared to us, however, that even under typical reversed-phase conditions (acetonitrile–water) the separations of the polyhydroxylated sterols did not proceed by an inclusion complex mechanism. We decided to perform some simple experiments using the sterols already isolated and characterized in order to determine what interactions were responsible for separating these compounds.

The first series of these polyhydroxylated sterols had identical oxidation patterns and side chains, but varying degrees of acetylation. The elution order on the β-cyclodextrin column, shown in Fig. 5, was: di-acetate (**1**), followed by mono-acetate (**8**), followed by the non-acetylated sterol (**2**). This was the opposite of the elution pattern observed for the same compounds injected onto a reversed-phase column where the elution order was most polar to non-polar, as expected (Fig. 6). These data seemed to indicate that the non-polar side chain of the sterols was not necessarily included into the hydrophobic cavity, if this were the case, the elution order should have approximated more closely that of the reversed-phase separation.



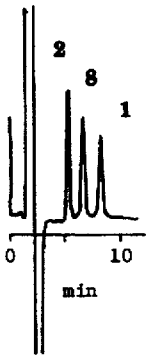


Fig. 5. β-Cyclodextrin separation of **1**, **2** and **8**. Acetonitrile–water (1:1); 1.0 ml/min.

Fig. 6. Reversed phase separation of **1**, **2** and **8**. Acetonitrile–water (1:1); 3.0 ml/min.

The next experiment involved the coinjection (Fig. 7) of a 5α- (**2**), and a 5β-polyhydroxysterol (**9**), both with the same substitution, acetylation, and side chain alkylation patterns. The conformations of these two molecules are so different about the A and B rings that an inclusion complex formed with this region of the molecule should have dramatically different characteristics for the two epimers. What was found, however, was that the two compounds had virtually the same retention times, and hence were not resolved at all. These two compounds were well separated on the reversed-phase column, with the *cis* compound eluting after the *trans*. It appeared that the hydroxyl groups of the sterols were hydrogen-bonding with the hydroxyl groups around the perimeter of the β-cyclodextrin molecule rather than forming

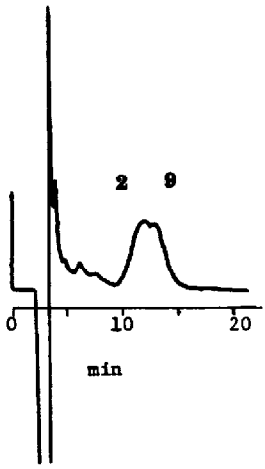
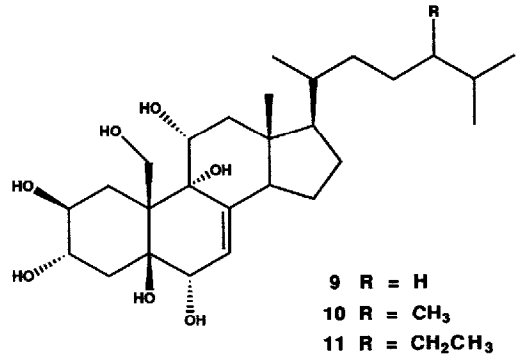
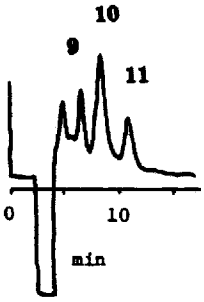


Fig. 7. β-Cyclodextrin separation of **2** and **9**. Acetonitrile–water (1:1); 1.0 ml/min.



inclusion complexes. Both of these experiments, therefore, pointed to the conclusion that polyhydroxylated sterols are retained on the β-cyclodextrin column through relatively non-specific hydrogen bonding interactions, and that inclusion complex formation with either the hydrophobic side chain or the sterol nucleus was not occurring. In this regard, the separation mechanism was analogous to that of a diol column. This behavior has already been observed for the normal-phase operation of β-cyclodextrin columns [15].

The third experiment involved the elution of a homologous series of 5β-hydroxysterols with the same ring substitutions but with slightly different side chains. The three sterols (**9**, **10** and **11**) differed by the degree of alkylation at the C-24 position. The sterols eluted in the order of increasing alkylation (Fig. 8), which was the same order as that observed for separation on a reversed phase column (Fig. 9). This observation was also in agreement with the separation mechanism proposed above, since hydrogen bonding of the hydroxyl groups of the sterols to the hydroxyl groups on the β-cyclodextrin molecule would allow the non-polar side chain to determine the elution order by partitioning into the mobile phase. If this were the case, then the more highly branched side chain would elute last in the polar mobile phase, as was observed. This by itself, however, does not preclude the possibility that the non-polar side chains are included into the hydrophobic cavity. When taken in conjunction with the previous



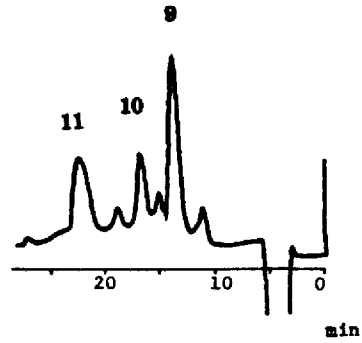


Fig. 8. β-Cyclodextrin separation of **9**, **10** and **11**. Acetonitrile–water (1:1); 1.0 ml/min.

Fig. 9. Reversed phase separation of **9**, **10** and **11**. Acetonitrile–water (2:1); 3.0 ml/min.

observations, though, the evidence seems to support overwhelmingly hydrogen bonding to the polar portions of the sterol molecules as the primary separation mechanism.

In the light of these data, it would be prudent to reexamine the interactions responsible for separations of other compounds by this versatile stationary phase, since insight into this might allow an even greater array of substances to be resolved. While there is no doubt that many separations, particularly those of enantiomers, are the result of inclusion complexing, it would be interesting to find out if enantiomeric or diastereomeric separations can occur in the absence of this mechanism. The β-cyclodextrin column would certainly seem to be another powerful tool for the natural products chemist confronted with challenging separation problems.

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