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Comparison of the liquid chromatographic behaviour of selected steroid isomers using different reversed-phase materials and mobile phase compositions

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ABSTRACT

The retention behaviour of steroid isomers in reversed-phase liquid chromatography was studied. Selectivity for the isomers changed as a function of both temperature and mobile-phase composition. In addition, the influence of the stationary phase on steroid retention and selectivity was studied for a number of monomeric and polymeric C_{18} columns and β-cyclodextrin columns. The selectivity of a polymeric C_{18} phase toward various androstane standards was similar to that for a liquid crystal phase used in supercritical fluid chromatography.

INTRODUCTION

Different modes of liquid chromatography (LC) have long been used for the analysis of steroid hormones. The techniques used include early studies with paper, partition, ion-exchange, adsorption and gel chromatography [1,2]. Today, high-performance liquid chromatography (HPLC) is becoming a widely used technique for the separation of steroids [3], although most applications still utilize other techniques such as gas chromatography (GC) or thin-layer chromatography. The applications of HPLC for the determination of steroids are numerous and have recently been reviewed [4]. A significant limitation to the use of HPLC for steroid analysis is detection, and many steroids require derivatization for detection with available LC detectors. Low efficiency is another limitation in the LC separation of steroids. On the other hand, enhanced separation selectivity can often be achieved for various steroids by selecting from the numerous packing materials and coatings available.

Steroid analyses by HPLC can be performed in both normal- and reversed-phase modes, the latter being more widely used. Bonded octadecylsilane (C_{18}) is the most commonly used reversed-phase material, although other supports and coatings can be utilized. It is well known that great differences exist among C_{18} stationary phases

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owing to differences in properties such as the silica matrix, pore diameter, phase synthesis and ligand density [5–7]. Reaction of octadecylsilane with the silica matrix can be carried out in different ways, yielding phases with different properties. Monomeric phases are prepared either with monofunctional silanes or with di- or trifunctional silanes in the absence of water. Polymeric phases, on the other hand, are prepared using trifunctional silanes in the presence of water [8,9]. Polymeric phases have enhanced shape selectivity towards polycyclic aromatic hydrocarbons (PAHs) compared to monomeric C_{18} phases. It has been suggested that this shape recognition ability is a result of ordering of alkyl chains within the polymeric phase, similar to liquid crystalline phases in GC [10]. Such liquid crystalline phases have been used in supercritical fluid chromatography (SFC) for the separation of isomeric steroids (*e.g.*, androstanes and estrogens), and better separations were possible than with non-shape-selective phases [11]. Shape recognition for isomeric compounds has also been demonstrated using cyclodextrin bonded phases [12,13]. Cyclodextrin has been reported as both a mobile phase additive [14,15] and a bonded phase [16] for the separation of steroid isomers.

As steroid hormones and their numerous metabolites are structurally very similar, enhanced shape selectivity is an important consideration when selecting a suitable system for their separation. Apart from the type of stationary phase used, selectivity is dependent on parameters such as mobile phase composition and temperature [17,18]. Because mobile phase composition has a dramatic effect on column selectivity for steroids, the need for careful investigation of composition parameters in method development is crucial.

Stationary phase selectivity towards steroids can be further modified by varying the column temperature. It has been shown recently that the column selectivity toward PAHs and carotenoids changes with temperature to the extent that the elution order may be reversed [19,20]. This trend has also been observed with certain steroid isomers. The use of subambient temperature for enhanced resolution of cortisol and cortisone on a μBondapak C_{18} column has been reported [21].

In this work, the retention behaviour of some selected steroid isomers was investigated. The stationary phases used in this study differed in phase type (*e.g.*, monomeric or polymeric) and ligand type. Dramatic changes in selectivity have been observed with changes in mobile phase composition and temperature.

EXPERIMENTAL[a]

Materials

Steroid standards were obtained from Sigma (St. Louis, MO, U.S.A.). Methanol, acetonitrile and water (all HPLC grade) and 1,4-dioxane and tetrahydrofuran (both analytical-reagent grade) were obtained from commercial suppliers.

[a] Certain commercial equipment, instruments or materials are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Columns

Standard solutions of steroid isomers were separated on the following columns: (1) polymeric C_{18} columns: Vydac 201 TP (Separations Group, Hesperia, CA, U.S.A.), Bakerbond wide-pore C_{18} (J. T. Baker, Phillipsburg, NJ, U.S.A.) and Erbasil C_{18}/H (Carlo Erba, Milan, Italy); (2) intermediate C_{18} columns: Vydac TP (custom synthesis, low load) and Bakerbond narrow-pore C_{18}; (3) monomeric C_{18} columns: Zorbax ODS (MAC-MOD Analytical, Wilmington, DE, U.S.A.), ODS Hypersil C_{18} (Keystone Scientific, State College, PA, U.S.A.) and Supelcosil LC-18-DB (Supelco, Bellefonte, PA, U.S.A.); and (4) cyclodextrin columns: Cyclobond I and Cyclobond I acetylated (β-cyclodextrin) (Astec, Whippany, NJ, U.S.A.). All the columns were 250 × 4.6 mm I.D. with 5-μm particle diameter silica.

Chromatography

A liquid chromatograph consisting of a reciprocating piston pump, a solvent programming system, a 20-μl loop injector and a 254-nm fixed-wavelength detector was used for the separation of the estrogen standards. For the androstane standards (which have very low UV absorbance), a refractive index detector was placed in series with the UV detector.

Retention data were determined using a chromatography data system (inital studies) and, in later studies, an integrator. All samples were run isocratically with aqueous organic mobile phases, and the solutes were dissolved in methanol prior to injection. Androstane standards were eluted with aqueous methanol mobile phases at 1.5 ml/min. The methanol content varied between 60 and 90% depending on the polarity of the solutes. The organic content in the mobile phases for elution of the estradiol standards in the column comparison studies was varied in order to achieve comparable capacity factors (k') between columns.

The column temperature was controlled either by placing the column in a block heater or, for the subambient studies, in an ice-bath where the column temperature was maintained at 0°C.

RESULTS AND DISCUSSION

The chromatographic selectivity of liquid crystalline phases in GC and SFC, for the separation of androstane isomers, is based on the size and shape of the steroids [11,22]. This shape discrimination ability results from the high degree of order in the liquid crystalline phase. Using similar stationary phases, selectivity is enhanced in SFC compared to GC because a lower operating temperature can be used. This produces a higher degree of order in the stationary phase and results in an increase in the strength of solute–stationary phase interactions [11].

Among LC columns, selectivity is often strongly influenced by the way in which the stationary phase is prepared. Most commercial C_{18} phases are prepared by reaction of monochlorosilanes with silica (monomeric C_{18} phases); however, a few (polymeric C_{18} phases) are prepared with polyfunctional silanes in the presence of water. These two types of bonded phases often have dissimilar properties, particularly when retention behaviour is compared for the separation of isomers or other compounds with similar overall shape. The relative monomeric and polymeric retention character of C_{18} columns can be characterized by Standard Reference

TABLE I

RETENTION DATA FOR ANDROSTANES (t_R/t_R)

Compound	Retention relative to	Monomeric C_{18} ($\alpha_{TBN/BaP} = 1.94$)	Polymeric C_{18} ($\alpha_{TBN/BaP} = 0.67$)	Monomeric C_{18} ($\alpha_{TBN/BaP} = 2.01$)
5β-Androstane-3β,17β-diol	5β-Androstane-3β,17β-diol	1.00	1.00	
5α-Androstane-3β,17β-diol		1.04	1.29	
5β-Androstane-3α,17β-diol		1.39	1.26	
5α-Androstane-3α,17β-diol		1.44	1.37	
5β-Androstane-3α,11β,17β-triol	5β-Androstane-3α,11β,17β-triol	1.00	1.00	1.00
5β-Androstane-3α,11α,17β-triol		1.24	1.28	1.22
5β-Androstane-3β,17β-diol		1.87	2.33	1.58
5α-Androstane-3β,17β-diol		1.91	3.24	1.58
5β-Androstane-3α,17β-diol		2.54	2.88	2.18
5α-Androstane-3α,17β-diol		2.71	3.40	2.28
5β-Androstane-3α-ol	5βAndrostane-3α-ol	1.00	1.00	
5β-Androstane-3β-ol		1.25	1.35	
5β-Androstane-17β-ol		1.29	1.21	
5α-Androstane-3β-ol		1.31	1.74	
5α-Androstane-17β-ol		1.52	1.90	

Material (SRM) 869. This material contains three PAH solutes that have planar and non-planar features {benzo[*a*]pyrene (BaP), phenanthro[3,4-*c*]phenanthrene (PhPh) and 1,2:3,4:5,6:7,8-tetrabenzonaphthalene (TBN)}, and is primarily intended for characterizing LC column selectivity for solute shape. The elution order of the three components changes with C_{18} phase type. Monomeric C_{18} phases give the elution order BaP $\leqslant$ PhPh $<$ TBN whereas polymeric C_{18} phases usually give the elution order PhPh $<$ TBN $\leqslant$ BaP. A quantitative measure of phase selectivity can be calculated to enable relative comparison between different C_{18} phases. The selectivity factor $\alpha_{TBN/BaP}$ is defined as k'_{TBN}/k'_{BaP}, where $k' = (t_R - t_0)/t_0$ and t_R and t_0 are the retention times of the analyte and void volume marker, respectively. Among commercial C_{18} columns, values for $\alpha_{TBN/BaP}$ range from about 0.6 to 2.0, with $\alpha_{TBN/BaP} \leqslant 1$ reflecting "polymeric-like" selectivity and $\alpha_{TBN/BaP} \geqslant 1.7$ reflecting "monomeric-like" selectivity (selectivity values $1 < \alpha_{TBN/BaP} < 1.7$ denote intermediate selectivity). In general, better separations of compounds with similar overall structure are possible with polymeric C_{18} phases compared to monomeric C_{18} phases. This trend is most often observed for non-polar solutes, in the absence of polar–polar (*i.e.*, silanol–solute) interactions.

Liquid crystalline phases in GC and polymeric C_{18} phases in HPLC exhibit remarkably similar selectivity towards PAHs. In both cases, this behaviour is based primarily on the shape and size of the solute [10]. Androstane isomers can exist in two structural conformations, which results from the position of the hydrogen atom at C-5: *trans* (α) or *cis* (β) conformations (see Fig. 1). Chang *et al.* [11] showed that the retention of these isomers on a liquid crystalline phase is clearly affected by this conformational difference, and the bulkier *cis*-isomer (β) is less retained than the more planar *trans*-isomer (α), which is more soluble in the ordered phase. This same trend was observed for LC separations performed on C_{18} bonded-phase columns. Androstane standards and relative retention data are listed in Table I. Zorbax ODS and Supelcosil LC-18-DB columns are monomeric C_{18} phases; Vydac 201 TP is a polymeric C_{18} phase. In each instance, the *cis*-isomer (5β-, nonplanar) elutes before the corresponding *trans*-isomer (5α-, planar). This trend was observed for both monomeric and polymeric C_{18} phases; however, resolution of 5α- and 5β-an-

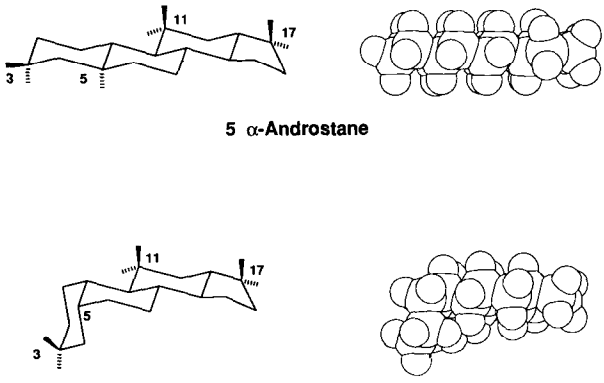


Fig. 1. Chemical structures and space-filling models for *cis* and *trans* conformational isomers of androstane. Wedge bonds denote α-stereochemistry; hatched bonds denote β-stereochemistry.

drostanediol isomers was only possible with polymeric C_{18} phases. The fact that α- and β-androstanediol isomers are separated on polymeric C_{18} phases, and are not separated on monomeric C_{18} phases, corresponds to similar observations reported for the separation of PAH isomers [7]. In general, polymeric C_{18} phases are more selective toward isomer shape and are more likely to resolve isomer mixtures than are monomeric C_{18} phases, at least when other effects such as silanol or other polar stationary phase interactions are insignificant.

The stereochemical position of the hydroxyl groups at positions 3 and 17 significantly affects the retention of androstanediols. The retention behaviour of four androstanediols was studied (3β,17β- and 3α,17β-diol isomer combinations of the 5α- and 5β- structures shown in Fig. 1). Separations of androstanediol isomers differing only in the position of the hydroxyl at C-3 were possible for both monomeric and polymeric C_{18} phases. For example, 5β-androstane-3β,17β-diol was fully resolved from 5β-androstane-3α,17β-diol regardless of column type. As the position of the hydroxyl group does affect polarity, but has little effect on the overall shape of the molecule, it seems reasonable to assume that the separation of these critical pairs depends more on solute polarity than on solute shape. Hydrogen bonding interactions with hydroxyl groups may also contribute to retention processes. The position of solute hydroxyl groups (*i.e.*, axial or equatorial) affects the accessibility and strength of any such interactions. This is consistent with the overall similar retention behaviour observed for these solutes on monomeric and polymeric C_{18} phases. Differences in retention behaviour would be expected between these two classes of columns, for solute pairs with dissimilar overall shape.

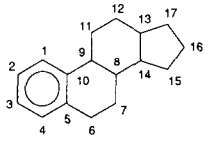
The effect of column temperature on the separation of various steroids was examined. Because the individual C_{18} ligands constituting the bonded phase straighten at reduced temperature, the shape recognition ability of alkyl bonded phases increases with decreasing column temperature. Stated differently, at subambient temperatures monomeric C_{18} phases gain some of the shape recognition properties exhibited by polymeric C_{18} phases at ambient temperature. At subambient temperatures, the shape recognition ability of polymeric C_{18} phases increases even more. Thus, shifts in retention behaviour for steroids are expected for the monomeric and polymeric phases. In fact, such changes in selectivity were observed when separations were carried out at 0°C, similar to the trends reported for PAHs [19]. The α- and β-androstanediol isomers were separated better at subambient than at ambient temperatures. This effect was also observed by Sheikh and Touchstone for various steroid isomers [23]. The opposite trend was observed when the polymeric C_{18} phase (Vydac 201TP) was heated to +50°C and the androstanediols were eluted as on the monomeric C_{18} phase. These results correspond with earlier observations concerning phase morphology [19]. At elevated temperatures the individual C_{18} ligands become more disordered than at ambient temperature, and the result is a loss in shape recognition ability. This is most dramatic for polymeric C_{18} phases, for which a high degree of shape recognition exists at ambient temperature. Correspondingly, when the polymeric C_{18} phase is cooled to 0°C, the distinction between α- and β- (planar and non-planar) isomers becomes even stronger.

The results for the androstane isomers are similar to trends in retention observed for PAHs, for which solute shape and phase morphology are important aspects of the retention mechanism. Because of the difficulties in the detection of androstane

steroids, methods other than LC may prove to be simpler and more practical for routine analysis (LC may prove most useful in preparative applications).

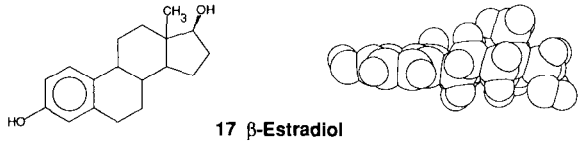
An analytical problem more suited for HPLC is the analysis of the UV-absorbing estrogens (Fig. 2). Estrogens are produced in large amounts in the female body and are also produced as pharmaceutical products for clinical therapy. Determination of the female hormones in both biological fluids and in other media is required and, owing to the close similarity of the isomers, chromatographic separation is difficult. The selectivity and resolution of a set of estrogen standards were investigated on a number of different C_{18} columns (see Table II). These columns ranged from heavily loaded polymeric C_{18} columns ($\alpha_{TBN/BaP} = 0.38$, normally associated with high shape discrimination) to monomeric C_{18} columns ($\alpha_{TBN/BaP} = 2.01$, associated with low shape discrimination). However, little or no difference in selectivity based on solute shape and size was observed between monomeric and polymeric C_{18} columns. This similarity in retention behaviour with different phases is perhaps not unexpected, as 17α- and 17β-estradiol differ only in the position of the hydroxyl group at C-17, and have very similar overall shapes (see Fig. 2). A more dramatic effect on selectivity has been observed with changes in the mobile phase composition [17,24,25].

During this investigation it was observed that 17α- and 17β-estradiol were strongly affected by mobile phase changes. They were selected as probe solutes,

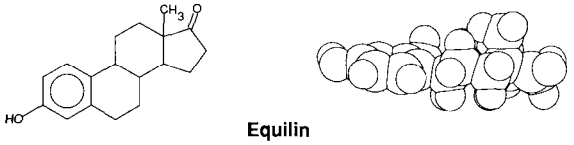


Estrogens

17 α-Estradiol



17 β-Estradiol



Equilin

Fig. 2. Chemical structures and space-filling models for 17α- and 17β-estradiol isomers and equilin. Separation of these solutes was strongly affected by the mobile phase composition.

TABLE II

RELATIVE RETENTION DATA FOR ESTROGENS WITH DIFFERENT MOBILE PHASES

These data were determined for individual columns and do not necessarily reflect representative column performance. Intended or unintended variations in the manufacturing process may alter retention behaviour between column types.

Mobile phase	Column	$\alpha_{TBN/BaP}$	$\alpha_{17\alpha\text{-estradiol/equilin}}$	$\alpha_{17\beta\text{-estradiol/equilin}}$
Aqueous methanol	Polymeric high load	0.38	1.29	1.26
	Polymeric int. load	0.54	1.30	1.30
	Polymeric int. load	0.60	1.15	1.30
	Polymeric normal load	0.67	1.22	1.41
	Polymeric low load	1.00	1.10	1.26
	Intermediate	1.12	1.28	1.41
	Intermediate	1.32	1.18	1.26
	Monomeric	1.94	1.28	1.12
	Monomeric	1.98	1.32	1.15
	Monomeric	2.01	1.33	1.23
Aqueous acetonitrile	Polymeric high load	0.38	0.84	0.72
	Polymeric int. load	0.54	0.85	0.79
	Polymeric int. load	0.60	0.82	0.80
	Polymeric normal load	0.67	0.84	0.84
	Polymeric low load	1.00	0.85	0.81
	Intermediate	1.12	0.90	0.85
	Intermediate	1.32	0.85	0.79
	Monomeric	1.94	0.82	0.65
	Monomeric	1.98	0.84	0.67
	Monomeric	2.01	0.85	0.72

together with equilin, which has a k' close to that of estradiol in the systems investigated. Equilin was chosen as a reference for relative retention data. The 17α- and 17β-estradiol isomers have identical functional groups and differ only in the stereochemical conformation at position 17. Even though the isomers are structurally very similar, the elution order was reversed when the percentage of methanol, acetonitrile and water was varied (see Table III). Changes in mobile phase composition

TABLE III

RELATIVE RETENTION DATA FOR ESTROGENS ON VYDAC LOW LOAD

Mobile phase			$\alpha_{17\alpha\text{-estradiol/equilin}}$	$\alpha_{17\beta\text{-estradiol/equilin}}$
Methanol (%)	Acetonitrile (%)	Water (%)		
0	30	70	0.89	0.85
5	25	70	0.95	0.91
15	20	65	1.00	1.00
25	15	60	1.11	1.11
35	10	55	1.19	1.22
45	5	50	1.21	1.29
50	0	50	1.24	1.38

caused changes in the elution order of 17α-estradiol and equilin (also 17β-estradiol and equilin) as well as the elution order of 17α- and 17β-estradiol (*e.g.*, the 17β-isomer elutes first when using aqueous acetonitrile and last when using aqueous methanol), as shown in Fig. 3.

The composition and properties of the mobile phase were further changed by adding different organic modifiers (acetonitrile, methanol, 1,4-dioxane and tetrahydrofuran) to an aqueous phase. The organic modifiers were selected so that both proton donors and proton acceptors would be represented [17,24,26]. Relative retention data are presented in Table IV. Considering the very subtle differences in the estradiol isomers, it is difficult to explain how the solvent environment can effect retention in such a significant manner. It is possible that the methyl group at position 13 partially shields the hydroxyl at position 17 from interaction with solvent molecules. The extent of any such interaction would, of course, be influenced by the proton-donating and -accepting properties of the mobile phase. Although actual retention mechanisms can only be speculated on at this point, the importance of the choice of the organic modifier in the development of a separation method is clear.

The retention behaviour of estradiols was also studied for a bonded β-cyclodextrin (β-CD) stationary phase. Peak tailing was severe, probably owing to hydrogen

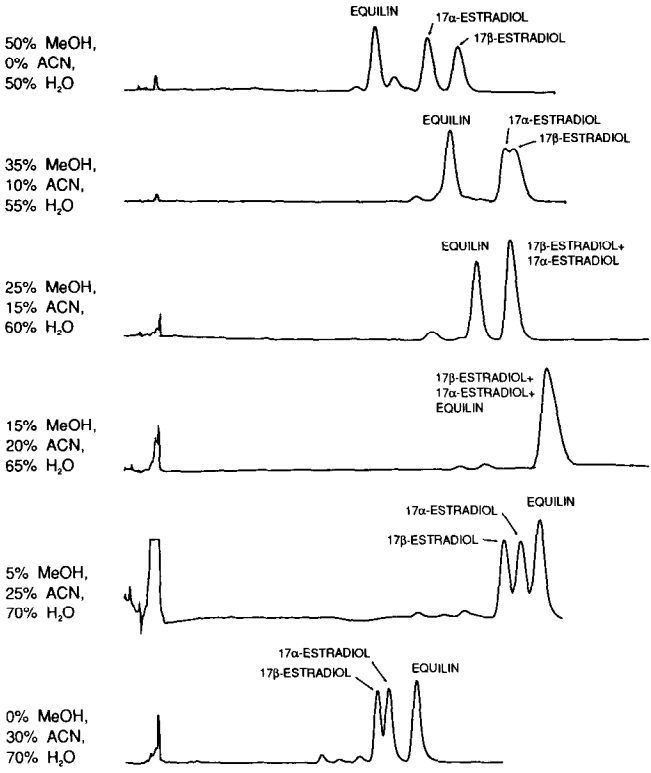


Fig. 3. Separation of α- and β-estradiol and equilin using various mobile phase compositions with a low-load polymeric C_{18} column. MeOH = methanol; ACN = acetonitrile.

TABLE IV

RELATIVE RETENTION DATA FOR ESTROGENS ON BAKERBOND NARROW-PORE

Mobile phase	$\alpha_{17\alpha\text{-estradiol/equilin}}$	$\alpha_{17\beta\text{-estradiol/equilin}}$
Acetonitrile–water (35:65)	0.79	0.77
Tetrahydrofuran–water (35:65)	1.05	0.83
Methanol–water (60:40)	1.17	1.20
Dioxane–water (50:50)	1.01	0.84

bonding between hydroxyls of the estrogens and hydroxyls on the outside of the cyclodextrin cavity. Relative retention data were collected on a β-CD and on an acetylated β-CD. The peak shape was slightly improved with the acetylated β-CD. The selectivity on the acetylated β-CD resembles that on C_{18} materials, and the same reversal of elution order was observed between the ketone and hydroxyl isomers when changing the mobile phase from aqueous methanol to aqueous acetonitrile. However, the relative retention of 17α- and 17β-estradiol did not change with mobile phase composition, as shown in Fig. 4. As demonstrated for 17α- and 17β-estradiol, specific solute–stationary phase interactions with cyclodextrin bonded phases can give rise to enhanced selectivity for isomers and other structurally closely related solutes.

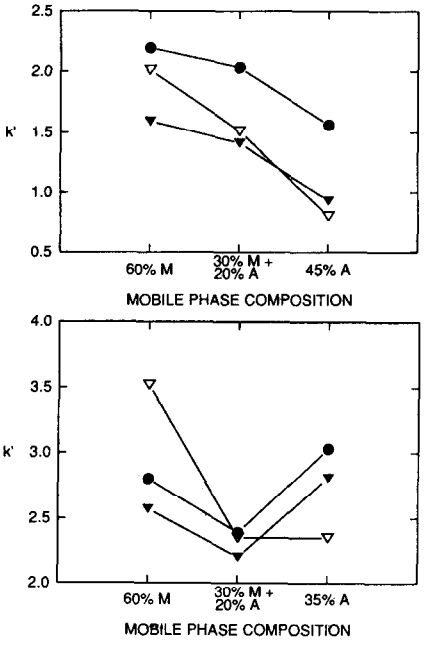


Fig. 4. Plots of retention *versus* mobile phase composition for aqueous methanol (M) and acetonitrile (A) mixtures, for cyclodextrin columns. Similar, but not identical, trends were observed for C_{18} phases. Top: β-cyclodextrin; bottom: acetylated β-cyclodextrin. ▼ = 17α-Estradiol; ● = 17β-estradiol; ▽ = equilin.

CONCLUSIONS

The retention behaviour of steroid hormones in LC is complex and is related to the overall shape and stereochemical positions of functional groups. Steroid isomers with similar functional group stereochemistry but different overall molecular shape are separated on the basis of shape, in a manner similar to that observed for polycyclic aromatic hydrocarbons (*i.e.*, planar solutes are retained longer than non-planar solutes). In such instances, phase type and column temperature are important separation parameters, and better separations of critical solute pairs may be possible with polymeric C_{18} phases and/or low column temperatures. For similarly shaped steroid isomers, functional group position is of importance, and the separation of these critical pairs is more strongly affected by mobile phase composition than phase type.

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