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COMPARISON OF THE CHROMATOGRAPHIC PROPERTIES OF STEROLS, SELECT ADDITIONAL STEROIDS AND TRITERPENOIDS: GRAVITY-FLOW COLUMN LIQUID CHROMATOGRAPHY, THIN-LAYER CHROMATOGRAPHY, GAS–LIQUID CHROMATOGRAPHY AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The chromatographic properties of approximately 100 sterols, select steroids of plant origin (sapogenins and steroidal alkaloids) and triterpenoids has been evaluated in this laboratory by monitoring their elution characteristics in adsorption (gravity column and thin-layer methods with and without the addition of silver nitrate), gas and reversed-phase high-performance liquid chromatography. The utility of each methodology to act in one or another chromatographic mode-separation, radiochemical purification, quantitation and structural elucidation, is discussed. The importance of the tilt of the –OH group at C-3 as well as the polarity, size, and shape of the rest of the molecule as it effects the hydrogen-bonding ability of the –OH group is demonstrated through changes in chromatographic behavior that result from the step-wise introduction of double bonds, methyl, bromo, oxygen, nitrogen and cyclopropyl groups into 5α-cholestanol. An independent aid in the structure identification and quantitation of the compounds was use of a multiple-wavelength diode array detector in which different wavelengths of the UV spectrum (200–400 nm) were simultaneously monitored following passage of the sample through a reversed-phase C_{18} column.

INTRODUCTION

There are four principal methodologies used for the chromatography of sterols, biogenetically derived steroids (steroidal alkaloids and sapogenins) and triterpenoids: gravity-flow column liquid chromatography (GCC), thin-layer chromatography (TLC), gas–liquid chromatography (GLC) and high-performance liquid chromatography (HPLC). Each chromatographic system serves a unique function in the separation, radiochemical purification, quantitation and structural elucidation of these compounds. Their chromatographic behavior is known to be influenced by the hydrogen-bonding character and other electronic attractions (*e.g.* van der Waals forces and dipole–dipole interactions) between the lipid and the adsorbent. The rate of

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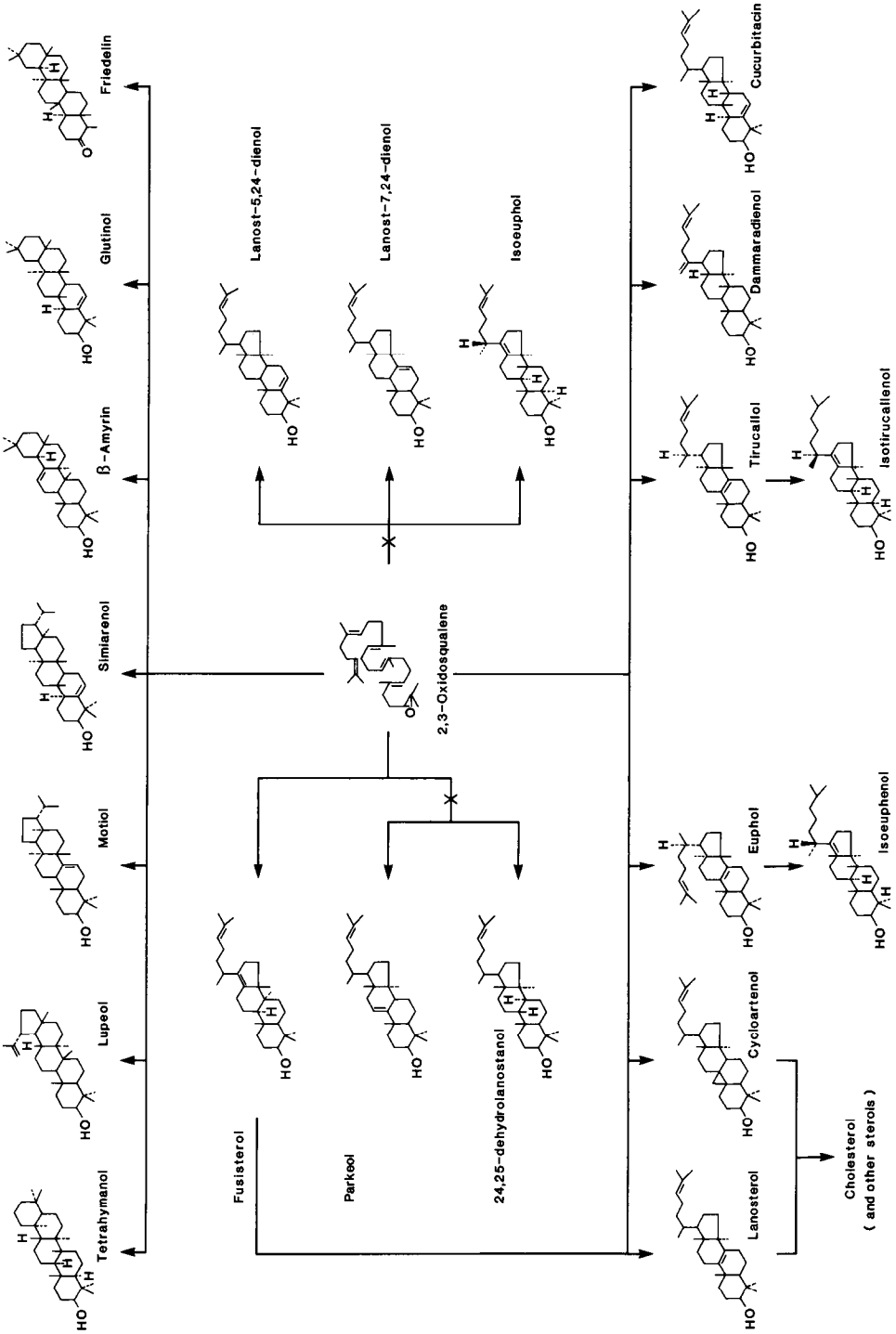


Fig. 1. Proposed routes of squalene oxide cyclization to tetracyclic and pentacyclic isopentenoids.

movement for each compound will depend on: (1) the stereochemistry and location of the polar substituents; (2) the solubility, partition coefficient and equilibrium constants of the compound in the solvent (and its polarity) used to develop the system; (3) the size and shape of the molecule; and (4) the degree of hydration and surface area of the adsorbent which affects intermolecular attraction between solvent, lipid and stationary phase. When the four systems are used in series; GCC → TLC → HPLC → GLC, not only is the probability of achieving homogeneity nearly 100% but important diagnostic information is gained about the location and geometry of select substituents and of the three-dimensional shape of the molecule as a whole.

Our interest in characterizing the chromatographic properties of steroids and triterpenoids is for several reasons. First, the physical chemistry involved in the lipid-stationary phase interaction may be similar to the kinds of associations which may be expected between these and other molecules in biomembranes. Therefore, how these compounds behave here may provide some predictive value of how they should behave in artificial and biological membranes. Second, the chromatographic behavior should reveal something about the preferred conformation of the molecule, as a result the confidence level regarding whether conformational transmission effects are realized outside of pure solution and solid state should be greatly increased. Third, to be able to predict *viz.*, to determine σ^G values, where compounds should elute in one or another system when authentic standards are not readily available for chromatography. Fourth, we have been interested for some time in the relationship between biosynthesis (Fig. 1) and function of steroids and triterpenoids throughout the evolutionary hierarchy. Because serious anomalies exist in this subject we have endeavored to prepare synthetically or isolate from natural sources numerous compounds which can be used in future metabolic, physiological and developmental studies. The chromatographic properties for many of these compounds have not before been reported in the literature (especially HPLC) and are given for the first time in this communication. In light of this volume of the *Journal of Chromatography* having been dedicated to Dr. Erich Heftmann we should like to point out at the outset that this field has evolved from the status of an art into a sophisticated science. Dr. Heftmann has, over a period of three decades, contributed much to its development[1-4]. One of us (W.D.N.) had the pleasure to receive post-doctoral training (1980–1981) in this area from Dr. Heftmann. The current effort extends and compliments previous findings from this[5-10] and other laboratories[11-24].

EXPERIMENTAL

Nomenclature

The numbering system that we have adopted for the tetracycles is shown in Fig. 2, although it may not be applicable for all sterols (*cf.* ref. 25 which examined the sterol side chain of marine sterols). The triterpenoid numbering system is different than the steroid numbering system even though the ring systems may be similar (Fig. 2). The α/β nomenclature for the chirality at C-24 of the sterol side chain is used in this study rather than the R/S nomenclature. It is important to remember that reference to α/β for the stereochemistry of nuclear substituents has a different meaning than its use for diastereoisomers in the side chain[12]. Unfortunately, the IUPAC system for numbering carbon atoms and rules for distinguishing the epimeric condition of chiral groups is not

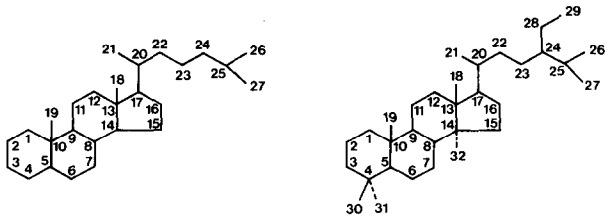


Fig. 2. Numbering system for sterols used in this study.

wholly satisfactory for the compounds examined in this study. This subject has been discussed elsewhere[1,2].

Chromatographic systems employed

Gravity column chromatography. GCC was performed in glass columns of varying dimensions depending on the sample load. The adsorbent (oven dried) was aluminum oxide (neutral Al_2O_3, Woelm Pharma) or silica gel (hydrated SiO_2, Mallinckrodt) having variable mesh size 60–200. An eluotropic series of solvents were employed to develop the systems. For alumina GCC the material was activated with water to 3% (v/w) and the eluent composition was changed in a discontinuous manner. The step-wise gradient was diethyl ether graded in 10% increments into Skelly Solve B (mixed hexanes). For the silica gel columns the series of solvents was added in the order of increasing polarity: hexane < hexane–benzene (1:1) < benzene < diethyl ether < methanol < methanol–chloroform. For elution of nitrogen compounds, the last solvent employed was triethylamine–ethylacetate.

Thin-layer chromatography. TLC was performed on 20 × 20 cm glass plates coated with 0.25 mm Silica Gel G (Analtech). TLC plates were standardized by preeluting the plates with benzene–ether. The plates were then oven dried at 110°C for 12 h. The plates were stored in a desiccator for at least 2 h prior to use. Four solvent systems were utilized in this study: S I, benzene–diethyl ether (9:1); S II, benzene–diethyl ether (85:15); S III, ethyl acetate–triethylamine (99:1); S IV, chloroform–diethyl ether (97:3).

Silver nitrate TLC. Compounds were chromatographed on silica gel G TLC plates and impregnated with silver ion by dipping the plates in a solution of 10-g silver nitrate in 25 ml water brought up to 100 ml with methanol. Prior to use the plates were dried overnight and allowed to cool for 30 min in a desiccator containing calcium carbonate. Plates were developed once in sealed TLC tanks containing 100 ml of chloroform–anhydrous diethyl ether (97:3, v:v). Test compounds were acetylated in sealed tubes with pyridine–acetic anhydride (1:1) at 55°C for 2 h. Compounds were loaded onto TLC plates at 5 μg per spot. The chromatographed sample was visualized by spraying plates with 50% methanolic sulfuric acid and slowly charred at 60°C.

Gas–liquid chromatography. GLC was routinely performed on a Hewlett-Packard Model 5890 gas chromatograph equipped with a HP 3393A computing integrator. Operating conditions for chromatography were as follows: column temperature, 245°C; detector temperature, 300°C; injector temperature, 275°C; helium carrier gas

flow-rate 20 ml/min. Attenuator and range were normally set at 4 and 10^{-11}, respectively. Retention times are reported relative to cholesterol (RRT_c). The elution time for cholesterol was standardized at 10 min.

Reversed-phase high-performance liquid chromatography. HPLC was performed on a Hewlett-Packard Model 1090 liquid chromatograph controlled by a ChemStation equipped with a Hewlett-Packard 9000 Series 300 computer. The C_{18} column (particle size 5 μm; 110 mm × 4.7 mm I.D. from Whatman) was interfaced with a guard column packed with Partisphere. Both columns were operated at 40°C with methanol–water (94:6, v/v) as the solvent. Flow-rate was maintained at 1 ml/min; pressure, 80 bar. Sample peak was monitored by a diode–array detector. Retention times $(\alpha_c)^9$ were relative to cholesterol. The retention time of cholesterol was maintained at about 10 min. About 10 μg of sample was dissolved in 10 μl of ethanol then loaded onto the column. Some compounds dropped out of solution (certain pentacycles) or failed to produce a UV response (saturated compounds). For these compounds we either changed the solvent for dissolving the compound (methanol or benzene) or increased the amount to be dissolved in the ethanol *e.g.*, sample load was increased to 100–200 μg of sample. The spectrum of each elution peak was fully characterized by continuously monitoring the absorbance between 200 and 400 nm. The UV cutoff for HPLC grade methanol and water was below 195 nm, the lowest wavelength which was used for monitoring absorption spectra of sterols. Solvents were HPLC grade (Burdick and Jackson) except for the diethyl ether, Skelly B and triethylamine which were purchased from American Scientific Products, Fisher and Eastman Kodak, respectively. The water for use in HPLC was obtained by glass distillation.

RESULTS AND DISCUSSION

Gravity column chromatography

This type of descending chromatography is usually employed as a preparative method to separate sterols from triterpenoids and to separate these polycyclic isopentenoids from other classes of lipids such as fatty acids and phospholipids. Fraction number is plotted relative to mass accumulated in test tubes. The elution profile is similar whether alumina or silica gel is the stationary phase. Preference for one system over the other may be predicated on the use of high levels of carcinogenic organic solvents such as benzene. A typical elution order is as follows: hydrocarbons (squalene) < ketones = esters < 4-monomethyl steroids < 4,4-dimethyl steroids = triterpenoids = primary long chain fatty alcohols (C_{28}–C_{32}) < 4-desmethyl sterols (cholesterol, ergosterol, etc.) < steryl glycosides = sapogenins < phospholipids < nitrogen containing steroids (elute with triethylamine). We have noted that dry-packed silica gel columns act to promote decomposition of nuclear poly-unsaturated systems *e.g.* of ergosterol. The extent of decomposition (hence of recovery) and double bond rearrangements depends on the sample size to silica gel ratio and the hydration of the silica gel. Obviously, for the investigator who is unaware of this phenomenon minor compounds could be lost in the sample work-up or novel compounds could be generated from a natural source which are in fact an artifact of the chromatographic method to purify the sample.

TABLE I

RELATIVE RETENTIONS OF TEST COMPOUNDS ON VARIOUS CHROMATOGRAPHIC SYSTEMS

= not examined.

Compound	*Source*	*System*				*Structure (based on 5α-cholestanol)*
		*TLC**, R_F	*TLC***, R_{ST}	*GC****, RRT_c	*RP-HPLC*§, α_c	
Cholestanol	Synthesis, this laboratory	0.18	1.22	1.03	1.10 (1.14)	Δ^0-3β-ol
Cholest-4-enol	Synthesis, this laboratory	0.25	1.12	0.96	0.93	Δ^4-3β-ol
Cholesterol	Steraloids	0.20 (0.47) [0.66]	1.00	1.00	1.00	Δ^5-3β-ol; 20*R*
Lathosterol	Synthesis, this laboratory	0.18 (0.43)	1.15	1.10	0.97	Δ^7-3β-ol
Cholest-8-enol	Synthesis, this laboratory	0.18 (0.43)	1.24	1.05	0.90 (0.93)	$\Delta^{8,9}$-3β-ol
Cholest-8(14)-enol	Synthesis, this laboratory	0.18	1.23	1.00	0.88	$\Delta^{8(14)}$-3β-ol
Cholest-14-enol	Synthesis, this laboratory	0.18	1.23	1.00	0.91	$\Delta^{14,15}$-3β-ol
Pregn-5-enol	W. R. Nes	0.18	–	0.16	0.18	Δ^5-3β-ol (no side chain)
20-Epicholesterol	W. R. Nes	0.20	–	0.93	0.84	Δ^5-3β-ol; 20*S*
(*E*)-17(20)-Dehydrocholesterol	W. R. Nes	0.20 (0.47)	0.25	0.94	0.64	$\Delta^{5,17(20)}$-3β-ol; (*E*)-17(20)
(*Z*)-17(20)-Dehydrocholesterol	W. R. Nes	0.20 (0.49)	0.19	0.86	0.61	$\Delta^{5,17(20)}$-3β-ol; (*Z*)-17(20)
Cholesta-5, (*E*)-20(22)-dienol	W. R. Nes	0.20	0.24	0.99	0.69	$\Delta^{5,20(22)}$-3β-ol; (*E*)-20(22)
Cholesta-5, (*E*)-22(23)	H. Kircher	0.20	0.44	0.92	0.81	$\Delta^{5,22(23)}$-3β-ol; (*E*)-22(23)
Zymosterol	Bakers yeast	0.20	N.E.	1.13	0.67	$\Delta^{8,24}$-3β-ol
Cholesta-5(*Z*)-22(23)-dienol	H. Kircher	0.20 (0.46)	0.33	0.90	0.74	$\Delta^{5,22(23)}$-3β-ol; (*Z*)-22(23)
Desmosterol	*Saprolegnia ferax*	0.20 (0.46)	0.36	1.10	0.72	$\Delta^{5,24(25)}$-3β-ol
Cholesta-5,25(27)-dienol	Synthesis, this laboratory	0.21	0.17	1.07	0.78	$\Delta^{5,25(27)}$-3β-ol
Cholesta-8,14-dienol	Synthesis, this laboratory	0.37	0.31	1.02	0.76	$\Delta^{8,14}$-3β-ol
7-Dehydrocholesterol	Steraloids	0.19	0.22	1.07	0.86	$\Delta^{5,7}$-3β-ol
26-Homocholesterol	M. J. Thompson	0.20	–	1.35	1.12	$\Delta^{5,26}$-Methyl-3β-ol
Campesterol	Cactus pollen	0.20	1.04	1.29	1.11 (1.15)	Δ^5-24α-Methyl-3β-ol
20(R)-*n*-Heptylpregn-5-en-3β-ol	W. R. Nes	0.20	–	1.47	1.24	Δ^5-3β-ol
22,23-Dihydrobrassicasterol	*Gibberella fujikuroi*	0.20	1.04	1.29	1.11 (1.15)	Δ^5-24β-Methyl-3β-ol
20(*R*)-*n*-Nonylpreg-5-en-3β-ol	W. R. Nes	0.20	–	2.61	1.82	Δ^5-3β-ol; extended side chain
Sitosterol	Sorghum	0.20	1.04	1.60	1.28	Δ^5-24α-Ethyl-3β-ol
Stigmasterol	Sorghum	0.20	0.78	1.40	1.11 (1.10)	$\Delta^{5,22(23)}$-24α-Ethyl-3β-ol; (*E*)-22(23)

Stigmastanol	M. J. Thompson	0.18	1.23	1.62	1.29	Δ^{0}-24α-Ethyl-3β-ol
Lophenol	H. Kircher	0.27	1.11	1.26	1.14	Δ^{7}-4α-Methyl-3β-ol
Obtusifoliol	*Gibberella fujikuroi*	0.27	–	1.49	0.96	$\Delta^{7,24(28)}$-4α,14α-Dimethyl-3β-ol
Ergosta-4,6,22-trienone	Synthesis, this laboratory	0.37	–	1.56	0.81	$\Delta^{4,6,22}$-24β-Methyl-3-one
Ergosterol	*Gibberella fujikuroi*	0.19 (0.43)	0.09	1.22	0.81 (0.76)	$\Delta^{5,7,22}$-24β-Methyl-3β-ol; (*E*)-22(23)
Ergosterol B_2	*Gibberella fujikuroi*	0.20	0.26	1.20	0.81 (0.71)	$\Delta^{6,8,22}$-24β-Methyl-3β-ol; (*E*)-22(23)
Brassicasterol	*Gibberella fujikuroi*	0.20	0.78	1.12	0.91	$\Delta^{5,22}$-24β-Methyl
Ergocalciferol	Sigma	0.19	–	1.10	0.60	$\Delta^{5,7,22}$-24β-Methyl with open B-ring
9(11)-Dehydroergosterol	Synthesis, this laboratory	0.18	0.09	1.12	0.57	$\Delta^{5,7,9(11),22}$- 24β-Methyl-3β-ol; (*E*)-22(23)
Ostreasterol	Cactus pollen	0.20	0.12	1.26	0.87	$\Delta^{5,24(28)}$-3β-ol
24-Dehydropollinastanol	Cactus pollen	0.20	–	1.26	0.75 (0.71)	Δ^{24}-9β,19-Cyclopropyl-3β-ol
24-Methyldesmosterol	Synthesis, this laboratory	0.20	–	1.45	0.93	$\Delta^{5,24(25)}$-24-Methyl-3β-ol
Fucosterol	*Saprolegnia ferax*	0.20 (0.47)	0.40	1.62	1.03 (1.00)	$\Delta^{5,24(28)}$-24-Ethylidene-3β-ol; *cis*/(*E*)-24(28)
Isofucosterol	Ulva sp.	0.20	0.46	1.65	1.05 (1.00)	$\Delta^{5,24(28)}$-24-Ethylidene-3β-ol; *trans*/(*Z*)-24(28)
Clerosterol	Codium	0.20	–	1.54	0.97	$\Delta^{5,25(27)}$-24β-Ethyl-3β-ol
25(27)-Dehydroporiferasterol	W. R. Nes	0.20	0.11	1.37	0.83	$\Delta^{5,22,25(27)}$-24β-Ethyl-3β-ol
14α,24β-Methyl-cholest-8(9)-3-ol	M. J. Thompson	0.18	1.46	1.36	1.09	$\Delta^{8(9)}$-14α,24β-Dimethyl-3β-ol
Cholesterol acetate	Synthesis, this laboratory	0.70	1.00	1.41	1.99	Δ^{5}-3β-ol-acetate
Epicholesterol	Synthesis, this laboratory	0.32	–	0.97	0.87	Δ^{5}-3β-ol
Cholesternone	Synthesis, this laboratory	0.42	–	1.34	1.07 (1.02)	Δ^{5}-3-one
Cholestanone	Synthesis, this laboratory	0.52	–	1.10	1.22	Δ^{0}-3-one
Stigmasta-4,22-dien-3-one	Aldrich	0.38	–	1.10	1.02	$\Delta^{4,22}$-24α-Ethyl-3-one
Cholest-4-en-3-one	Synthesis, this laboratory	0.38	–	1.31	0.91 (0.87)	Δ^{4}-3-one
24-Methylenelanosterone	Synthesis, this laboratory	0.66	–	1.96	1.33	$\Delta^{8,24(28)}$-4,4,14-Trimethyl-3-one
4,4-Dimethylcholest-5-en-3-one	Synthesis, this laboratory	0.66	–	1.27	1.48	Δ^{5}-4,4,-Dimethyl-3-one
4,4-Dimethylcholesta-8,14-dienol	Synthesis, this laboratory	0.31	–	1.59	1.07	$\Delta^{8,14}$-4,4-Dimethyl-3β-ol
20α-Hydroxycholesterol	W. R. Nes	0.15	–	1.49	0.27	Δ^{5}-3β,20α-diol

(*continued on p. 384*)

TABLE I (*continued*)

Compound	*Source*	*System*				*Structure (based on 5α-cholestanol)*
		*TLC**, R_F	*TLC***, R_{ST}	*GC****, RRT_c	*RP-HPLC*§, α_c	
22-R-Hydroxycholesterol	H. Kircher	0.15	–	1.71	0.25	Δ^5-3β,22R-diol
22-S-Hydroxycholesterol	H. Kircher	0.10	–	1.70	0.21	Δ^5-3β,22S-diol
25-Hydroxycholesterol	Steraloids	0.04	–	1.63	0.28	Δ^5-3β,25-diol
26-Hydroxycholesterol	E. Heftmann	0.04	–	2.20	0.21	Δ^5-3β,26-diol
29-Hydroxyfucosterol	T. McMorris	0.10	–	2.99	0.31	Δ^5-24-Ethylidene-3β,29-diol; (*E*)-24(28)
Cycloartenol	Sorghum	0.31 (0.63)	0.88	1.89	1.16	See Fig. 1
Lanosterol	*Gibberella fujikuroi*	0.31 (0.62) [0.77]	0.86	1.65	1.04	See Fig. 1
Lanosta-7,24-dienol	Synthesis, this laboratory	0.31	0.83	1.83	1.01	See Fig. 1
4,4-Dimethyl-cholest-5-enol (Δ^5-Lanosterol)	E. J. Parish	0.33 (0.66)	1.16	1.44	1.39	Δ^5-4,4-Dimethyl-3β-ol
24,25-Dihydrolanosterol	Synthesis, this laboratory	0.31	1.28	1.53	1.32	Δ^8-4,4,14-Trimethyl-3β-ol
24-Methyllanosterol	Synthesis, this laboratory	0.31 [0.77]	–	2.20	1.34	$\Delta^{8,24(25)}$-24-Methyl-3β-ol
Agnosterol	Commercial lanosterol	0.31	–	1.56	0.86	$\Delta^{7,9(11),24(25)}$-4,4,14-Trimethyl-3β-ol
24,25-Dihydroagnosterol	Commercial lanosterol	0.31	–	1.42	1.07	$\Delta^{7,9(11)}$-4,4,14-Trimethyl-3β-ol
Lanostanol	Synthesis, this laboratory	0.31	1.28	1.92	1.44	Δ^0-4,4,14-Trimethyl-3β-ol
24,25-Dehydrolanosterol	Synthesis, this laboratory	0.31	0.88	2.04	1.18	Δ^{24}-4,4,14-Trimethyl-β-ol
Euphol	*Euphorbia tirucalli*	0.33	0.75	1.44	0.99 (1.06)	See Fig. 1
Tirucallol	*Euphorbia tirucalli*	0.30	0.80	1.57	1.03 (1.06)	See Fig. 1
Isotirucallenol	Synthesis, this laboratory	0.31	1.22	1.02	0.95	See Fig. 1
Dammaradienol	E. Spencer	0.22	–	2.61	0.30	20β-Hydroxy-dammaradienol
Lupeol	Sorghum	0.31	0.60	1.82	0.81	See Fig. 1
Lupeol methyl ether	Synthesis, this laboratory	0.72	–	1.92	1.44	See Fig. 1, methyl ether
Simiarenol	Sorghum	0.39	0.87	2.16	1.25	See Fig. 1
Epifriedalanol	Synthesis, this laboratory	0.65	1.20	2.19	2.66	See Fig. 1; 3β-ol
β-Amyrin	Sorghum	0.29	1.23	1.62	1.03	See Fig. 1.
Glutinol	T. Itoh	0.42	0.88	1.85	1.03	See Fig. 1

Friedelin	*Kalmia latifolia*	0.39	–	2.33	1.42	See Fig. 1
Tetrahymanol	*Tetrahymena pyriformis*	0.29	1.29	2.50	–	See Fig. 1
Motiol	Sorghum	0.28	1.09	2.43	1.50	See Fig. 1
α-Amyrin	Sorghum	0.31	1.23	1.79	1.13	See Fig. 1
24,25-Epiminolanosterol	Synthesis, this laboratory	0.00 [0.29]	0.29	2.263	–	Δ^8-4,4,14-Trimethyl, 24(*RS*)-25-epimino-3β-ol
25-Azalanosterol	Synthesis, this laboratory	0.00 [0.25]	0.25	1.65	–	Δ^8-4,4,14-Trimethyl-5-aza-3β-ol
25-Aminolanosterol	Synthesis, this laboratory	0.00 [0.14]	0.14	2.34	–	Δ^8-4,4,14-Trimethyl-25-amino-3β-ol
24,(*S*)25-Oxidolanosterol	E. J. Parish	0.23 [0.67]	0.67	2.52	0.22	Δ^8-4,4,14-Trimethyl-24(S)oxido; 3β-ol
24(*R*)25-Oxidolanosterol	E. J. Parish	0.23 [0.62]	0.67	2.52	0.24	Δ^8-4,4,14-Trimethyl-24(R)oxido; 3β-ol
24-Hydroxylanosterol	Synthesis, this laboratory	0.16 [0.73]	–	2.24	0.33	Δ^8-4,4,14-Trimethyl-3β,24-diol
25-Hydroxylanosterol	Synthesis, this laboratory	0.06	0.62	2.69	0.30	Δ^8-4,4,14-Trimethyl-3β,25-diol
24-Bromolanosterol	Synthesis, this laboratory	0.31 [0.77]	0.77	1.61	1.02	$\Delta^{8,24}$-4,4,14-Trimethyl-24-bromo-3β-ol
25-Keto,26-norcholesterol	Kurt Spira Co.	0.13	–	1.46	0.29	Δ^5-25-One-3β-ol
Solasodine	*Soslanum khasianum*	0.02 [0.60]	–	1.88	–	See Fig. 4
Soladulcidine	E. Heftmann	0.01 [0.52]	–	1.92	–	See Fig. 4
Solanidine	E. Heftmann	0.25 [0.66]	–	1.13	–	See Fig. 4
Diosgenin	E. Heftmann	0.15 [0.67]	–	1.40	0.38	See Fig. 4
Tigogenin	E. Heftmann	0.14 [0.66]	–	1.44	0.41	See Fig. 4
Demissidine	E. Heftmann	0.07 [0.67]	–	1.12	–	See Fig. 4
25-Azacholesterol	M. J. Thompson	0.00 [0.41]	–	1.11	–	See Fig. 4
Tomatidine	D. Johnson	0.01 [0.61]	–	2.03	–	See Fig. 4
Solasodiene	D. Johnson	0.19 [0.78]	–	1.18	–	See Fig. 4
Squalene	Aldrich	0.85	0.02	0.51	2.14	–
Sitosteryl glycoside	*Solanum khasianum*	0.00	–	–	0.24	–
Triacontanol	Wheat	0.28	1.23	1.88	2.15	30-Carbon fatty alcohol

* Adsorption TLC; plates were developed in S I; in parenthesis, S II; in brackcts, S IV.

** Silver nitrate TLC; plates were developed in S III; R_F values are relative to cholesteryl acetate; $R_{ST} = R_{F(\text{compound})}/R_{F(\text{cholesterol})}$

*** Gas–liquid chromatography was performed on 3% SE-30 packed columns operated at 245°C. Retention times are relative to cholesterol.

§ Reversed-phase HPLC was performed at 40°C with 6% aq. methanol as the eluent. Retention times are relative to cholesterol.

Thin-layer chromatography

TLC has become a multipurpose workhorse in the analysis of steroids and triterpenoids. TLC may be used not only to purify samples but the R_F value provides a clue to the compounds structural identification. Total lipid extracts of biological specimens normally contain a mixture of polycyclic isopentenoids. These compounds can be separated into various classes based on the compound's movement off the origin. Cholesterol is used as the reference marker and has an R_F of 0.18 with S I (Table I). The structural feature which most contributes to the chromatographic behavior of cholesterol in adsorption TLC is the presence of a free 3β-OH group. Addition of one or two methyls to C-4, inverting the A/B ring juncture or converting the C-3 OH to an acetoxy, methoxy, keto, or 3α-OH which occurs during routine metabolism will result in a steroid having a less polar R_F value relative to the R_F value obtained for cholesterol. The distal portion of the molecule plays less of a role in mediating the chromatographic behavior of steroids in adsorption TLC than it does in silver nitrate or reversed-phase TLC [15,26]. Thus triterpenoids which possess geminal methyls at C-4 behave in TLC like the 4,4-dimethyl steroids (Table I) although they are structurally very different from lanosterol.

The total lipid extract is oily, as a result only bulk separations of steroids is generally achieved in the first run. In contrast to the diminished TLC sensitivity for compounds mixed in with the total lipid extract, fine-tune chromatography of the sort shown in Fig. 3 can be obtained with pure compounds. The rate of movement for each compound will depend on the hydrogen bonding strength of the polar group at C-3. The hydrogen bonding strength in turn will be influenced by the tilt of the C-3 –OH group and the proximity of double bonds, steric hindering agents (alkyl groups) and neighboring polar groups (hydroxyl, keto, nitrogen, etc.) to the C-3 –OH. Polar groups introduced into the side chain (Figs. 3 and 4) decrease the mobility of the steroid in an additive manner. The stereochemistry and position of the oxygenated function are also important to the compound's rate of movement. The polarity of the solvent can be changed to permit separation of compounds *e.g.*, nitrogen-containing steroids, which in benzene–ether failed to migrate off the origin (Table I). Good separation was achieved with triethylamine in ethyl acetate for the various nitrogen-containing steroids shown in Table I. The order of R_F values from more to less polar for the nitrogen groupings was as follows: 25-amino(tertiary amine) < 25-aza < 24,25-epimino (aziridine) < solasodane (26-azasteroid). The isomeric steroidal alkaloids at C-22, tomatidine and soladulcidine were easily separated by using S IV. The difference in migration is due to the stereochemistry of the F-ring which places the nitrogen closer or farther away from the E-ring oxygen and the stationary phase. The stereochemistry of the ring systems effect the migration of steroids and triterpenoids on TLC.

As shown in Fig. 5 the tilt of the C-3 –OH group differs between cycloartenol and lanosterol and between these steroids and cholesterol. On the assumption that the sterol interacts with the gel so that the C-18 and C-19 angular methyls are directed toward the gel (β-face binding) then the hydrogen-bond vector between the C–OH and the gel can be influenced in a predictable manner depending on the spatial orientation of the C-3 –OH group. For instance, the three-dimensional shape observed in Dreiding models and X-ray crystallographs of 3-epi and 3-keto steroids[27] indicate that the polar groups are directed up from the plane of the nucleus and therefore, assuming β-face binding, away from the gel. Thus, the hydrogen-bond vector is weakened and the

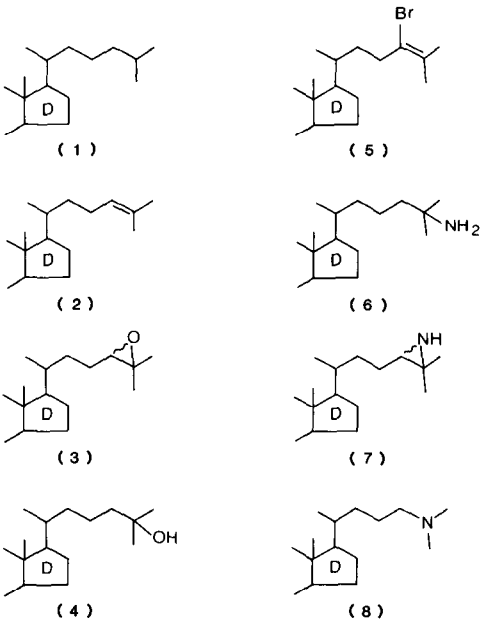


Fig. 3. Sterol side chain variations.

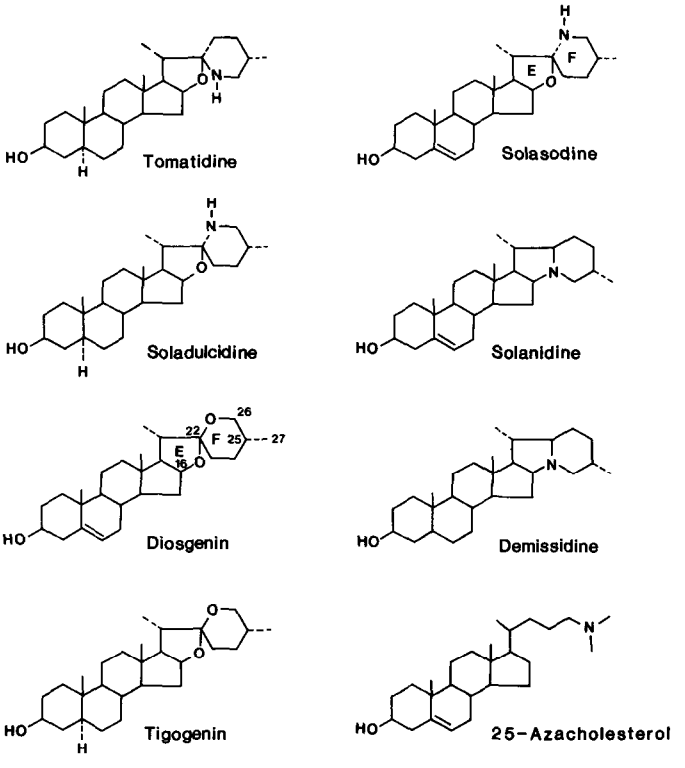
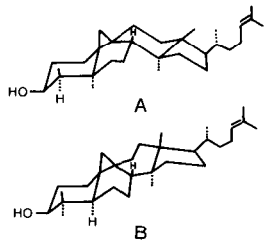
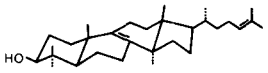


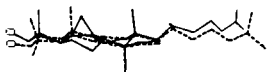
Fig. 4. Structures of steroidal alkaloids and sapogenins.



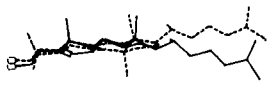
TWO POSSIBLE CONFORMATIONS OF CYCLOARTENOL



ESTABLISHED CONFORMATION OF LANOSTEROL



STEREOSTICK DRAWING OF LANOSTEROL SUPERIMPOSED ON CYCLOARTENOL



STEREOSTICK DRAWING OF LANOSTEROL SUPERIMPOSED ON CHOLESTEROL

Fig. 5. Different conformations of steroids in which the tilt of the 3β-OH is observed.

compounds should and experimentally do run up the plate compared with 3β-OH sterols. The significance of conformational transmission effects on TLC behaviour is shown in the pairs of "left-handed" and "right-handed" sterols *viz.*, 20-epicholesterol–cholesterol and euphol–tirucallol, where the 20-*S* compound is slightly separated from the 20-*R* compound. Not only is the molecular volume changed by inverting the configuration at C-20 which alters the conformation of the side chain but the orientation of the 3β-OH group is changed and hence the hydrogen-bond vector (*cf.* X-ray crystallographs given in refs. 28 and 29).

Argentation chromatography

Silver nitrate TLC is a method to separate compounds based on the number and position of double bonds in the molecule. Silver nitrate on the surface of the silica gel coordinates with the double bond due to coulombic attractions between the pi electrons and the silver ion. The greater the accessibility (lack of hindrance) of the double bond to bind with the silver ion the more polar the compound's R_F value. As shown in Fig. 6 and Table I, tetrasubstituted double bonds have a small affinity for complexing with the silver ion while trisubstituted double bonds have a strong affinity for the silver ion. Double bonds in the nucleus are shielded to a greater extent than

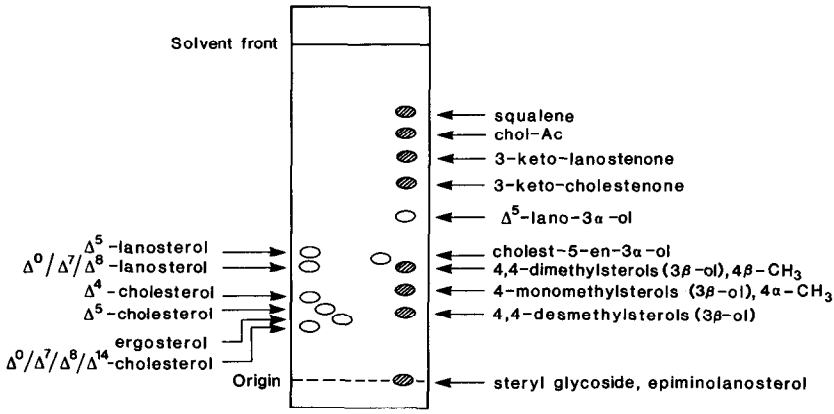


Fig. 6. Chromatographic behavior of sterols in adsorption TLC. Plates were developed with benzene–diethyl ether (9:1). Chol = cholesterol. Ac = acetate.

isolated double bonds in the acyclic side chain, therefore good separation is achieved with steroidal dienols in which one double bond is located in the nucleus and the other located in the side chain. A number of steryl acetates have been separated from one another by silver nitrate TLC[30–33]. We preferred to use the solvent system employed by Goad[15] to provide a standardized silver nitrate TLC system for sterols and triterpenoids. In other studies we[6,7] and others[30–32] have used benzene–hexane as the solvent to develop the plates. In this study we noted some variability from run to run in the R_F value of cholesteryl acetate (mean $R_F = 0.51$, $n = 7$), however the relative R_F value for each compound to the R_F value of cholesteryl acetate was constant from run to run. The order of elution based on double bond position for steroids was not directly correlatable to triterpenoids, since the triterpenoids possessed much greater nuclear

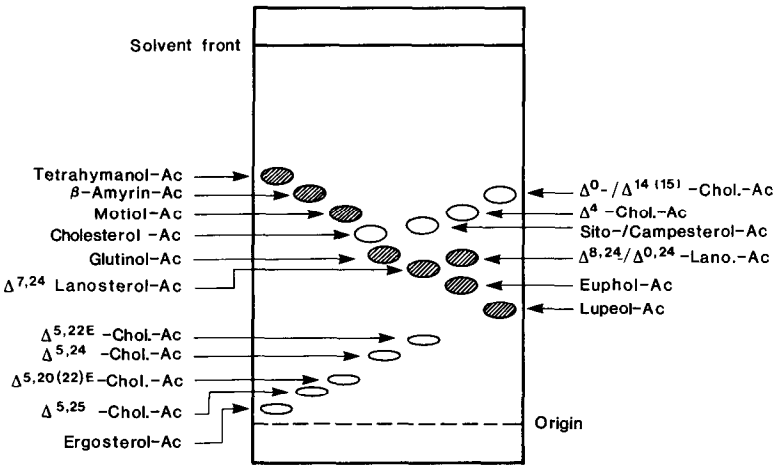


Fig. 7. Chromatographic behavior of steryl acetates in silver nitrate TLC. Plates were developed with chloroform–diethyl ether (97:3). Chol. = cholesterol; Lano. = lanosterol; Ac = acetate.

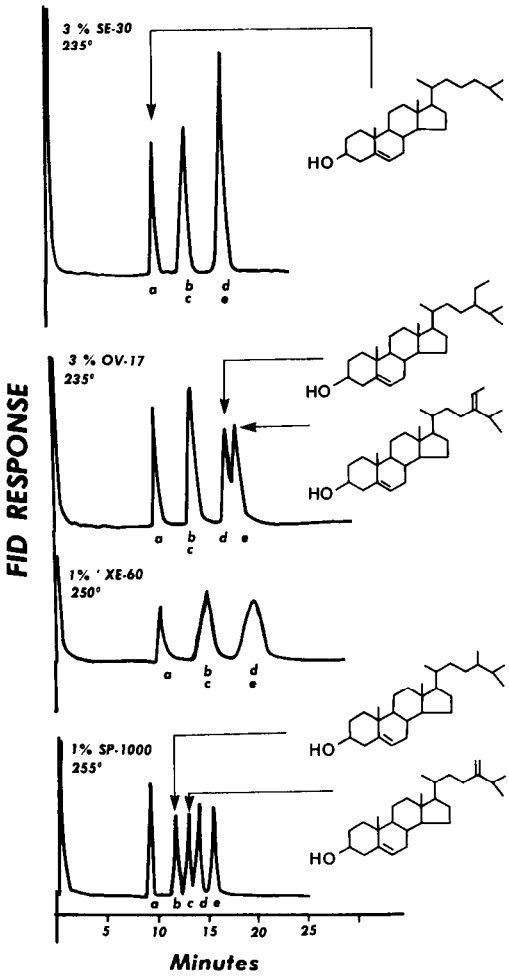


Fig. 8. Chromatographic behavior of structurally similar sterols on four GLC columns. The column packings exhibit the range of McReynolds constants from non-polar (SE-30) to polar (SP-1000).

variations and hence conformational differences due to 1–2 shifts of methyl groups and hydrogen atoms that resulted from the cyclization process (Fig. 1).

Gas–liquid chromatography

GLC is a powerful tool in structure determination and quantitation. In this study the vaporized compounds in the effluent gas were detected by a flame-ionization detector. The rate of movement for each compound in GLC was dependent on the polarity of the column packing. As shown in Fig. 8, sterols which cochromatograph on non-polar stationary phases can be separated from one another on polar stationary phases. Long polar capillary columns (100 m) where the number of theoretical plates has been greatly increased have been used to separate isomeric C-24 sterols which cannot be resolved on 6 ft. columns[34]. Extensive correlations between structure and

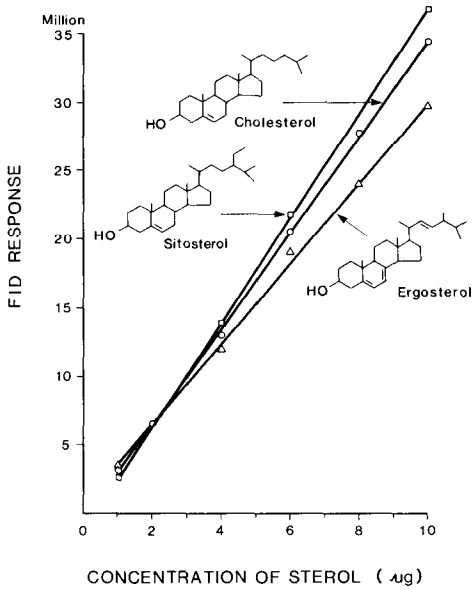


Fig. 9. Flame ionization detection (FID) in GC using 3% SE-30 packed columns to increasing concentrations of cholesterol, sitosterol and ergosterol.

retention time for sterols[17,20,21] and triterpenoids[17,18,35,36] have been reported using similar or the same column packings shown in Fig. 8. Because 4,4,14-trimethyl steroids, tetracyclic and pentacyclic triterpenoids possess nuclear conformations which may place angular methyl groups in spatial orientations that differ from what might be expected for such additions onto a cholestanol skeleton, it is not always possible to calculate *a priori* the retention time of the unknown steroid or triterpenoid by comparison with the features added to cholestanol (*cf.* refs. 11,19,21). For instance, the structural jump from cholestanol to lanosterol is too great to accurately calculate the RRT_c for lanosterol based on the retention time of the basal sterol structure (5α-cholestanol) and the retention factors contributed by each additional group *i.e.*, three methyls at C-4 and C-14 and two double bonds at C-8 and C-24. However, it is possible, to use the contribution factor (σ^G) when a single feature is common between pairs of structurally similar compounds. Thus, the RRT_c for the C-20 epimer of tirucallol (a tetracyclic triterpenoid) can be determined based on the retention times for the sterol pair cholesterol and 20-epicholesterol (Table I). Similarly the influence of Δ^0–Δ^5 can be determined for sapogenins and steroidal alkaloids based on the cholestanol–cholesterol pair of sterols.

The retention time is also influenced by the amount of the injection. While the retention time remains constant between 20 ng to 2 µg/ml solvent, as the detector response goes off scale due to an increase in the concentration of sample above 2 µg/ml, the retention time increases (in a linear manner) by as much as 0.5 min at 10 µg/ml. Furthermore, as shown in Fig. 9, the ability to quantitate sterols at the same concentration injected into the instrument becomes structurally dependent at the higher sample load. These facts should be considered at times when the investigator

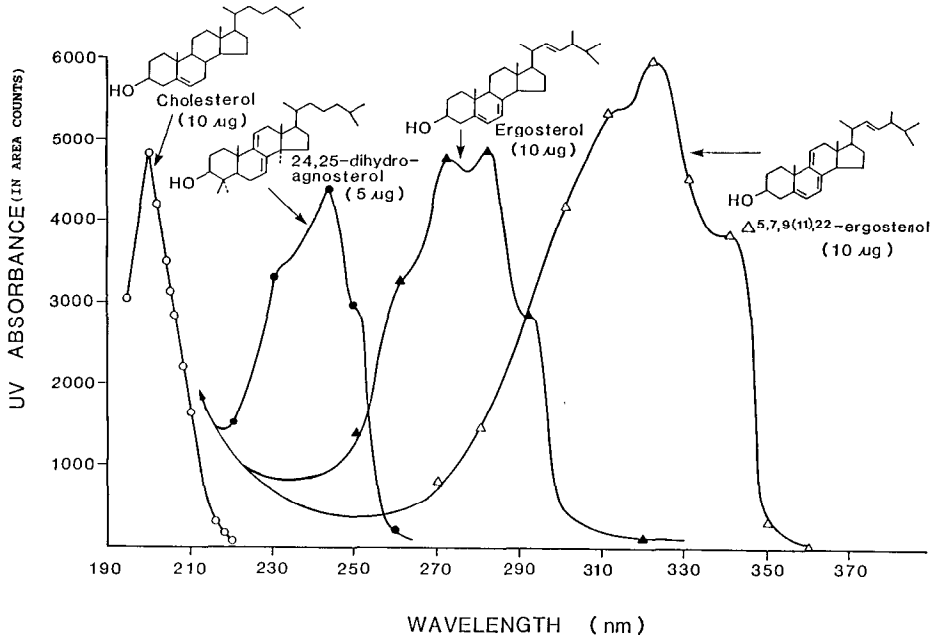


Fig. 10. Detector response at a given wavelength to the chromophore condition. The points that compose the UV fingerprints for each sterol were determined by the peak area (mAU × time) obtained at select wavelengths between 195 and 400 nm. For 10 μg of cholesterol monitored at 205 nm the peak area (in mAU) gave an area count of 3119. The shape of the individual curves *i.e.*, the shape between the data points, was drawn as they appeared in UV spectra obtained on the respective compound before chromatography.

has observed a major compound present in a mixture and it is off scale, then changes the attenuator to bring this peak on scale to quantify it or to obtain its RRT_c. Neither the retention time nor counts given by the computer may reflect the true RRT_c or amount of the unknown compound relative to readings based on cholesterol. It is best, as we have found, to dilute the sample and reinject it into the gas chromatograph in order to quantify the off scale peak.

Reversed-phase (RP) high-performance liquid chromatography

RP-HPLC is a recent chromatographic system that has great utility for steroid analysis. Its origin was in the lipophilic (LH-20) gravity Sephadex columns that were developed in the early part of the last decade to separate sterols differing in the number of C-24 alkyl groups and on the number of double bonds in the nucleus and side chain[37–39]. RP-HPLC has the advantage over the LH-20 columns in the amount of time involved for chromatography (min *versus* days) and in resolving sterols that were unseparable with the LH-20 columns. Another advantage is the ability to interface the column with a multiple-wavelength diode array detector. This detector will produce a signal for any sterol that passes through the aperture. The response or peak height measured in mAU is dependent on the amount loaded onto the column, number and kind of chromophores in the molecule *e.g.*, tetrasubstituted, trisubstituted, heteroanular, or homoanular, and on peak purity. Even though cholesterol will not be detected by UV absorbance above 220 nm (Fig. 10) all sterols including stanols exhibit

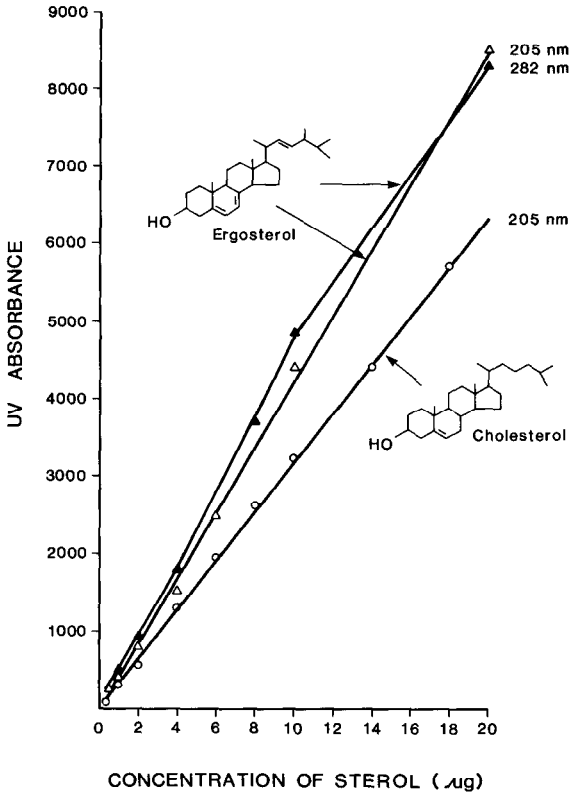


Fig. 11. UV detector response to increasing concentrations of cholesterol and ergosterol.

an end absorption spectra. Thus, the instrument can be dialed to 205 nm for routine analysis, however it should be kept in mind that similar peak heights may represent vastly different levels of sterols. As shown in Fig. 11, there is a linear response that is structurally dependent between 20 ng and 20 μg with increasing sterol concentration. In the present study we used a 10-cm C_{18} column and operated the instrument at 40°C while in earlier studies we used a 25-cm C_{18} column and operated the instrument at ambient temperature[5–9]. In our earlier studies we observed that cholesterol and lanosterol cochromatographed. However, as shown in Fig. 12 the two compounds were partially separated by elevating the operating temperature for chromatography. The compounds were distinguished by their end absorption spectra.

In addition to changing the temperature conditions other chromatographic "tricks" can be used to separate structurally similar sterols. For instance, the polarity of the eluant will influence the hydrogen bonding character of the sterol *i.e.*, the sterol will act either as a hydrogen donor or hydrogen acceptor. Therefore, it is possible to induce chromatographic frameshifts on a given set of compounds based on the use of a normal phase and reversed-phase solvents. In fact, the rate of movement of campesterol, stigmasterol, 7-dehydrocholesterol, ergosterol, 24(28)-methylene cholesterol and cholesta-5,22(*E*)-dienol relative to cholesterol is demonstrably different on C_{18}-columns eluted with acetonitrile[40], acetonitrile–water[16], propanol in hexane[41], methanol[42] or methanol–water (this study).

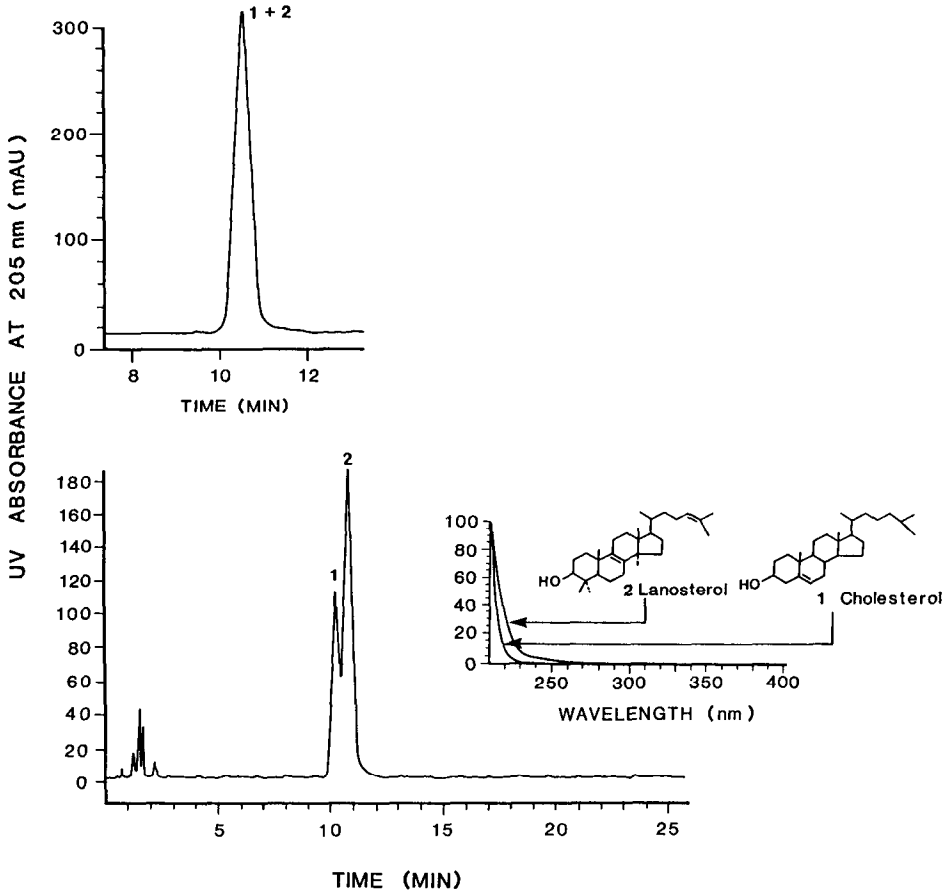


Fig. 12. Temperature dependent chromatographic separation of cholesterol and lanosterol by RP-HPLC eluted with 6% aq. methanol. (A) Column operated at 24°C; (B) column operated at 40°C.

The multiple-wavelength diode array detector was used in the purification of commercial lanosterol, a sterol mixture which contains four sterols; lanosterol, 24,25-dihydrolanosterol, agnosterol and 24,25-dihydroagnosterol[43]. We found that neither silver nitrate TLC nor the chemical procedure of forming the dibromide alone will produce pure lanosterol as implied in the literature[44]. As shown in Fig. 13 neither GLC (using the standard packed column) nor RP-HPLC in which the monitor is set at 205 nm would have indicated the presence of the impurity of 24,25-dihydroagnosterol mixed in with lanosterol. However, by monitoring several wavelengths simultaneously (Fig. 14) the presence of contaminating levels of 24,25-dihydroagnosterol in the tail of the lanosterol peak was evident. The utility of this instrumentation was demonstrated in our separation of lanosterol from the contaminant sterol by collecting the fractions corresponding to the leading edge of the peak with repeated injections using an auto injector.

We have found the C_{18}-column coupled to a multiple-wavelength diode array

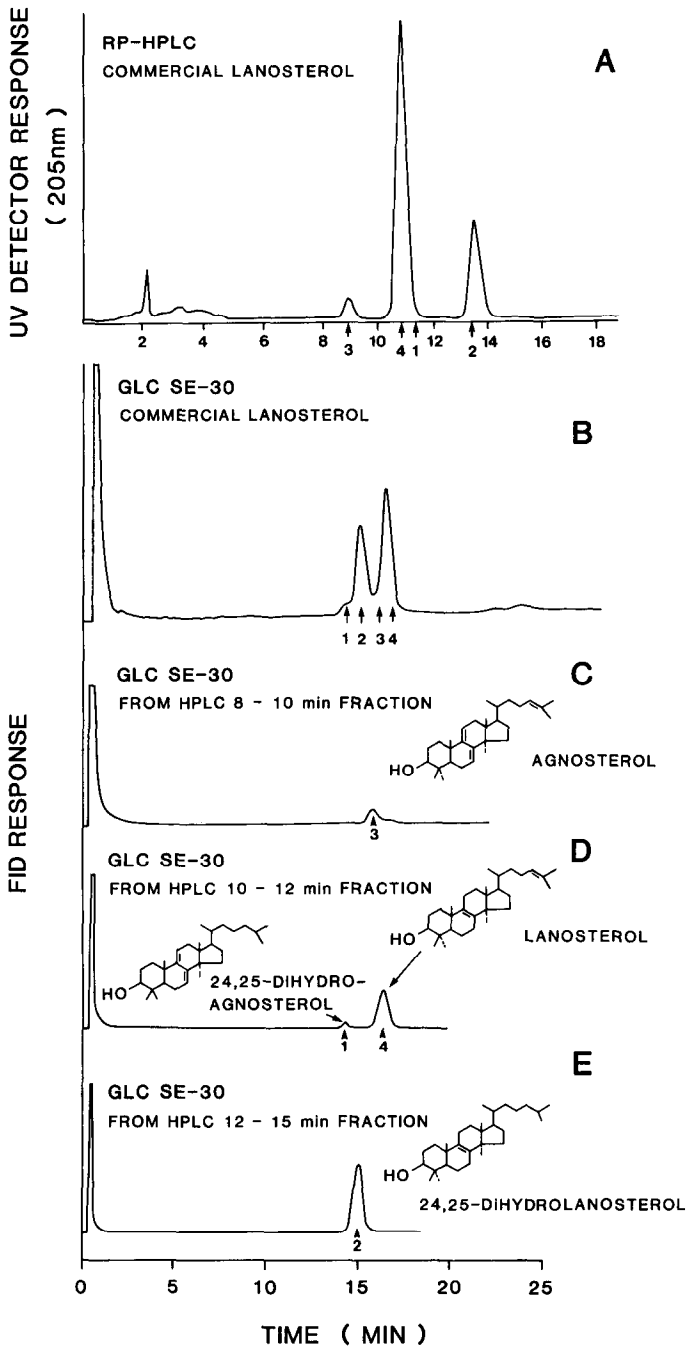


Fig. 13. Chromatograms of commercial lanosterol in RP-HPLC and GLC. The sample was fractionated by RP-HPLC and the fractions re-examined by GLC (C–E).

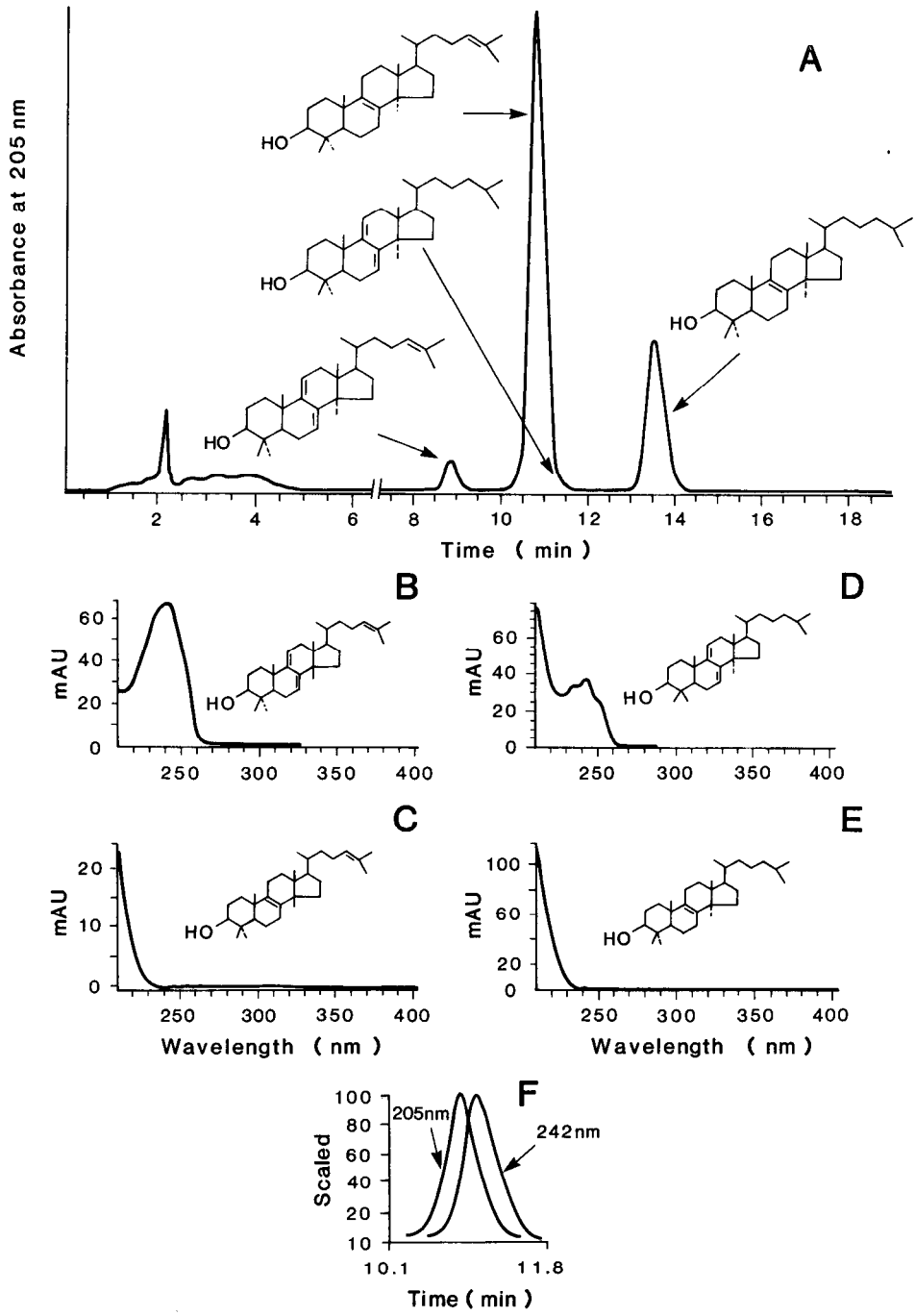


Fig. 14. Chromatogram of commercial lanosterol in RP-HPLC. UV spectra were recorded for each of the compounds eluting between 8 and 15 min. Peak purity (F) for the compound eluting between 10 and 12 min indicated two compounds with different UV spectra.

detector provides an elucidation of chemical purity which cannot be done with other chromatographic modes even GLC packed columns coupled to mass spectrometry.

CONCLUSIONS

The structural features which influence chromatographic mobility vary between adsorption and partition systems and on the polarity of each analytical system. Therefore, the way a compound behaves in TLC relative to cholesterol may be quite different in another chromatographic system. This attribute is particularly useful in the radiochemical purification of structurally similar sterols[1,6,9,42]. Moreover, from the data compiled in Table I and consideration of molecular models of steroids and triterpenoids it should be possible to make rational choices for the chromatographic steps that will be required in the separation of a given set of compounds isolated from natural sources or prepared through chemical synthesis.

The two principal structural components that act as the chromatographic determinant are the hydrogen bonding capability of the C-3 OH-group and the stereochemistry of the molecule. For TLC, the degree of methylation at C-4 and the number and position of double bonds proximal to the –OH group affect mobility. In GLC and RP-HPLC, the methylation condition at C-4 plays a small role in the rate of movement while the flatter a sterol is the slower it moves (*cf.*, ref. 11 for a deeper discussion of this point). By comparison of the RRT_c and α_c of triacontanol and tetrahymanol the effect of cyclization of an acyclic molecule (formally with squalene) on chromatographic mobility was apparent. Interestingly, on a polar GLC column (1% SP-1000)[45], triacontanol frameshifts, to RRT_c 0.71 while tetrahymanol RRT_c remains about the same as its RRT_c on 3% SE-30 packed columns. Oxygenated and nitrogen-containing compounds such as diosgenin and solasodine will move as sterols depending on the chromatographic system employed. In general, however, the presence of polar groups has a strong effect on chromatographic mobility, but the direction and magnitude of the kind of effect depend strongly on the method. Additional double bonds in the sterol produce a more polar R_F, however, a saturation level is obtained in silver nitrate TLC after three double bonds are introduced into the molecule. In RP-HPLC we have separated sterols with base-line resolution having as many as five double bonds[46]. Compounds which have a low solubility in methanol–water such as triterpenoids with very high melting points produce a broad late peak in RP-HPLC. Unfortunately, the chromatographic rules applicable for sterols may not operate for sterol-like molecules *e.g.*, triterpenoids. More data is required on the non-steroidal polycyclic isopentenoids before rules for structure–retention can be formulated as has now been constructed for sterols[11].

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