SEPARATION OF C_{27}, C_{28} AND C_{29} STEROLS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON SMALL PARTICLES

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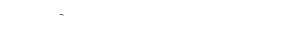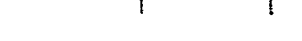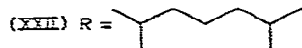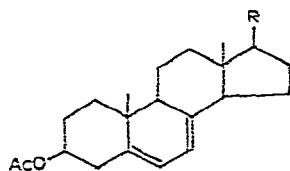
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SUMMARY

The application of reversed-phase high-performance liquid chromatography to the analytical- and preparative-scale separation of sterols has been evaluated. The capacity factors, k', for a number of compounds chromatographed on a μBondapak C_{18} (<10 μm) column are presented. C_{27}, C_{28} and C_{29} sterols and also sterols differing in degree of unsaturation could be readily separated as their acetates in this system. The present reversed-phase chromatographic method is apparently not as selective as silver nitrate–silica gel thin-layer chromatography for the position of unsaturation in the sterol molecule.

INTRODUCTION

During the past few years, there has been rapid progress in the development of high-performance liquid chromatography (HPLC). The application of this technique to the separation of various steroid hormones has been well documented[1–7]. In some cases, the sensitivity of detection of non-ultraviolet-absorbing steroids has been improved by benzoylation of hydroxy-steroids to form an ultraviolet (UV)-absorbing derivative[3], and by the formation of 2,4-dinitrophenylhydrazine derivatives in the case of keto steroids[2].

The application of HPLC to the separation of the relatively non-polar sterols related to cholesterol (I) and the phytosterols has not been documented. Isolation of such sterols from biological materials is commonly accomplished by fractionation of lipid extracts by column chromatography, followed by thin-layer chromatography (TLC) and in many cases by silver nitrate–silica gel TLC of either the free sterols or their acetates[8]. Phytosterols are typically characterised by the presence of either an extra C_1 or C_2 alkyl group at C-24. However, the above chromatographic procedures will not separate different C-24 homologues of sterols, *e.g.* cholesterol (I; C_{27}), campesterol (II; C_{28}), and sitosterol (III; C_{29}). Such sterols can be readily separated by gas–liquid chromatography (GLC) on an analytical scale, but present certain problems on a preparative basis. In particular, many of the sterols are thermally decomposed, especially in the presence of heated metal. Separation of appreciable amounts of C_{27}, C_{28} and C_{29} sterols is often necessary, *e.g.* during the complete struc-

(I)   R¹ = OH, } R² =
(IV)   R¹ = OAc

(II)   R¹ = OH, } R² =
(VII)   R¹ = OAc

(III)   R¹ = OH, } R² =
(XII)   R¹ = OAc

(V)   R¹ = OAc, R² =

(VI)   R¹ = OAc, R² =

(VIII) R¹ = OAc, R² =

(IX)   R¹ = OAc, R² =

(X)   R¹ = OAc, R² =

(XI)   R¹ = OAc, R² =

(XIII) R¹ = OAc, R² =

(XIV) R¹ = OAc, R² =

(XV)   R¹ = OAc, R² =

(XVI) R¹ = OAc, R² =

(XVII) R¹ = OAc, R² =

(XVIII) R¹ = OAc, R² =

(XIX)   R =

(XXI)   R =

(XX)   R =

(XXII) R =

(XXIII) R =
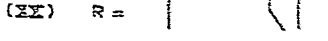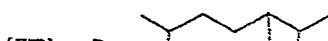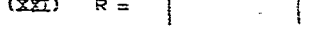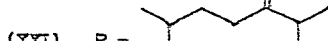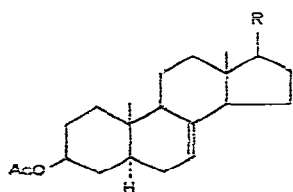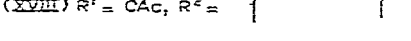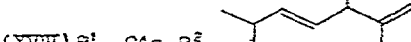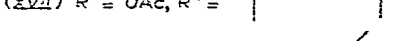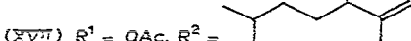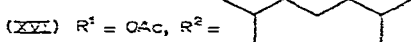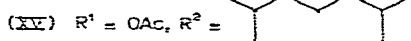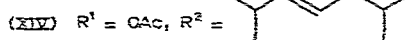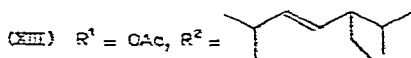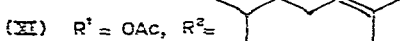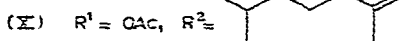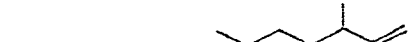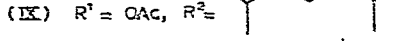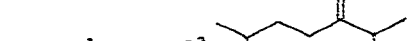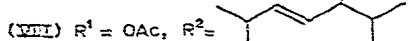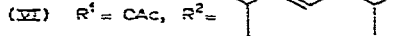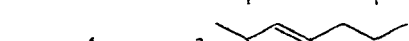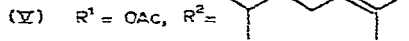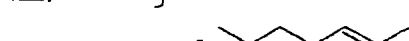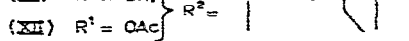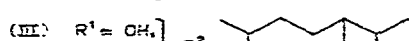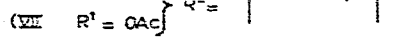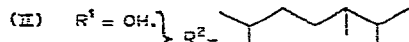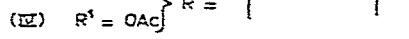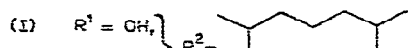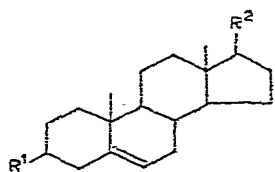
tural elucidation of sterols from plants, during investigation of their biosynthesis and metabolism, and during studies on the metabolism of dietary phytosterols in insects or other invertebrates. It is also often necessary to separate sterols differing in the number and/or position of double bonds. This can often be accomplished by silver nitrate–silica gel TLC, with careful selection of the solvent system[6].

The separation of C_{27}, C_{28} and C_{29} phytosterols and of sterols differing in the position and/or number of double bonds has been accomplished by various reversed-phase TLC methods[9]. These methods, in general, when used on a preparative scale, have the disadvantages of a low loading capacity, and also the stationary phase is eluted from the plates together with the separated solutes. Partial separation of sitosterol (III) and campesterol (II) on columns of Sephadex LH-20 after 20–25 cycles has been reported[10]. Similarly, liquid gel chromatography on columns of hydroxyalkoxypropyl-Sephadex has been used for group separation of biological extracts, including sterols and other terpenoids[11]. This column packing material consists of C_{11}–C_{14} hydrocarbons chemically attached to Sephadex LH-20. Although separation of microgram quantities of cholesterol (I), campesterol (II) and sitosterol (III) on the latter type of columns has been achieved at very low solvent flow-rates[12], complete separation of a 25-mg mixture of campesterol (II) and sitosterol (III) has been reported on such columns after 3–6 cycles[13]. Some separation of cholesterol (I), campesterol (II) and sitosterol (III) has also been achieved on Sephadex LH-20 chemically substituted with hydroxy-5β-cholanyl residues[14]. All these column chromatography methods for resolution of C_{27}, C_{28} and C_{29} sterols suffer from the disadvantage that a long time is required for the separation.

In an attempt to develop a more rapid method for separating C_{27}, C_{28} and C_{29} sterols, as well as ones differing in the number and/or position of double bonds, we have examined an HPLC approach using chemically bonded reversed-phase packings. Chemically bonded stationary phases have the major advantages that they can be made mechanically, thermally and hydrolytically stable, and can be readily used for gradient elution. Owing to the absence of column bleed, sample recovery is possible merely by evaporation of the eluent.

EXPERIMENTAL

Apparatus

A Model ALC/100 liquid chromatograph fitted with both a Model R401 refractive index detector and a UV monitor (254 nm) coupled in series, was used throughout this work, all from Waters Assoc. (Stockport, Great Britain). The instrument was modified so as to reduce to an absolute minimum the dead volume between the end of the column and the detectors. A Waters Assoc. M6000 pumping system was used, capable of delivering up to 9.9 ml/min at pressures up to 6,000 p.s.i.

Sample injection

Injections were made using the Waters Assoc. Model U6K septumless high-pressure valve injector. Samples dissolved in chloroform were introduced into the 2-ml sample loading loop of the injector using a 50-μl syringe having a 2-in. 25-gauge needle (Precision Sampling, Baton Rouge, La., U.S.A.). Usually for analytical separations at least 100 μg of each component was injected in a total sample volume of 10–

20 µl. Prior to injection, all sample solutions were filtered through a Millipore Fluoropore (PTFE) membrane filter of 0.5-µm pore size held in a stainless-steel Swinny filter holder attached to a glass syringe having a Luer-type outlet fitting. This removes any possible fine insoluble materials from the sample solution and thus prevents clogging of syringes, injection system, and in particular the column inlets, with consequent prolonged column life.

Columns

Bondapak C₁₈/Corasil (37–50 µm) and Bondapak C₁₈/Porasil B were purchased from Waters Assoc., whereas Permaphase ODS was purchased from DuPont (Hitchin, Great Britain). All three chromatographic materials were packed into 2 ft. × 1/8 in. O.D. stainless-steel columns. which were joined in series by 0.02-in. stainless-steel tubing to give greater lengths as required. Pre-packed columns of µBondapak C₁₈ in drilled 30 cm × 4 mm I.D. tubing fitted with 5-µm end fittings were purchased from Waters Assoc.

Solvents

AnalaR grade methanol and chloroform were purchased from BDH, and carefully re-distilled prior to use. In the case of methanol, the fraction boiling between 64.5° and 65.5° was collected, whereas in the case of chloroform, which contained 2% (v/v) ethanol as preservative, the fraction boiling between 60° and 62° was used. Water was deionised and then distilled in glass apparatus. All solvent mixtures for elution were degassed under vacuum immediately prior to use.

Determination of recovery of [4-¹⁴C]-cholesteryl acetate from a µBondapak C₁₈ column

A standard solution of [4-¹⁴C]-cholesteryl acetate in toluene was prepared (10 mg/ml; 104,250 cpm/ml) and a series of dilutions made from this stock solution. After accurate radioassay of each solution, different loadings (0.1–1.0 mg) of the [4-¹⁴C]-cholesteryl acetate were chromatographed on the µBondapak C₁₈ column (30 cm × 4 mm I.D.), with a mobile phase of methanol–chloroform–water (71:16:13) at a flow-rate of 1.5 ml/min. The complete peaks were collected in scintillation counting vials and the solvent evaporated to dryness under nitrogen. After addition of 10 ml of scintillation fluid consisting of 8 g butyl PBD in 1 l toluene, the samples were radioassayed in an Intertechnique ABAC-SL40 scintillation spectrometer.

RESULTS AND DISCUSSION

The reversed-phase separation of sterols as their acetates was initially attempted on an 8 ft. × 1/8 in. column of Bondapak C₁₈/Corasil using a variety of mobile phases. Bondapak C₁₈/Corasil consists of a monomolecular layer of C₁₈ straight-chain hydrocarbon chemically bonded to the pellicular packing material Corasil (37–50 µm diameter) via Si–C bonds[15]. This is prepared by reaction of octadecyltrichlorosilane with Corasil. Fig. 1 shows the best separation of the acetates of cholesterol (IV) and stigmasterol (XIII) which could be achieved on this type of packing material. Since this is clearly not a practical separation of these two components, the properties of other packing materials were explored.

Separation of the acetates of cholesterol (IV) and stigmasterol (XIII) could
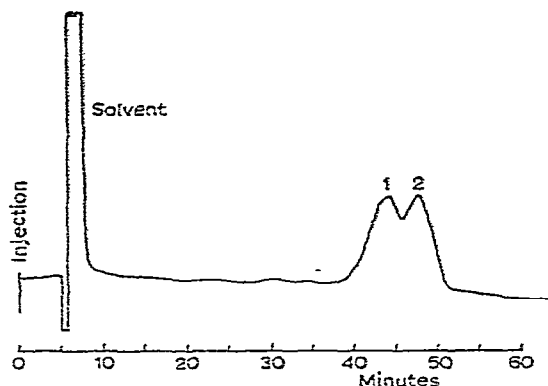
Fig. 1. Separation of the acetates of cholesterol (1) and stigmasterol (2) on Bondapak C_{18}/Corasil. Column, 8 ft. × 1/8 in. O.D.; mobile phase, methanol–chloroform–water (85:6:9); flow-rate, 1 ml/min.

not be achieved under the conditions used on Bondapak C_{18}/Porasil B (8 ft. × 1/8 in. O.D., particle diameter, 36–75 μm; Waters Assoc.)[16] nor on Permaphase ODS (8 ft. × 1/8 in. O.D.; DuPont, Hitchin, Herts., Great Britain)[17]. Bondapak C_{18}/Porasil B is designed as a high capacity packing material for preparative reversed-phase chromatography, and consists of heavy coating of C_{18} hydrocarbon permanently bonded to a high-surface-area porous silica substrate. Although it is chemically similar to the pellicular support, Bondapak C_{18}/Corasil, we considered that the heavier coating of C_{18} hydrocarbon in Bondapak C_{18}/Porasil B might yield better resolution of steryl acetates. This was evidently not the case, and this packing material appeared to give broader peaks with our solutes, which might be explained by inadequate rates of solute mass transfer. Such solute mass transfer effects might also account for the acute peak spreading observed on Permaphase ODS. This packing material consists of multi-layer, cross-linked, polymeric octadecylsilane bonded to a pellicular support. Since we were unable to perform the desired separations on any of the above packing materials, we explored the use of μBondapak C_{18} columns, which became available at that time. μBondapak C_{18} differs from Bondapak C_{18}/Corasil in that the C_{18} hydrocarbon is chemically bonded to the porous small diameter (<10 μm) packing material μPorasil[18]. Such material is reported to give much improved column efficiencies as compared with larger-diameter packing material[19].

Although nearly complete resolution of cholesterol (I), campesterol (II) and sitosterol (III) in the free form was observed on a μBondapak C_{18} column (Fig. 2), complete separation of the corresponding acetates could be achieved (Fig. 3a). Steryl acetates were used in most of this work in view of this and since we aimed to develop a rapid HPLC method which could be used to complement the commonly used silver nitrate–silica gel TLC methods for separation of sterols, usually as acetates.

Table I gives some chromatographic data for a μBondapak C_{18} column when used with steryl acetate solutes and a mobile phase of methanol–chloroform–water (71:16:13), which was employed in most of this work. It is now well documented[19,21] that decreasing packing particle size increases column efficiency in all modes of liquid chromatography and gives higher sample capacity. This increased efficiency is due to
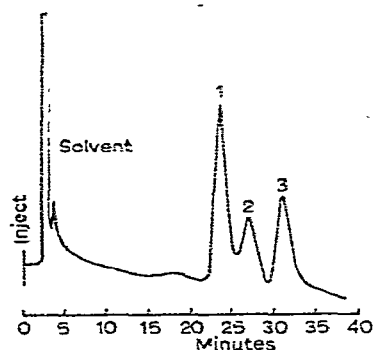
Fig. 2. Separation of free sterols on μBondapak C_{18}. Column, 30 cm × 4 mm I.D.; mobile phase, methanol–chloroform–water (71:12:17); flow-rate, 1.5 ml/min. 1 = Cholesterol; 2 = campesterol; 3 = sitosterol.
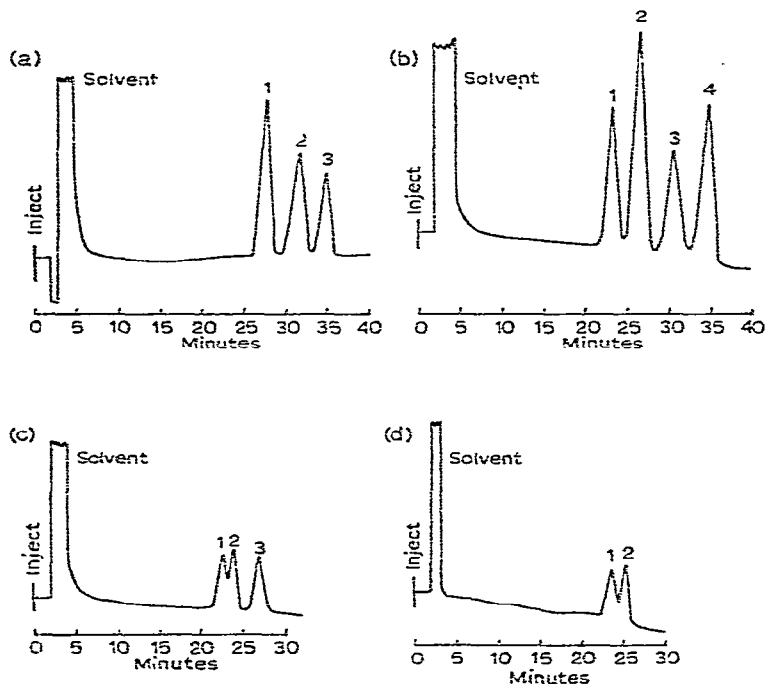
Fig. 3. Separation of various steryl acetates on μBondapak C_{18}. Column, 30 cm × 4 mm I.D.; mobile phase, methanol–chloroform–water (71:16:13); flow-rate, 1.5 ml/min. (a) 1 = Cholesteryl acetate; 2 = campesteryl acetate; 3 = sitosteryl acetate. (b) 1 = Ergosteryl acetate; 2 = brassica-steryl acetate; 3 = campesteryl acetate; 4 = sitosteryl acetate. (c) 1 = Desmosteryl acetate; 2 = cholesta-5,7-dien-3β-yl acetate; 3 = 5α-cholest-7-en-3β-yl acetate. (d) 1 = 24-Methylcholesta-5,25-dien-3β-yl acetate; 2 = 24-methylcholesta-5,24-dien-3β-yl acetate.

more rapid solute mass transfer into and out of small porous particles. The data in Table I show that the H values are essentially independent of the k' values, suggesting that rapid mass transfer occurs in the bonded phase. Although more than 3,000 theoretical plates had been obtained under certain conditions on the 30-cm column

TABLE I

CHROMATOGRAPHIC DATA FOR A μBONDAPAK C_{18} COLUMN WHEN USED WITH STERYL ACETATE SOLUTES

Column, 30 cm × 4 mm I.D.; mobile phase, methanol–chloroform–water (71:16:13) at a flow-rate of 1.5 ml/min.

Solute	Parameter[20]					
	k'	N	$H(mm)$	$N_{eff.}$	$N_{eff.}/sec$	R_s
Cholesteryl acetate (IV)	12.7	2191	0.14	1881	1.14	} 1.52
Campesteryl acetate (VII)	} 14.4	1888	0.16	1650	0.87	}
Stigmasteryl acetate (XIII)						} 1.30
Sitosteryl acetate (XII)	16.0	4381	0.07	3879	1.86	
5β-Cholestan-3β-yl acetate	12.2	1943	0.15	1659	1.02	}
5α-Cholestan-3β-yl acetate	14.5	1918	0.16	1678	0.88	} 1.77
5α-Cholest-7-en-3β-yl acetate (XIX)	12.5	3388	0.09	2904	1.75	} 1.88
Desmosteryl acetate (V)	10.0	2645	0.11	2185	20.81	} 0.71
Cholesta-5,7-dien-3β-yl acetate (XXII)	10.6	2947	0.10	2460	23.41	

which was used for most of this work, Table I shows that under our particular conditions of solute, mobile phase and flow-rate, N was somewhat lower than this for most samples. Since N varies to some extent with the solute retention time or capacity factor k', a better way of expressing column efficiency for comparison of plate numbers for different solutes is the number of effective plates, $N_{eff.}$:

$$N_{eff.} = N \left(\frac{k'}{1 + k'} \right)^2$$

The $N_{eff.}$ values observed in Table I are similar to those previously reported for this type of packing material[19].

The capacity factors (k') for a number of C_{27}, C_{28} and C_{29} steryl acetates chromatographed on a 30-cm μBondapak C_{18} column are compared in Table II. The mobile phase used for this column was more polar than that employed for the Bondapak C_{18}/Corasil. Excellent separation as their acetates of the Δ^5-C_{27}, -C_{28} and -C_{29} sterol homologues, cholesterol, campesterol and sitosterol was obtained (Fig. 3a). The acetates of cholesterol (IV)–campesterol (VII), and campesterol (VII)–sitosterol (XII) were separated with separation factors, α, of 1.14 and 1.11, respectively. Introduction of additional unsaturation into the molecules results in earlier elution (Fig. 3b). Stigmasteryl acetate (XIII) and campesteryl acetates (VII) are not resolved in this reversed phase system. Δ^5 and Δ^7 steryl acetates are separated in neither the C_{27} nor the C_{29} series. However, complete resolution as the acetates of 5α-cholestan-3β-ol (Δ^0) from a mixture of cholesterol and 5α-cholest-7-en-3β-ol was achieved. 5α- and 5β-cholestan-3β-yl acetates were well separated. The acetates of desmosterol (V) and cholesta-5,7-dien-3β-ol (XXII) were only partly resolved (Fig. 3c), but nearly complete separation was observed after three recycles. Use of two columns in series coupled with recycling should readily give complete resolution of this pair of compounds. The acetates of desmosterol (V) and 22-*trans*-cholesta-5,22-dien-3β-yl acetate (VI) were also resolved. Although the differences in k' values between $\Delta^{5,24(28)}$ and $\Delta^{5,25}$-steryl

TABLE II

COMPARISON OF THE CAPACITY FACTORS (k') FOR A NUMBER OF STERYL ACETATES CHROMATOGRAPHED ON A μBONDAPAK C_{18} COLUMN

Column, 30 cm $\times$ 4 mm I.D.; void volume, 3.08 ml; mobile phase, methanol–chloroform–water (71:16:13) at a flow-rate of 1.5 ml/min.

Solute	Grouping	k'
Cholesteryl acetate (IV)	C_{27} Δ^5	12.7
5β-Cholestan-3β-yl acetate	C_{27} Δ^0 5β-H	12.2
5α-Cholestan-3β-yl acetate	C_{27} Δ^0 5α-H	14.5
5α-Cholest-7-en-3β-yl acetate (XIX)	C_{27} Δ^7	12.5
Desmosteryl acetate (V)	C_{27} $\Delta^{5,24}$	10.0
Cholesta-5,7-dien-3β-yl acetate (XXII)	C_{27} $\Delta^{5,7}$	10.6
22-cis-Cholesta-5,22-dien-3β-yl acetate	C_{27} $\Delta^{5,22}$	9.2
22-trans-Cholesta-5,22-dien-3β-yl acetate (VI)	C_{27} $\Delta^{5,22}$	10.2
Campesteryl acetate (VII)	C_{28} Δ^5	14.4
Brassicasteryl acetate (VIII)	C_{28} $\Delta^{5,22}$	12.0
24-Methylenecholesteryl acetate (IX)	C_{28} $\Delta^{5,24(28)}$	10.9
24-Methylcholest-5,25-dien-3β-yl acetate (X)	C_{28} $\Delta^{5,25}$	10.5
24-Methylcholest-5,24-dien-3β-yl acetate (XI)	C_{28} $\Delta^{5,24}$	11.4
Ergosteryl acetate (XXIII)	C_{28} $\Delta^{5,7,22}$	10.4
Sitosteryl acetate (XII)	C_{29} Δ^5	16.0
5α-Stigmast-7-en-3β-yl acetate (XX)	C_{29} Δ^7	15.3
Stigmasteryl acetate (XIII)	C_{29} $\Delta^{5,22}$ (24S)	14.4
Poriferasteryl acetate (XIV)	C_{29} $\Delta^{5,22}$ (24R)	14.4
Fucosteryl acetate (XVI)	C_{29} $\Delta^{5,24(28)}$ (24E)	13.6
28-Isofucosteryl acetate (XV)	C_{29} $\Delta^{5,24(28)}$ (24Z)	13.6
(24Z)-5α-Stigmasta-7,24(28)-dien-3β-yl acetate (XXI)	C_{29} $\Delta^{7,24(28)}$ (24Z)	13.0
24-Ethylcholest-5,25-dien-3β-yl acetate (XVII)	C_{29} $\Delta^{5,25}$	13.0
24-Ethylcholest-5,22,25-dien-3β-yl acetate (XVIII)	C_{29} $\Delta^{5,22,25}$	11.4

acetates were insufficient to give a separation in both the C_{28} and C_{29} series, partial resolution of $\Delta^{5,25}$ and $\Delta^{5,24}$ steryl acetates was obtained (Fig. 3d). Neither the acetates of the 24Z and 24E isomers, fucosterol (XVI) and 28-isofucosterol (XV), nor the 24S and 24R epimers, stigmasterol (XIII) and poriferasterol (XIV), could be separated by this reversed-phase method.

Recovery of material from a μBondapak C_{18} column was assessed by chromatography of samples of [4-^{14}C]-cholesteryl acetate ranging from 0.1 mg–1.0 mg. The peaks were collected, and then radioassayed. The results (Table III) indicate that with-

TABLE III

RECOVERY OF [4-^{14}C]-CHOLESTERYL ACETATE FROM A μBONDAPAK C_{18} COLUMN AT DIFFERENT LOADINGS

Column, 30 cm $\times$ 4 mm I.D.; mobile phase, methanol–chloroform–water (71:16:13) at a flow-rate of 1.5 ml/min.

Load (mg)	Recovery (%)
0.1	99
0.5	97
0.8	98
1.0	97

in the limits of the experimental error the recovery of cholesteryl acetate at each loading is practically quantitative.

Small porous particles are reported to have higher sample capacity than pellicular materials[19]. The capacity of μBondapak C_{18} was examined by chromatography of varying amounts (0.1 mg–1.0 mg) of a mixture of the acetates of sitosterol (XII) and campesterol (VII) (3:2). Whereas baseline separation was maintained up to a load of approximately 0.6 mg, beyond that there was a rapid loss of resolution. However, the total volume of the μBondapak C_{18} column is sufficient to make recycling feasible. For example, when 9 mg of a mixture of the acetates of sitosterol and campesterol (3:2) were recycled, practically complete separation of the components could be achieved after the fourth recycle despite marked peak distortion. Collection of the entire individual peaks after the fourth recycle and analysis by GLC (3 % OV-17) showed that the compounds were practically pure. Improved resolution of sitosteryl and campesteryl acetates was obtained on two μBondapak C_{18} columns (30 cm $\times$ 4 mm I.D. each) joined in series, although the separation required a longer time. Although the feasible loading depends on the resolution, R_s, of a pair of solutes, this system should allow preparative separation of much larger samples in the region of 15–20 mg. Analytical scale separation of the acetates of campesterol (VII) and stigmasterol (XIII) could not be achieved on this 60-cm-long column.

The useful lower limit of detection of steryl acetates, with the mobile phase we mostly used for this work, was of the order of 50 μg, using the refractive index detector. This limit is rather high in view of the similarity in refractive index of the steryl acetates and the mobile phase; the free sterols show a greater difference. The sensitivity of detection could be greatly increased using the steryl benzoates, which can be detected by an UV monitor (Fig. 4). However, the resolution in this case may not be quite as good, at least with the particular mobile phase used.

μBondapak C_{18} gives excellent separations of sterols differing in number of double bonds, and also of C_{27}, C_{28} and C_{29} sterols. The latter C-24 homologues cannot be separated by silver nitrate–silica gel TLC, which has the added disadvantage of usually yielding poor recoveries due to autoxidation. However, silver nitrate–silica gel TLC, which involves specific binding between the silver and π electrons of the double bonds, appears to be slightly more selective than the present reversed-phase
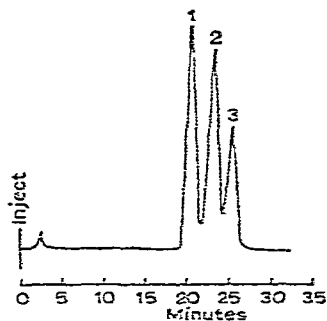

Fig. 4. Separation of steryl benzoates. Column, μBondapak C_{18}, 30 cm $\times$ 4 mm I.D.; mobile phase, methanol–chloroform–water (72:16:12); flow-rate 2.0 ml/min. 1 = Cholesteryl benzoate; 2 = campesteryl benzoate–stigmasteryl benzoate; 3 = sitosteryl benzoate.

method for the position of the double bonds in the sterol molecule. This is often critically dependent upon the purity and composition of the developing solvent. For example, using a 30-cm μBondapak C_{18} column, the acetates of desmosterol (V) and cholesta-5,7-dien-3β-ol (XXII) are only partly resolved, whereas fucosteryl (XVI) and 28-isofucosteryl (XV) acetates are not separated. The former pair of compounds can be readily separated by silver nitrate–silica gel TLC ,whereas the latter pair can be resolved with care.

There is some doubt as to the exact retention mechanism of solutes on monomolecular layers of bonded stationary phases[1,17,22]. It is argued that in the case of "brush" type (non-cross-linked) bonded packings, the solute–stationary phase interactions cannot be true solution, and may extensively involve simple adsorption of the solute on the stationary phase. However, for similar solutes, selectivity is dependent upon the relative solute solubilities in the mobile phase[17].

The reversed-phase separation of sterols on μBondapak C_{18} can be applied to the preparative separation of sterol mixtures isolated from biological materials by the conventional techniques of column and thin-layer chromatography. This technique used analytically provides a criterion based upon another principle (reversed phase) for checking the purity of samples isolated by liquid–solid chromatography. Similarly, the method may be used to provide additional evidence for the identity of a particular sterol by comparison with an appropriate standard. In this connection, it is important to take into account possible variation in the observed capacity factors, k', which can be appreciable in HPLC. Additionally, slight variation in the composition of the mobile phase can be very critical. In view of this potential variability, the capacity factors alone cannot be used as valid criteria of positive identification, and should only be used in conjunction with other methods, such as GLC, mass and nuclear magnetic resonance spectrometry. This preparative separation of C_{27}, C_{28} and C_{29} sterols on μBondapak C_{18} can be achieved in very much shorter time than by previously reported methods of conventional reversed-phase column chromatography. The present method is also readily applicable to the separation of individual 4,4-dimethyl and also 4α-methyl sterols.

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