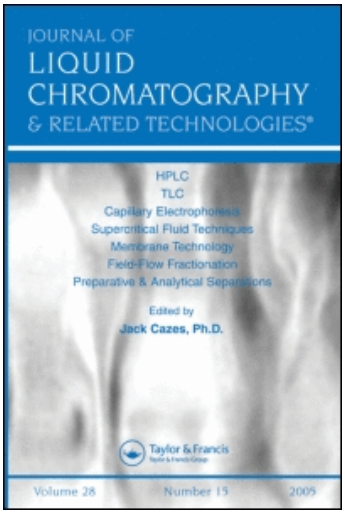

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Behavior of Stereoisomeric Steroids in Liquid Chromatography

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BEHAVIOR OF STEREOISOMERIC STEROIDS IN LIQUID CHROMATOGRAPHY

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ABSTRACT

From literature data on the behavior of diastereoisomers, the author derives empirical rules governing their relative mobilities in liquid-solid and liquid-liquid chromatographic systems. Applications of these rules to the resolution of steroid mixtures, belonging to the various classes of steroids, are cited.

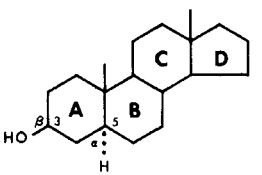
INTRODUCTION

The separation of steroids by chromatography is of considerable interest, not only because they are compounds of major biological importance, but also because they constitute a large group of organic molecules with a rigid backbone and numerous possibilities for isomerism. This makes them ideal models on which chromatographic methods and theories can be tested. No wonder that an immense volume of literature on the chromatography of steroids has accumulated. The older literature has been reviewed by Neher (1), and the literature since 1964 has been summarized by Heftmann (2). The development of this field has been surveyed at intervals since 1961 (3-5).

In this article I hope to develop a coherent picture of the present state of knowledge concerning the effect of stereoisomerism on mobility in liquid chromatographic systems. Having witnessed the failure of premature attempts at constructing an all-embracing theoretical framework (6), I shall be careful to limit myself to an essentially empirical and qualitative approach. To my knowledge, this subject has not been dealt with before, but references to review articles and papers on various other aspects of the liquid chromatography of steroids will be found in the bibliographies of the books cited so far (1-6). Instead of covering the literature exhaustively, I shall only cite a few examples of the separation of diastereoisomers, partly on the basis of my own experience. The bibliography of these articles will lead the reader to the older literature.

CONFORMATION OF STEROIDS

The conventional representation of the steroid nucleus provides no clue to the differences in chromatographic behavior



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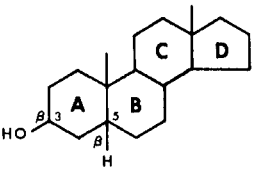

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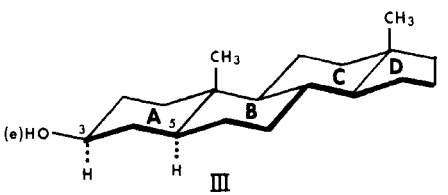

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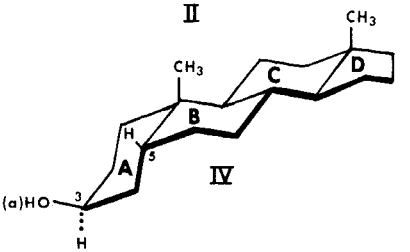


IV

between compounds belonging to the A/B trans-(I) and A/B cis-(II) series. But the inspection of the corresponding conformational structures III and IV, respectively, or, better yet, the construction of molecular models reveals the stereochemical details responsible for those differences.

Theoretically, the four rings which constitute the cyclopentanoperhydrophenanthrene nucleus could be in either the chair or boat conformation. In all natural steroids, they are in the chair conformation. Rings A and B may be cis- or trans-fused, but in all natural steroids the B/C ring juncture is trans and in most of them (with the notable exception of the cardenolides and bufadienolides) the C/D juncture is also trans. When Rings A and B are trans-fused (III), a flat, lath-like structure results, but in the A/B cis-steroids (IV) Ring A forms an angle with the rest of the molecule. Steroids which are unsaturated at C-5 (Δ^4-, Δ^5-compounds and steroids with aromatic Ring A) assume a shape similar to the A/B trans-series.

The A/B cis-steroids are usually less adsorbed on polar adsorbents than the A/B trans-steroids and, in adsorption chromatography, the A/B cis-steroids are usually eluted before the A/B trans-steroids, provided, of course, that all the other groups in the two compounds being compared are the same.

The 3-hydroxyl group, present in most natural steroids, presents a special problem in this respect. As is evident from the conformational formulae, the 3β-hydroxyl group is equatorial in the A/B trans-steroids (III), but it is axial in the A/B cis-

steroids (IV). As it happens, the effect of the conformation of that hydroxyl group on chromatographic behavior may be at least as great as that of the fusion between Rings A and B.

Usually, the equatorial hydroxyl group is more strongly adsorbed that the axial one. Thus, in the case of two A/B trans-steroids epimeric at C-3, the 3β-hydroxy compound may be more strongly adsorbed than the 3α-hydroxy compound, whereas under the same conditions, the analogous 3α-hydroxy steroid may be more strongly adsorbed than the 3β-hydroxy steroid if they belong to the A/B cis-series. The prediction of the chromatographic sequence of two analogs belonging to different series may thus be complicated by the relative intensity of the two factors if they have opposite effects on adsorption.

The separation of stereoisomers by partition chromatography is generally poorer than by adsorption chromatography, and no rules can be established for the relative mobilities of the A/B cis- and A/B trans-series. However, steroids with an equatorial hydroxyl group at C-3 can usually be relied upon to be more polar than analogous steroids with an axial hydroxyl group at C-3, regardless of the stereochemical configuration at C-5.

STEROLS

The sterols, which may have up to 32 carbon atoms, offer a multitude of opportunities for the chromatographic comparison of diastereoisomers, but there is a paucity of information on their behavior in liquid chromatography. This is due partly to the fact

that not many pure sterols are generally available and partly to the superiority of gas chromatography for this application.

The effect of stereochemical changes at C-5 on the adsorption of sterols is well illustrated by the elution pattern of their 3-keto analogs, which we have observed in high-pressure liquid chromatography (HPLC) (Fig 1) (7). When a mixture of 3-keto analogs was chromatographed on a 50-cm column of silica gel with a mixture of dichloromethane-n-hexane-ethyl acetate (94:5:1), 5β-cholestan-3-one was eluted first, followed by 5α-cholestan-3-one, then 5-cholesten-3-one, and 4-cholesten-3-one last. Thus, the conjugated

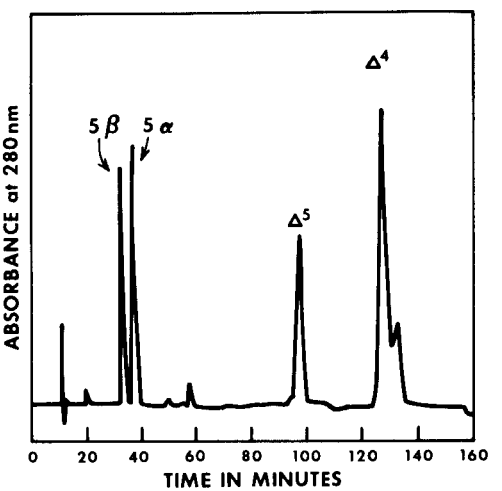


FIGURE 1 HPLC of the 3-Keto Derivatives of C_{27} Sterols

Silica gel column chromatogram developed with dichloromethane-n-hexane-ethyl acetate (94:5:1); 5β = 5β-cholestan-3-one, 5α = 5α-cholestan-3-one, Δ^5 = 5-cholesten-3-one, Δ^4 = 4-cholesten-3-one.

Reprinted from Hunter et al. (7) by permission of the American Oil Chemists' Society.

ketone was most strongly adsorbed, and the A/B cis-analog was least adsorbed on silica.

An instructive example of the behavior of C_{27} sterols epimeric at C-3 as well as C-5 in liquid-solid systems is the chromatography on lipophilic dextran gels (8). Table 1 gives the per cent total bed volume (PTV), i.e. the milliliters of benzene required to elute the individual sterols from a 100-ml column of the cross-linked dextran gel, Sephadex LH-20, substituted with hydroxycyclohexyl residues. The sorption mechanism is incompletely understood, but appears to be based largely on hydrogen bonding between the hydroxyl group of the sterol and the ether

TABLE 1

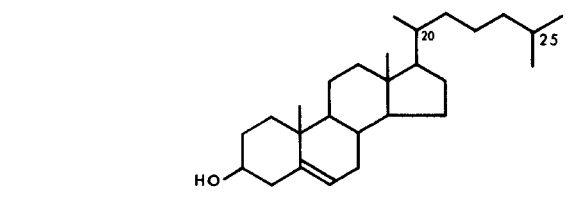
Elution of C_{27} Sterols from a Sephadex LH-20 Column by Benzene

Steroid		OH*	PTV**
Systematic name	Trivial name		
5-Cholesten-3α-ol	Epicholesterol	a	70.1
5α-Cholestan-3α-ol	Epicholestanol	a	82.9
5β-Cholestan-3α-ol	Epicoprostanol	e	87.0
5β-Cholestan-3β-ol	Coprostanol	a	87.0
5α-Cholestan-3β-ol	Cholestanol	e	88.4
5-Cholesten-3β-ol	Cholesterol	e	94.4

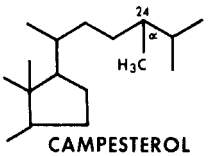
* Conformation: a = axial, e = equatorial
** Per cent total bed volume

linkage of the gel. Evidently, the effect of 3-isomerism on chromatographic behavior is greater than the effect of 5-isomerism, but it depends on the nature of the A/B ring junction. The equatorial hydroxyl group in cholesterol (V) makes it considerably more polar than epicholesterol, which has an axial hydroxyl group. The 5α-sterols show a behavior similar to that of the Δ^5-sterols, but the difference in PTV values between the two epimers, cholestanol and epicholestanol, is smaller. However, in the 5β-series there is no difference at all. Among the sterols with an equatorial hydroxyl group the A/B cis-steroid is eluted before the A/B trans-steroid, but the reverse is true for the sterols with an axial hydroxyl group.

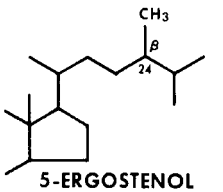
Stereoisomerism among sterols may result not only from the differential orientation of various nuclear substituents. Of much greater interest, from the standpoint of both biology and chromatography, are the differences in side-chain substituents, particularly the alkyl radicals at C-24. To avoid confusion, we shall, in this case, employ Fieser's nomenclature (9) rather than the IUPAC nomenclature (10). When the side chain of sterols is drawn as shown for cholesterol (V), we shall designate the methyl group at C-20 as β-oriented, and we shall picture other β-oriented side-chain substitutes above the zig-zag chain. Oppositely oriented substituents will be represented below the side chain and designated as α-oriented. In the partial structural formulae (VI-IX), the rest of the molecule is the same as in cholesterol (cf. V).



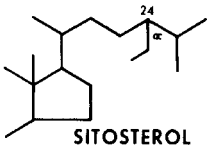
CHOLESTEROL
V



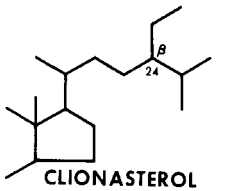
CAMPESTEROL
VI



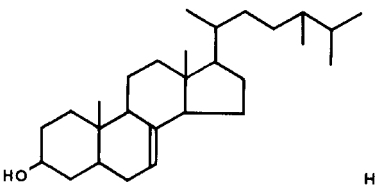
5-ERGOSTENOL
VII



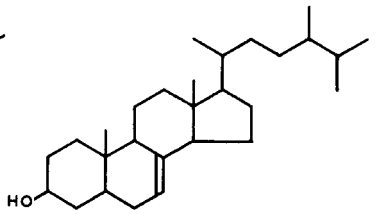
SITOSTEROL
VIII



CLIONASTEROL
IX



X



XI

Knights (11) has compared the mobilities of some 24-epimers in thin-layer chromatograms on Anasil B plates. When the sterols were chromatographed in the form of their acetates by continuous development with 1% ether in petroleum ether, it was found that the two 24α-methyl sterols (VI, X) moved faster than the corre-

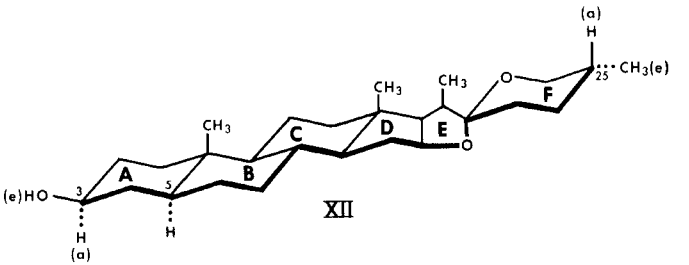
sponding 24β-methyl sterols (VII, XI), but the 24β-ethyl sterol (XI) was slightly more mobile than the corresponding 24α-ethyl sterol (VIII).

When one of the geminal CH_3 groups in the side chain of cholesterol (V) is oxidized to CH_2OH, C-25 becomes optically active. The resolution of the epimeric 25α(R)- and 25β(S)-26-hydroxycholesterols is a recent example of the power of HPLC (12). When a mixture of their diacetates was recycled 10 times through a 60-cm Microporasil column with 2.5% ethyl acetate in n-hexane, the less polar 25β-epimer emerged before the 25α-epimer.

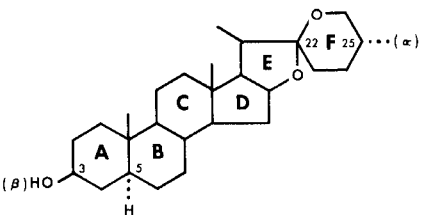
C_{27} SAPOGENINS AND ALKALOIDS

All C_{27} sapogenins have the same steric configuration at C-22, but the methyl group at C-25 may be either axial or equatorial (XII), being represented in the projection formula by a full or dotted line and designated as β- or α-oriented, respectively (XIII). In their nitrogen analogs, the C_{27} alkaloids, conversely, the configuration at C-25 is always the same (α), whereas two series of 22-epimers exist in nature, exemplified by soladulcidine (XIV) and tomatidine (XV). The chromatographic problems to be considered thus focus on the asymmetric carbons 3, 5, 22, and 25.

A method of separating sapogenins by thin-layer chromatography (TLC), which we described in 1962 (13), will serve to illustrate the behavior of epimeric sapogenins in adsorption and partition systems. The Δ^5-sapogenins are generally difficult to separate from the 5α-sapogenins, but a partition system, which we



XII



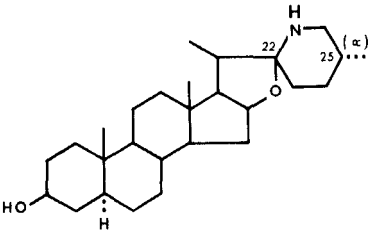
TIGOGENIN

XIII

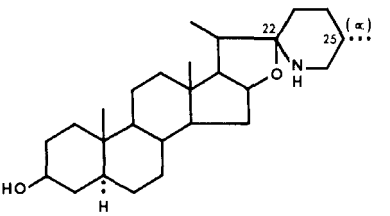
had devised for paper chromatography (PC) in 1952 (14), successfully resolved the 5β-, 5α-, and Δ^5-analogs. Development of Kieselguhr G plates with n-hexane-toluene-ethanol-water (20:10:1:9) moved the A/B cis-sapogenin smilagenin (5β, 25α-spirostan-3β-ol) faster than the A/B trans-sapogenin tigogenin (5α, 25α-spirostan-3β-ol), which was well separated from its Δ^5-analog diosgenin (25α, 5-spirosten-3β-ol). In the 25β-series, similarly, the A/B cis-compound sarsasapogenin (5β, 25β-spirostan-3β-ol) moved well ahead of its A/B trans-analog neotigogenin (5α, 25β-spirostan-3β-ol). Although adsorption systems did not separate other pairs of 5α- and Δ^5-sapogenin analogs, the separation of hecogenin (3β-hydroxy-5α, 25α-spirostan-12-one) from gentrogenin (3β-hydroxy-25α, 5-spirosten-12-one) was curiously successful in most adsorption systems.

Most natural sapogenins are 3β-hydroxy steroids, and the separation of cis/trans-isomers by adsorption chromatography presented no difficulty, because the hydroxyl group of the trans-analog, being equatorial, is usually more strongly adsorbed. As expected, 3-episarsasapogenin, having an equatorial hydroxyl group was also more strongly adsorbed than sarsasapogenin. The separation of 25-epimers required the acylation of the sapogenins. TLC on Silica Gel G with chloroform-toluene (9:1) as the developer separated the acetates and trifluoroacetates in the following order of decreasing mobility: tigogenin (5α, 25α) > smilagenin (5β, 25α) > neotigogenin (5α, 25β) > sarsasapogenin (5β, 25β). Thus, the equatorial (25α) epimers were less adsorbed than the axial (25β) epimers.

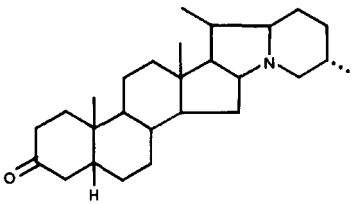
In the steroidal alkaloid group, we have been limited to the comparison of the behavior of 3 pairs of epimers in TLC (15). Soladulcidine (XIV) was not well separated from its Δ^5-analog solasodine, and tomatidine (XV) was not well separated from its Δ^5-analog tomatidenol, but the separation of 22-epimers presented no difficulty. On Silica Gel G plates, the tomatidine analogs migrated faster than the epimeric soladulcidine analogs in n-hexane-ethyl acetate (1:1), dichloromethane-acetone (4:1), and other solvent systems. In most of our adsorption systems 5β-solanidan-3-one (XVI) was less adsorbed than 4-solaniden-3-one (XVII), which assumes a conformation similar to the A/B trans-steroids. Oddly, demissidine (5α-solanidan-3β-ol) was readily separable from its Δ^5-analog solanidine (5-solaniden-3β-ol) by several adsorption systems.



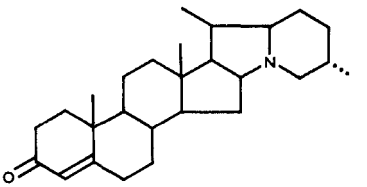
SOLADULCIDINE
XIV



TOMATIDINE
XV



5β-SOLANIDAN-3-ONE
XVI

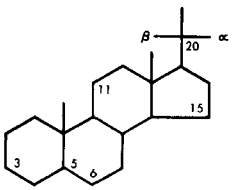


4-SOLANIDEN-3-ONE
XVII

In HPLC on a silica column, tomatidine was eluted well ahead of solasodine by a 2:1 mixture of acetone and n-hexane (16), confirming our observations made by TLC.

PREGNANE DERIVATIVES

There has been more interest in the chromatography of the C_{21} steroids than in that of any other group of steroids, because they include the progestational and the adrenocortical hormones. Intensive research activity in this area has yielded, as a by-product, a lode of semisynthetic stereoisomers with known chromatographic properties. From the voluminous collection of data on PC and TLC by Lisboa (17, 18), certain generalizations may be gleaned concerning the behavior of pregnane derivative in partition and adsorption systems. Referring to the pregnane skeleton



PREGNANE
XVIII

(XVIII), we shall examine the effect of isomerism at the numbered centers on chromatographic behavior.

The effect of A/B cis/trans-isomerism on adsorption is very slight, as judged from TLC data on the saturated 3,20-diketones (cf. Table 2). If a hydroxyl group is present at C-3, its effect on adsorption exceeds the influence of the isomerism at C-5, as shown in Table 2. In the 5α-pregnane derivatives as well as in the 5β-pregnanes, the compounds with an equatorial 3-hydroxyl group are more strongly adsorbed than the axial 3-epimers, but the influence of cis/trans-isomerism on the pair with an axial 3-hydroxyl group and on the pair with an equatorial 3-hydroxyl group is slight and variable. If a hydroxyl group is present at C-11, its orientation has a definite effect on adsorption, and 11β-hydroxyprogesterone invariably outdistances 11α-hydroxyprogesterone (Table 2). It is also generally true for the 6-, 15-, and 20-hydroxy analogs of progesterone that the β-isomer moves faster than the α-isomer.

The 21-hydroxylated pregnanes are related to the adrenocortical hormones. From an extensive list of R_F values in TLC with 16 solvent systems (19), it is evident that among the natural

TABLE 2

hR_F Values of Pregnane Derivatives in TLC

Steroid		Solvent System*	
Systematic name	Trivial name	1	2
5α-Pregnane-3,20-dione	Allopregnanedione	66	53
5β-Pregnane-3,20-dione	Pregnanedione	64	50
3α-Hydroxy-5α-pregnan-20-one		59	36
3β-Hydroxy-5α-pregnan-20-one		55	33
3α-Hydroxy-5β-pregnan-20-one		52	29
3β-Hydroxy-5β-pregnan-20-one		56	39
11α-Hydroxy-4-pregnene-3,20-dione	11α-Hydroxyprogesterone	31	6
11β-Hydroxy-4-pregnene-3,20-dione	11β-Hydroxyprogesterone	46	14
20α-Hydroxy-4-pregnen-3-one		50	24
20β-Hydroxy-4-pregnen-3-one		56	27

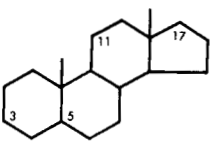
* Silica Gel G; solvents: 1 = cyclohexane-ethyl acetate-ethanol (9:9:2), 2 = cyclohexane-ethyl acetate (1:1).

corticosteroid metabolites with a 3α-hydroxyl group the A/B trans-isomers move uniformly faster than their A/B cis-analogs. Among the hydroxyl-substituted analogs of desoxycorticosterone (21-hydroxy-4-pregnene-3,20-dione) the β-isomer is generally more mobile than the α-isomer.

The chromatographic behavior of C_{21} alkaloids, in which $-NH_2$ or $-NMe_2$ groups are substituted for the -OH groups of 3,20-pregnanediols, is analogous to that of the other pregnane derivatives (20).

ANDROSTANE DERIVATIVES

The C_{19} steroids (XIX) available for testing chromatographic behavior exhibit stereoisomerism at Carbons 3, 5, 11, and 17. The



ANDROSTANE
XIX

generalizations derived for the C_{21} steroids also apply to the C_{19} series. In the absence of a hydroxyl group at C-3, the 5α- and 5β-androstane derivatives are difficult to separate by liquid chromatography. However, Galletti (21) succeeded in separating 5α-androstane-3,17-dione from 5β-androstane-3,17-dione by TLC on alumina by triple development with benzene-ether (1:1).

The relative mobilities for several pairs of epimeric C_{19} steriods in typical partition and adsorption systems are shown in Table 3. Their polarity generally decreases in the order 3β, 5α $\geq$ 3α, 5β > 3β, 5β $\geq$ 3α, 5α. A 17β-hydroxyl group usually makes C_{19} steroids more polar than does a 17α-hydroxyl group, but an 11β-hydroxyl group makes them less polar than does an 11α-hydroxyl group.

TABLE 3

Relative Mobilities of Androstane Derivatives in PC and TLC

Steroid		Paper*		SiO_2**	
Systematic name	Trivial name	1	2	3	4
3β-Hydroxy-5-androsten-17-one	Dehydroepiandrosterone	150	118	53	20
3α-Hydroxy-5α-androstan-17-one	Androsterone	160	140	56	29
3β-Hydroxy-5α-androstan-17-one	Epiandrosterone	145	126	59	23
3α-Hydroxy-5β-androstan-17-one	Etiocholanolone	150	126	53	27
3β-Hydroxy-5β-androstan-17-one		170	135	57	33
17α-Hydroxy-4-androsten-3-one	Epitestosterone	113	107	51	23
17β-Hydroxy-4-androsten-3-one	Testosterone	101	95	50	23
11α-Hydroxy-4-androstene-3,17-dione		---	---	37	13
11β-Hydroxy-4-androstene-3,17-dione		---	---	47	21

* Mobilities relative to desoxycorticosterone = 100; solvents: 1 = benzene-cyclohexane (1:1)/formamide, 2 = petroleum ether-benzene-methanol-water (33:17:40:10).

** hR_F values; solvents: 1 = cyclohexane-ethyl acetate-ethanol (9:9:2), 2 = benzene-ethanol (19:1).

ESTROGENS

Because in the estrogen series Ring A is aromatic, the stereoisomerism of practical interest is only that involving Carbons 16 and 17 (XX). Table 4 shows the relative mobilities of

TABLE 4

Relative Mobilities of Estrogens in PC and TLC

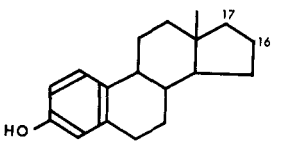
Steroid		Paper		SiO_2 ††	
Systematic name*	Trivial name	1**	2†	3	4
E-3,17α-diol	Estradiol-17α	108	416	61	55
E-3,17β-diol	Estradiol(-17β)	100	391	61	52
3,16α-Dihydroxy-E-17-one	16α-Hydroxyestrone	70	335	57	58
3,16β-Dihydroxy-E-17-one	16β-Hydroxyestrone	59	340	--	--
E-3,16α,17α-triol	17-Epiestriol	39	---	45	30
E-3,16β,17α-triol	16,17-Epiestriol	7	---	34	18
E-3,16α,17β-triol	Estriol	7	100	29	21
E-3,16β,17β-triol	16-Epiestriol	32	286	43	26

* E = 1,3,5(10)-Estratrien(e).

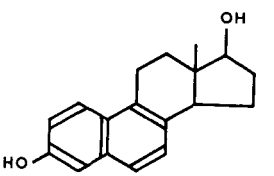
** Mobilities relative to estradiol = 100, Solvent 1 = chloroform/formamide.

†Mobilities relative to estriol = 100, Solvent 2 = toluene-ethyl acetate-methanol-water (9:1:5:5).

††hR_F values; solvents: 3 = ethyl acetate-cyclohexane- ethanol (9:9:2), 4 = chloroform-ethanol (9:1).



XX



DIHYDROEQUILENIN

XXI

some isomeric estrogens in typical partition and adsorption systems. As in the C_{19} series, the 17β-hydroxy steroids of the

C_{18} series are more polar than the 17α-epimers, at least in partition systems. The separation of estradiol-17α from estradiol-17β was, incidentally, the first reported separation of stereoisomers by partition chromatography in the steroid field (22). Dihydroequilenin (XXI) is also more polar than its 17α-epimer. The separation of the four epimeric estriols is difficult by paper partition chromatography, but relatively successful by TLC. The trans-diols are more strongly adsorbed than the cis-diols.

Epimeric estrogens were also separated when 0.02 N NaOH was percolated through a column of Sephadex LH-20 (23). At 25°C, estradiol-17α was eluted before estradiol-17β, and at 55°C, 16-epiestriol was eluted before estriol, which was followed by a mixture of 17-epiestriol and 16,17-epiestriol.

Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

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