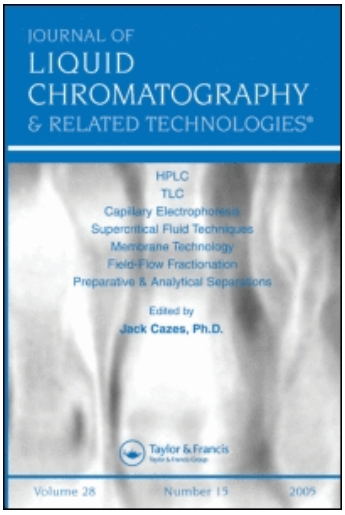

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PHARMACEUTICAL APPLICATIONS USING CHOLESTERYL-10-UNDECENOATE BONDED PHASE IN MICROCOLUMN LIQUID CHROMATOGRAPHY

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ABSTRACT

The separation behaviors of four different groups of pharmaceuticals were investigated using cholesteryl-10-undecenoate and ODS phases. The cholesteryl phase, which has liquid crystal properties, showed different selectivity and retention behavior from those of ODS phase. The difference is on its apparent ability to recognize specific structures in the molecules, discriminate among derivatives, or subsequently recognize modifications done on these structures. However, on ODS phase the hydrophobicity effects seem to take precedent as the main factor in its retention mechanism. This enhanced selectivity of the cholesteryl phase is attributed to three contributing structures found in its structure; the long hydrocarbon chain that gives added

mobility to the phase, the carbonyl group which can interact with a variety of substituent groups, and the cholesteric moiety which can invoke π-π interaction as well as steric effects. The use of the cholesteryl phase for these kinds of pharmaceutical analyses is therefore warranted and showed interesting possibilities for future applications.

INTRODUCTION

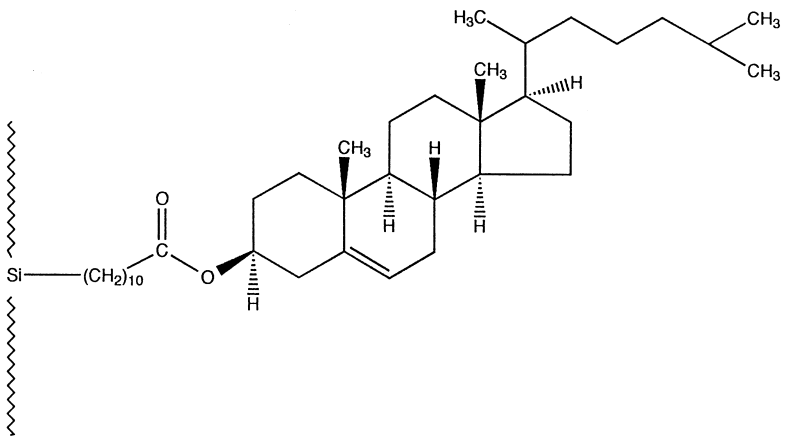
The applicability of the cholesteryl-10-undecenoate bonded phase in the analysis of some polycyclic aromatic hydrocarbons and benzodiazepines have been previously demonstrated in Microcolumn Liquid Chromatography.[1-3] In the analysis of the benzodiazepines the retention mechanism seemed to indicate an entirely different behavior to that exhibited by the ODS phase. It appears that separation on this type of column is based primarily on its ability to recognize specific structures in the molecules and on the modifications done on these structures thereafter. Although the samples' hydrophobicity contribute to the degree of retention, it is indicated that the cholesteryl-10-undecenoate phase is less dependent than the ODS phase on the hydrophobicity effects.

As an extension of our previous work, we evaluated further this retention behavior by using other samples, especially pharmaceuticals, which are structurally similar or analogous and again compare the retention behavior using ODS phase. The results seemed to support our claim that this bonded phase has molecular recognition capability. We report here the separation of four groups of drugs namely barbiturates, steroidal compounds, xanthines, and a cold medicine mixture. They were chosen primarily on the basis of their related structures as well as on their importance to pharmacologic, toxicologic, and forensic studies.

The separation of these components were successfully obtained and compared with the ODS phase. The separation and the molecular recognition capability by the cholesteric phase are attributed to the presence of three contributing structures; the long hydrocarbon chain, the carbonyl group, and the cholesteric moiety. The structure of the bonded phase is given in Figure 1.

The cholesteryl-10-undecenoate bonded phase, due to the twisted stacking of the molecules exhibits liquid crystalline properties and thus, is expected to exhibit high selectivity toward structurally-related compounds.[4] Although it is not well understood whether the crystalline property is fully retained when it is bonded to silica surface, we surmised that it still possesses enough selectivity for this kind of separations as our results indicated.

A



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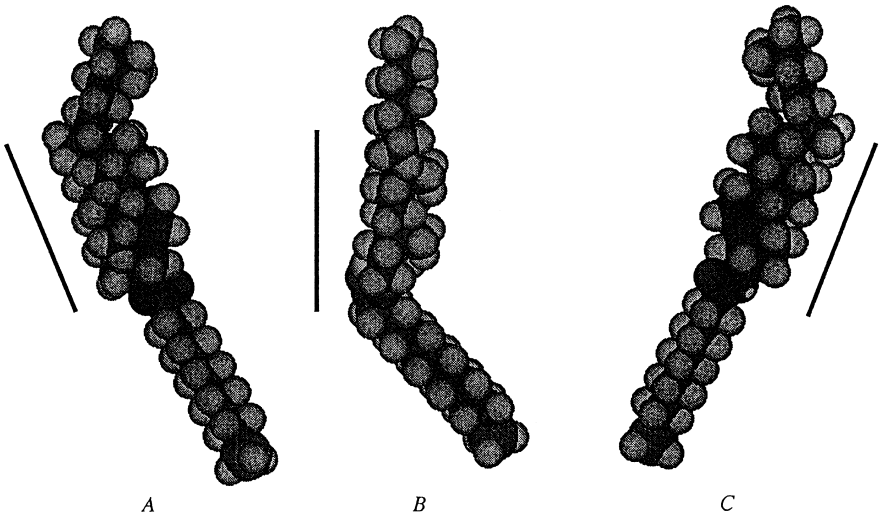


Figure 1. Two-dimensional structure of the cholesteryl-10-undecenoate bonded phase. Note the long alkyl chain, the carbonyl group and the bulky cholesteric moiety, **A**. Three-dimensional structure shows the (A) front view, (B) side-view and (C) back-side-view. Black lines indicate planar part of the phase, **B**.

Liquid crystals have been extensively employed in GC and SFC for difficult separations because of their strong shape selectivity.[5-7] They can be physically coated on a solid support or on the inner wall of the capillary such that they can have high stability even without chemical bonding of the stationary phase unlike when they are employed in HPLC wherein it is necessary for the liquid crystal to be linked to a solid support to increase column stability.

The use of liquid crystals for RPLC is not really new[8-10] although its application is somewhat limited by the lack of appropriate compounds that can be used. Pesek et al. in the late 1980's introduced a new format when they bonded chemical moieties on particulate silica surface which retained most of their crystalline properties and were subsequently employed in liquid chromatographic studies.[11-14]

Although ODS and other alkyl bonded phases have shown a wide range of selectivities for most applications, the search for new phases with special selectivities continue. Therefore the use of this cholesteric phase offers a challenging alternative and as it has initially shown, very interesting possibilities for molecular recognition, this continuing study seem to be in order.

EXPERIMENTAL

The microcolumn liquid chromatographic system consisted of a microfeeder (MF-2, Azuma Electric, Japan) serving as pump with flow rate set at 2 μL/min, a UV detector (UVIDEC-100-III, Jasco, Japan) set at wavelengths of maximum absorbances for the corresponding groups of compounds, and a microloop injector (Model 7619 Rheodyne, USA) for sample injection.

Columns used were fused silica capillaries with 0.53 mm internal diameter and 150 mm in length, laboratory made and packed with the bonded phases using conventional slurry method.

The cholesteryl-10-undecenoate bonded phase was synthesized at the Department of Chemistry, San Jose State University, USA and the ODS-5 (Develosil) was from Nomura Chemicals, Japan.

The barbiturates and the components of the cold mixture were kindly provided by Dr. M. Hayashida of Nippon Medical School, Tokyo. Barbituric acid was obtained from Kishida Chemicals, Japan. The steroidal compounds were obtained from Tokyo Kasei Kogyo Co. Ltd., Japan and the xanthines were obtained from Sigma Chemical Company, USA (theobromine), Kishida Chemicals, Japan (caffeine) and Tokyo Kasei, Japan (theophylline).

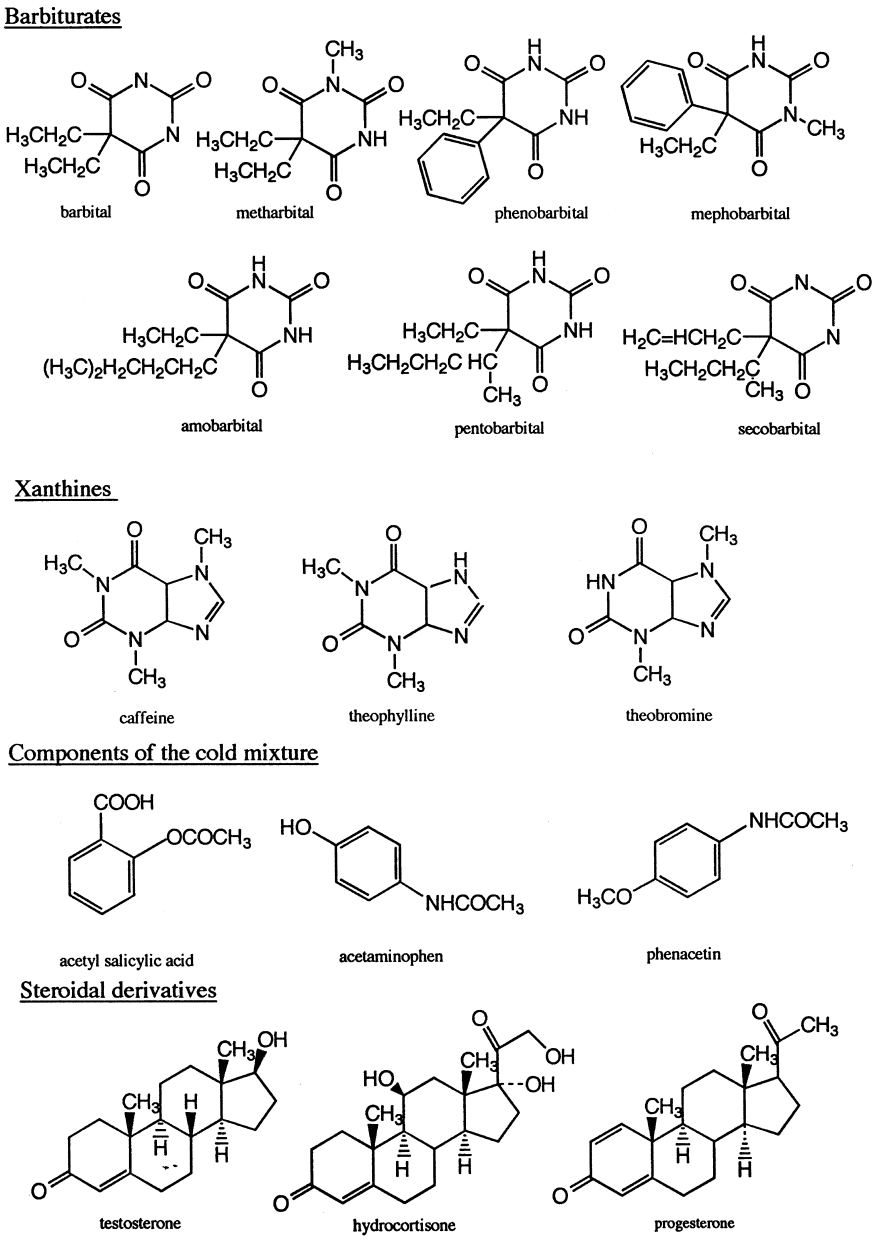
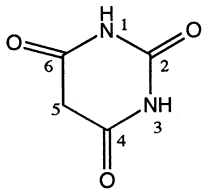
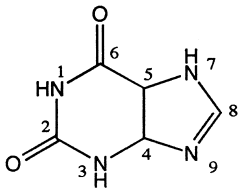


Figure 2. The structures of the four groups of pharmaceutical compounds used in this study.

Barbituric acid: parent structure of the barbiturates



Parent structure of the xanthines



Parent structure of the steroidal compounds

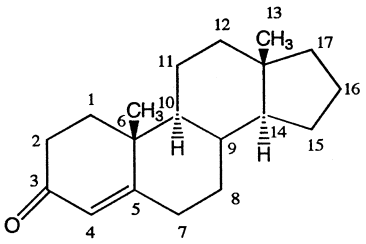


Figure 3. Parent structures of each group of compounds used in this study identifying atomic positions by numbers.

Mobile phases were prepared from chromatographic grade acetonitrile and deionized water from a Milli-Q water system (Millipore, Tokyo, Japan). Samples were dissolved in high grade methanol to the concentration of 100ppm. Other reagents used are of highest purity. Uracil was used as the column dead-volume marker.

Results reported are averages of triplicate determinations. Molecular modeling was carried out using Chem 3D Plus software (Cambridge Scientific Computing Inc., MA, USA).

RESULTS AND DISCUSSION

Barbiturate Analysis

Six barbiturates and barbituric acid with corresponding structures given in Figures 2 & 3, were subjected to chromatographic analysis using cholesteryl-10-undecenoate and ODS phases. The elution order was slightly different. For cholesteryl, using 10% acetonitrile in water, amobarbital and mephobarbital co-eluted while on the ODS phase, using 35% acetonitrile in water, these two were successfully resolved as shown in Figure 4A and 4B. However, note that for ODS, metharbital and phenobarbital were unresolved but were separated on the cholesteryl phase. Decreasing the amount of acetonitrile, did not improve the separation for the cholesteryl phase. However, for the ODS, separation of the two unresolved peaks were possible at increased water concentration but the retention times were increased considerably.

It is interesting to note that there is an elution order reversal for the two peaks, metharbital and phenobarbital with increasing water content in the mobile phase, Figure 4B and 5B. This is possibly due to solvophobic interactions, i.e., the increased polarity of the mobile phase contributes to the increased sorption of the less polar solute molecule with the stationary phase. The degree of solvation of the two samples, or the partitioning between the mobile phase and the stationary phase, at this mobile phase composition may have changed and thus the reversal in the elution order.

Barbituric acid had the least retention possibly due to the presence of the three carbonyl groups, C=O, at positions 2-, 4- and 6- which make it highly polar, see numbered positions in Figure 3. The presence of alkyl groups at position 5- increased the retention as in the case of barbital which is a 5,5-diethyl derivative. Metharbital which differs only with barbital in that it has the methyl group at N-1 eluted next. This is consistent with the findings we have earlier reported[1] that is, the presence of a CH_3- substituted amine will have increased interaction with the cholesteryl phase. Phenobarbital eluted next and it is characterized by the presence of an aromatic ring in place of the second ethyl group at position 5. This aromatic ring can undergo π-π interaction with the cholesteric moiety in the bonded phase. Amobarbital eluted after phenobarbital. Instead of the second ethyl group at position 5, it is characterized by the presence of a branched alkyl chain, 3-methylbutyl. The longer the hydrocarbon chain, the greater is the interaction with the cholesteric phase. Mephobarbital, which is similar in structure with phenobarbital except for the additional CH_3- at N-3 eluted next. Again the presence of a CH_3-substituted amine increased the retention. However, separation of amobarbital and phenobarbital in the cholesteryl phase was not possible unless a highly polar mobile phase is used which can make the retention time of the other barbiturates

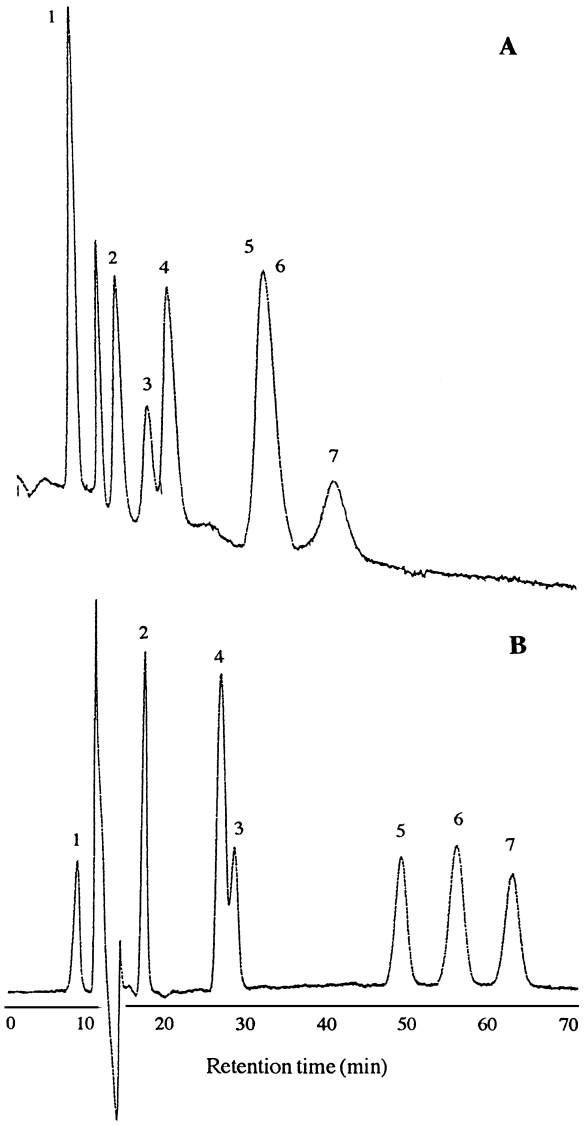


Figure 4. Separation of the barbiturates in cholesteryl-10-undecenoate bonded phase using 10% acetonitrile in water, **A** and in ODS phase using 35% acetonitrile in water, **B**. Barbiturates used are as follows: 1, barbituric acid; 2, barbital; 3, metharbital; 4, phenobarbital; 5, amobarbital; 6, mephobarbital; and 7, secobarbital.

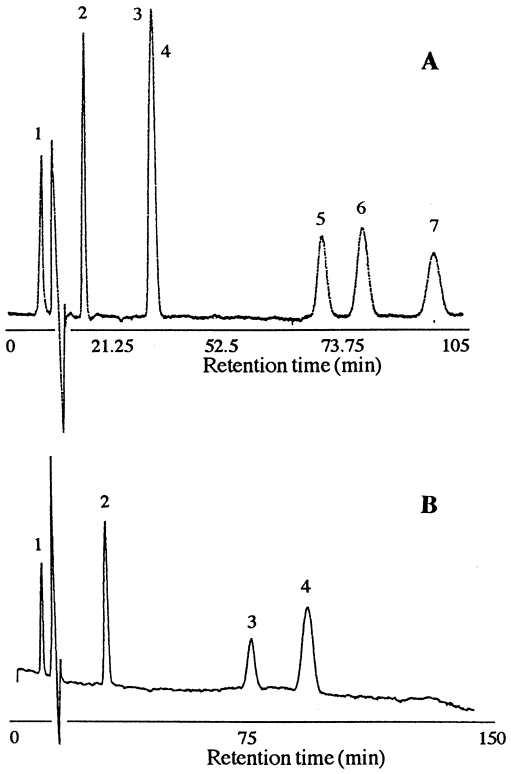


Figure 5. Chromatograms showing elution order reversal for 3, metharbital, and 4, phenobarbital in ODS phase using 30 % acetonitrile in water, **A** and 20% acetonitrile in water, **B**. Peaks are numbered as in Figure 4.

too long. Finally, secobarbital has the highest retention due to the presence of the allyl group at position 5- which can undergo possible π-π interaction with the cholesteric moiety and a branched alkyl chain, 1-methylbutyl, which has greater interaction with the long hydrocarbon arm of the cholesteryl phase.

We can therefore conclude that the presence of alkyl substituents at N-1 or N-3 can increase retention of the barbiturates. The longer the alkyl chain substituent at C-5 the longer is the retention. Barbiturates containing branched alkyl chains are retained over those containing unbranched alkyl chains. Presence of an aromatic substituent at C-5 also affects the retention through possible π-π interaction with the cholesteric moiety.

Separation of Xanthine Derivatives

The three xanthine derivatives used in this study are also shown in Figure 2. They were successfully separated by the cholesteryl phase using 10% acetonitrile in water, Figure 6A. In the ODS phase the theophylline and theobromine co-eluted, as shown in Figure 6B, even at lower organic modifier concentration. Again the presence of the methyl groups determined the order of elution. Caffeine which is a 1,3,7-trimethylxanthine has the greatest retention. Theobromine and theophylline are both dimethyl xanthines with methyl groups at positions N-3 and N-7 and at positions N-1 and N-3 respectively, see Figure 3B. Theophylline eluted after theobromine. The presence of the methyl groups at N-1 and N-3 of the theophylline can make the carbonyl groups at C-2 and C-6 less polar and thus may translate into enhanced retention for this compound compared with theobromine.

In the ODS phase, the dimethyl derivatives were poorly resolved even with increasing water content. The molecular recognition capability of the cholesteryl phase seem to be greater than the ODS phase for isomeric pairs in this case as shown by the retention behavior of theobromine and theophylline.

Analysis of the Cold Medicine Mixture

The cold medicine mixture consisted of aspirin, acetaminophen, caffeine and phenacetin, see Figure 2. At 20% acetonitrile concentration in water, complete separation was achieved with the cholesteryl phase, Figure 7A. Meanwhile in ODS phase at 40% acetonitrile concentration, they co-eluted, Figure 7B. Acetaminophen and caffeine can be separated using ODS phase at 15% acetonitrile concentration, Figure 7C. However at this low acetonitrile concentration phenacetin was retained strongly by the ODS phase with an elution time of 150 minutes as compared to cholesteryl with 26 minutes at 20% acetonitrile concentration.

Aspirin has very small interaction with both the ODS and cholesteryl phase eluting almost at the same time with uracil (dead-volume marker). This is because it is highly polar at this condition. The presence of the two carbonyl groups also contributed to its decreased interaction with the cholesteryl phase. Acetaminophen which is less polar than aspirin eluted next. The presence of a p-methoxy group in phenacetin, instead of a phenolic -OH as in acetaminophen enhanced the retention of phenacetin. Caffeine has intermediate retention to that of acetaminophen and phenacetin although it is characterized by the presence of two fused ring systems. The cholesteryl phase therefore showed better resolving power than ODS in this separation.

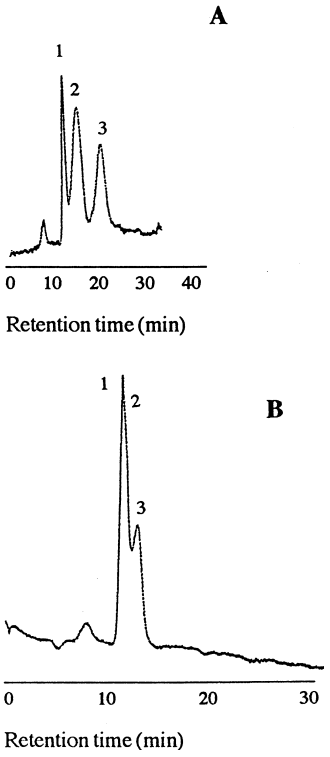


Figure 6. Chromatograms showing the separation of the xanthines in cholesteryl-10-undecenoate bonded phase at 10% acetonitrile in water, **A** and in ODS phase at 40% acetonitrile in water, **B**. Xanthines used are as follows: 1, theobromine; 2, theophylline; and 3, caffeine.

Analysis of Steroidal Compounds

The separation of the steroidal compounds, structures also given in Figure 2, in cholesteryl and ODS phases are comparable basing it on the elution order, Figure 8A and 8B. However, note that the amount of the organic modifier using ODS phase is twice that used in the cholesteryl separation. The retention times are almost similar indicating that hydrophobic effects are in greater effect when ODS phases are utilized although better peak symmetries were noted.

The order of elution is based on subsequent modifications done of the structure, specifically on C-17, see Figure 3. For example hydrocortisone acetate, 17-acetyl derivative of hydrocortisone eluted after hydrocortisone and

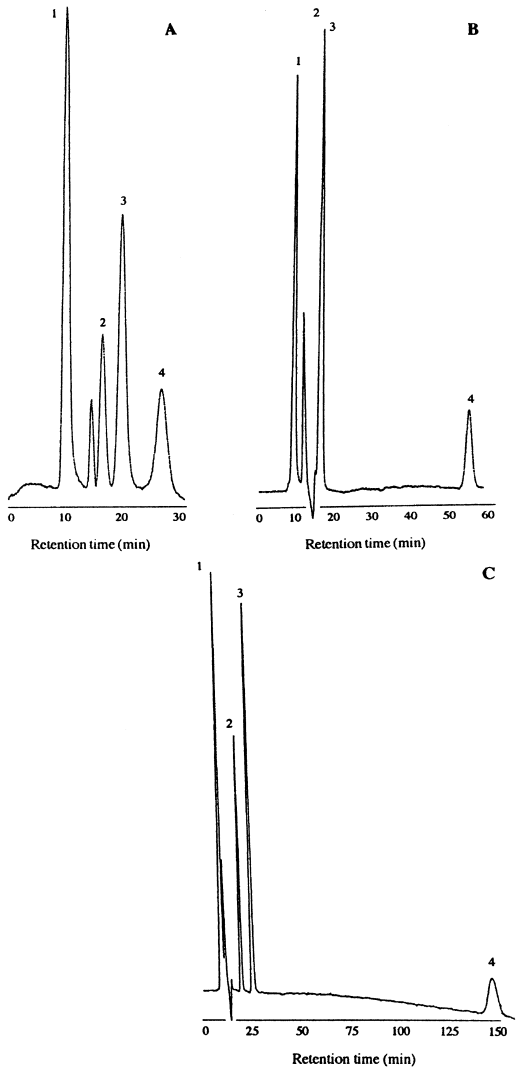


Figure 7. Chromatograms showing separation of the components of a cold medicine mixture in cholesteryl-10-undecenoate bonded phase at 20% acetonitrile in water, **A;** in ODS phase at 40% acetonitrile in water, **B** and at 15% acetonitrile in water, **C.** Cold medicine mixture components are as follows: 1, aspirin; 2, acetaminophen; 3, caffeine; and 4, phenacetin.

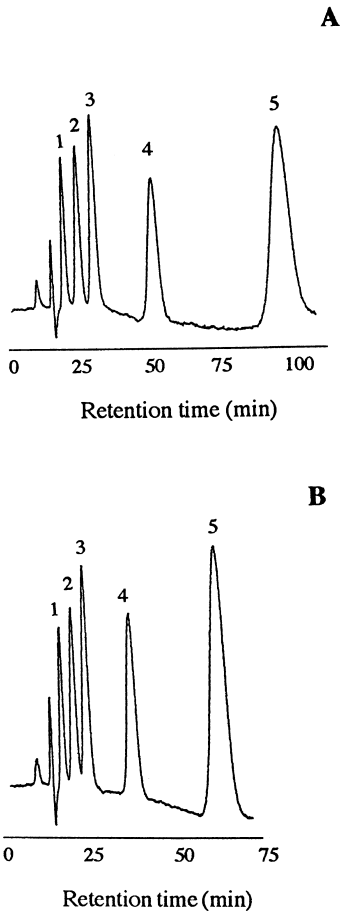


Figure 8. Chromatograms showing separation of steroidal compounds in cholesteryl-10-undecenoate bonded phase at 35% acetonitrile in water, **A** and in ODS phase at 70% acetonitrile in water, **B**. Steroidal compounds used are as follows; 1, hydrocortisone; 2, hydrocortisone acetate; 3, testosterone; 4, progesterone; and 5, testosterone propionate.

testosterone propionate, 17-propionyl derivative of testosterone, after testosterone. It would have been interesting to test further using other derivatives but samples were not available at present time. This will be the subject of another study using testosterone derivatives, specifically phase 2 metabolites, produced in-vitro by enzyme assisted conjugation. But, suffice it to say at this juncture, that the cholesteryl phase is a good alternative, if not better, to ODS phase in steroidal separations.

CONCLUSION

This follow-up study on the retention behavior of the cholesteryl phase indicate that this phase has special selectivity which is entirely different from that of ODS phase. For special applications involving compounds which are structurally similar and differing only in additional substituents either they be analogous, isomeric, or with other structural modifications the cholesteryl-10-undecenoate bonded phase may yet prove to be a better alternative to the various alkyl bonded phases already in use. It has high molecular recognition capability and thus it shows high selectivity. The presence of three specific structures in the cholesteryl phase contribute to the selectivity; the long hydrocarbon chain, the carbonyl group, and the cholesteric moiety. The liquid crystalline property of this phase also enhances the selectivity. The implication to the over-all separation performance of all these contributing factors is therefore considerable and thus makes this bonded phase ideal for these types of pharmaceutical applications.

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