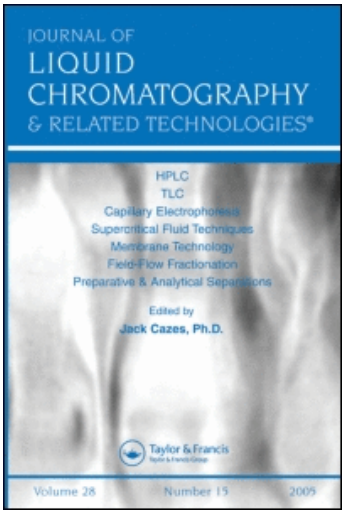

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SEPARATION OF EPIMERIC PAIRS OF C-24 ALKYLSTEROLS BY REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF THE FREE STEROLS AT SUBAMBIENT TEMPERATURE

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ABSTRACT

Separation of most of the common biologically occurring epimeric pairs of C-24 alkylsterols was obtained by reversed-phase HPLC of non-derivatized free sterols at subambient temperatures. Base line separation occurred in most cases with an analysis time frequently as rapid as 30 minutes with a solvent system of methanol and isopropanol. Several pairs of C-24 ethylsterols were separated poorly by this system but were satisfactorily resolved with a mobile phase of methanol, although analysis times were as long as 90 minutes. A method was also developed for concurrent resolution of important metabolic intermediates in cholesterol metabolism: cholesterol, desmosterol, lathosterol, and 7-dehydrocholesterol.

INTRODUCTION

The sterols biosynthesized by plants predominantly contain C-24 alkyl substituents with frequently divergent stereochemical orientation (1). The separation of these pairs of C-24 epimeric sterols has been achieved with remarkable difficulty. Although nuclear magnetic resonance (NMR) spectroscopy offers an unequivocal means of identification and quantification of mixtures of C-24 epimeric sterols (2,3), the instrumentation involved is often inaccessible to many researchers. Of course, NMR cannot be employed to preparatively fractionate a mixture of compounds for further experimentation and is also less sensitive than high-performance liquid chromatography (HPLC) or gas-liquid chromatography (GLC). Although the latter technique has been used for the separation of trimethylsilyl ethers of several C-24 epimers (4), analyses require 2-3 hr and have limited preparative value. More recently, separation of epimeric pairs of 24-alkylsteryl benzoates has been achieved with reversed-phase HPLC (5,6) at 20°C. Because the preparation of steryl benzoates involves an additional derivatization step and because mass spectral data bases for free sterols are more voluminous than those for the less frequently characterized steryl benzoates, we began an investigation to determine the feasibility of fractionating these important biomolecules as free sterols. Through the utilization of subambient (10°C-14°C) temperatures, base line resolution of most epimeric pairs occurred within reasonable periods of analysis.

MATERIALS AND METHODS

All HPLC fractionations were obtained with a TSK-Gel ODS 120A column, 4.6-mm i.d. x 25 cm, 5μm particle size (Toyo Soda Manufacturing Co., Tokyo). In most cases, the column was contained in a water jacket (Rainin Instrument Co., Woburn, MA) and cooled with tap water (10°C-14°C). For comparative purposes, the column was

occasionally inserted in an ice-water bath or in a column heater (Waters, Milford, MA) operated at 30°C. A Spectra-Physics SP-8700XR solvent delivery system was used to isocratically pump methanol:isopropanol 4:1 or methanol (for epimeric pairs that were difficult to separate) at a flow rate of 1.0 ml/min. Absorbance was monitored at 214 nm with a Waters model 441 detector connected to a Shimadzu model C-R3A recording integrator. Approximately 5 μg of each compound was injected by itself, with its C-24 epimer, and with a cholesterol internal standard for accurate RRT determination. All HPLC peaks were trapped and analyzed by GLC (J&W DB-1 capillary column, 14 m x 0.25 mm, 0.25-μm film, 240°C) as a safeguard against artifact.

Sterols (Fig. 1) were the same samples employed in a previous investigation (4). Nomenclature is generally with reference to campestane (24α-methylcholestane), ergostane (24β-methylcholestane), stigmastane (24α-ethylcholestane) and poriferastane (24β-ethylcholestane). Some trivial and systematic names are as follows: cholesterol, cholest-5-en-3β-ol; 7-dehydrocholesterol, cholesta-5,7-dien-3β-ol; desmosterol, cholesta-5,24-dien-3β-ol; lathosterol, 5α-cholest-7-en-3β-ol; campesterol, 24α-methylcholest-5-en-3β-ol; dihydrobrassicasterol, 24β-methylcholest-5-en-3β-ol; epibrassicasterol, 24α-methylcholesta-5,22(*E*)-dien-3β-ol; brassicasterol, 24β-methylcholesta-5,22(*E*)-dien-3β-ol; sitosterol, 24α-ethylcholest-5-en-3β-ol; clionasterol, 24β-ethylcholest-5-en-3β-ol; stigmasterol, 24α-ethylcholesta-5,22(*E*)-dien-3β-ol; poriferasterol, 24β-ethylcholesta-5-22(*E*)-dien-3β-ol; spinasterol, 24α-ethyl-5α-cholesta-7,22(*E*)-dien-3β-ol; chondrillasterol, 24β-ethyl-5α-cholesta-7,22(*E*)-dien-3β-ol.

RESULTS

We selected methanol:isopropanol mixtures for initial examination of the feasibility of resolving campesterol and its 24β-epimer, 22-

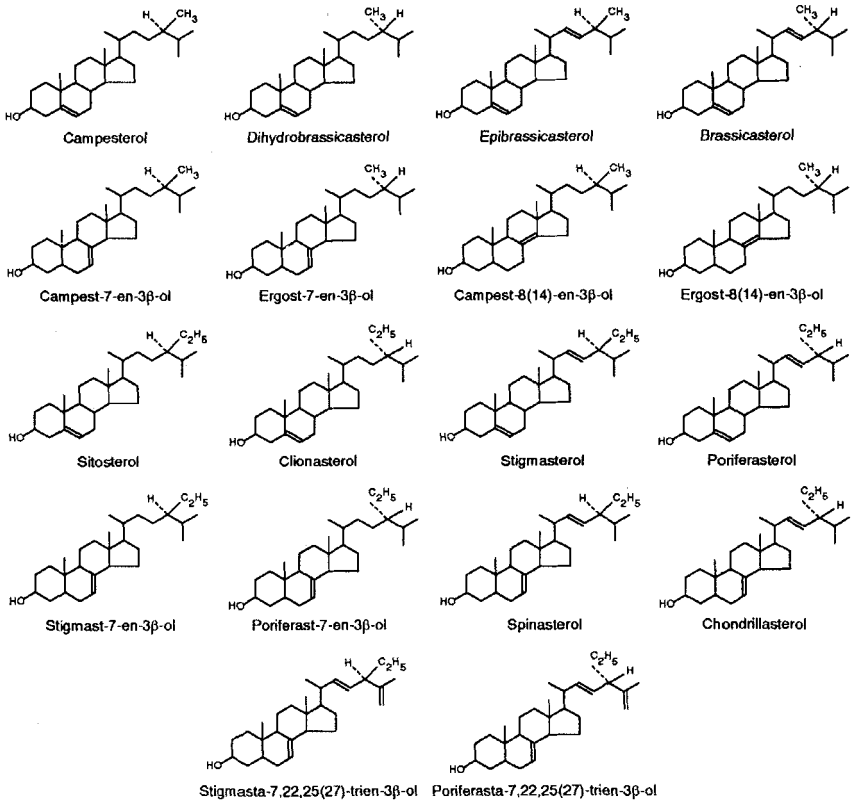

FIGURE 1. Structures of some pairs of C-24 epimeric sterols.

dihydrobrassicasterol. The respective retention times in minutes of these two epimers with solvents of 0, 10, 20, 30, 40 and 50% isopropanol in methanol were 78.99 and 87.33, 59.72 and 66.33, 44.64 and 50.62, 30.98 and 34.24, 25.23 and 27.99, and 17.39 and 18.95. Base line resolution was obtained even at the lattermost solvent strength. Several sterol epimers were separated with a solvent system of methanol:isopropanol 4:1 (Fig. 2, Table 1), including all C_{28}-sterols. Several C_{29}-sterols separated poorly with this solvent system but were

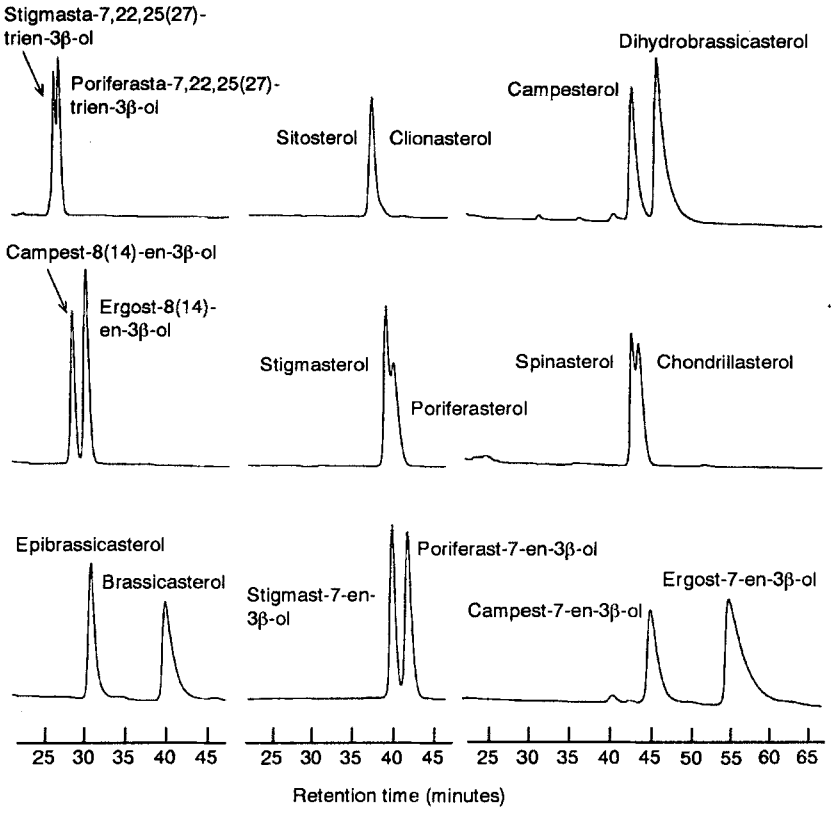

FIGURE 2. HPLC chromatograms of pairs of C-24 epimeric sterols at 12°C with a solvent system of methanol:isopropanol 4:1 at 1.0 ml/min through a TSK-Gel ODS 120A column, 4.6-mm i.d. x 25 cm, 5μm particle size.

resolved better with a solvent of methanol (Fig. 3). Sitosterol and clionasterol were notable exceptions.

Because of the difficulty in separating sitosterol from clionasterol, a few other procedures were investigated. Solvent systems consisting of various mixtures of hexane and methanol, acetonitrile and methanol, and acetonitrile and isopropanol did not improve resolution of the other

TABLE 1

High Performance Liquid Chromatographic Relative Retention Times (RRTs) for Sterols, Expressed Relative to Cholesterol.

Sterol	Orientation at C-24	RRT
5α-Stigmasta-7,22,25(27)-trien-3β-ol	α	0.52
5α-Poriferasta-7,22,25(27)-trien-3β-ol	β	0.54
5α-Campest-8(14)-en-3β-ol	α	0.59
5α-Ergost-8(14)-en-3β-ol	β	0.64
Epibrassicasterol	α	0.64
Brassicasterol	β	0.84
Sitosterol	α	0.77
Clionasterol	β	0.77
Stigmasterol	α	0.82
Poriferasterol	β	0.84
5α-Stigmast-7-en-3β-ol	α	0.85
5α-Poriferast-7-en-3β-ol	β	0.90
Campesterol	α	0.89
Dihydrobrassicasterol	β	1.02
Spinasterol	α	0.88
Chondrillasterol	β	0.91
5α-Campest-7-en-3β-ol	α	0.95
5α-Ergost-7-en-3β-ol	β	1.16
Desmosterol	†	0.63
7-Dehydrocholesterol	†	1.12
Lathosterol	†	1.13

Analyses were performed on a TSK-Gel ODS 120A column, 4.6-mm i.d. x 25 cm, 5μm particle size, at 12°C with a solvent system of methanol:isopropanol 4:1.

† Not applicable.

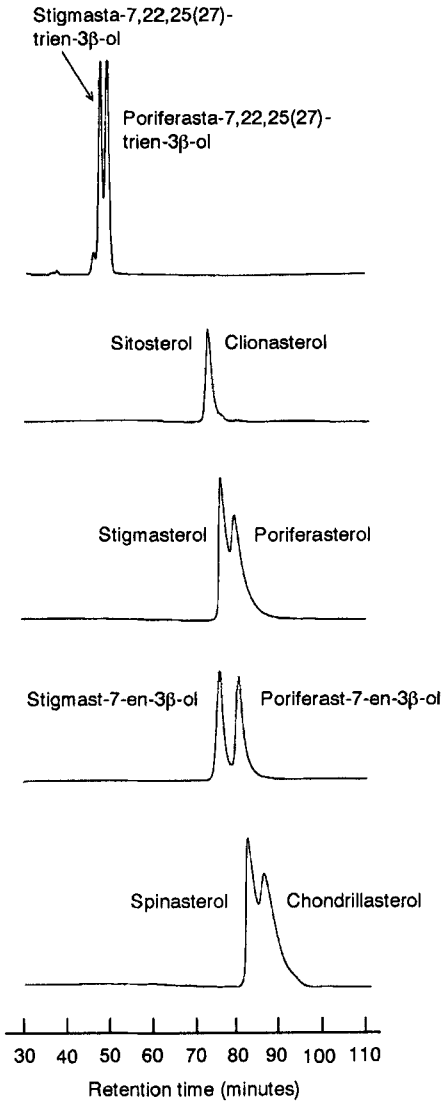

FIGURE 3. HPLC chromatograms of epimeric pairs of 24-ethylsterols at 12°C with a solvent system of methanol at 1.0 ml/min through a TSK-Gel ODS 120A column, 4.6-mm i.d. x 25 cm, 5μm particle size..

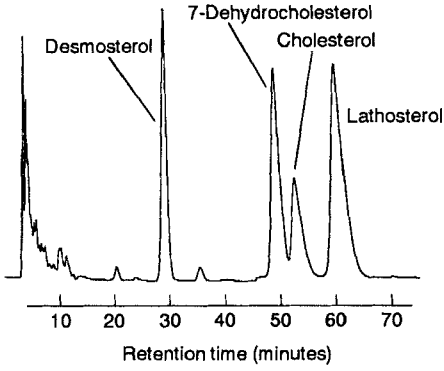

FIGURE 4. HPLC chromatogram of separation of four common 24-desalkylsterols at 12°C with a solvent of acetonitrile:isopropanol:hexane 57:40:3 at 1.0 ml/min through a TSK-Gel ODS 120A column, 4.6-mm i.d. x 25 cm, 5μm particle size..

difficult-to-separate epimeric pairs and also did not partially resolve sitosterol and clionasterol. In addition, a few separations were attempted in an ice-water bath with mixtures of methanol and isopropanol or acetonitrile and isopropanol. In all cases, excessive peak tailing occurred. In a final attempt to separate clionasterol and sitosterol, the acetate derivatives of each compound were prepared and chromatographed with a solvent of methanol:isopropanol 4:1 or methanol. As with the free sterols, only a single peak appeared following coinjection of sitosteryl and clionasteryl acetate.

In addition to 24-alkylsterols, a few 24-desmethylsterols were chromatographed. The methanol:isopropanol solvent system did not separate 7-dehydrocholesterol and lathosterol (Table 1). However, cholesterol (HPLC RRT 1.00), lathosterol (RRT 1.12), desmosterol (RRT 0.56), and 7-dehydrocholesterol (RRT 0.93) were successfully separated with a solvent of acetonitrile:isopropanol:hexane 57:40:3 (Fig.4).

DISCUSSION

During the past several years, we have been investigating the biochemistry and phylogenetic distribution of sterols in nematodes (7,8) and algae (9). We have been particularly interested in the metabolism of sterols from higher plants by nematodes. The 24-ethylsterols of higher plants are usually α-epimers, but the 24-methylsterols are often mixtures of α- and β-epimers (1). There is some evidence for C-24 dealkylation by nematodes of the most common 24-methylsterols in higher plants, i.e., campesterol and dihydrobrassicasterol (10). Therefore, our main priority was to develop procedures for separation of common C_{28} phytosterols.

Although Ikekawa et al. reported the best separations of steryl benzoates with chloroform/acetonitrile mixtures, use of chloroform precludes the use of free sterols, which have λ_{max} below the UV cutoff of chloroform. Also, acetonitrile supplies were fairly erratic during the course of the investigation. Therefore, initial fractionations of campesterol and dihydrobrassicasterol were performed with mixtures of methanol, isopropanol, and hexane, although we did attempt numerous fractionations with acetonitrile-based mobile phases as well. The four C_{28} epimeric pairs and a few C_{29} epimers were separated. Resolution of the C_{29} epimers improved with a more polar mobile phase at the expense of increased retention time.

Temperature-induced changes in relative retention of sterols and steroids have been reported previously (11-13). Ikekawa et al. (6) obtained the best resolution of C-24 epimeric steryl benzoates at 20°C. In our investigations, resolution of epimeric pairs improved with further decreases in column temperature, with maximum resolution at about 10-12°C. The use of subambient temperatures facilitated the use of less polar solvents and permitted more rapid chromatographic analysis. At

lower temperatures, substantial peak tailing outweighed any improvements in resolution.

We also attempted to improve the resolution of difficult-to-separate pairs by using mobile phases consisting of mixtures of methanol and acetonitrile, methanol and hexane, or acetonitrile and isopropanol, but resolution did not improve. More importantly, no mobile phase even partially separated clionasterol from sitosterol, one of the three most common sterols of higher plants. In addition, none of the mobile phases could partially resolve sitosteryl acetate and clionasterol acetate.

The molecular basis for resolution of these epimeric pairs is unknown. Other C_{18} HPLC systems do not resolve C-24 epimeric pairs (3,13,14), and we could not separate epimeric pairs with two other C_{18} columns (Shandon ODS Hypersil, 25 cm x 4.6 mm i.d., 5 μm particles; Alltech Adsorbosphere HS, 25 cm X 4.6 mm i.d., 5μm particles, 20% C_{18} load). The TSK column is the only one of the three columns not fully endcapped. Obviously, other chemical or physical properties of the packing surface could also result in differential selectivity. Also interesting is the difference in relative retentions among sterols with the TSK column in comparison to other reversed phase systems. The most obvious example is in the effect of side chain alkylation on retention; on our other C_{18} systems as well as in the literature (3,13-15), an increase in retention accompanies an increase in extent of alkylation of the side chain. In contrast, sitosterol emerged before campesterol, which emerged before cholesterol during HPLC on the TSK column. Similarly, Δ^7-stigmastenol emerged before Δ^7-campestenol, which emerged before Δ^7-cholestenol (i.e., lathosterol). When a Δ^{22}-bond was present, however, this unusual retention did not occur; e.g., $\Delta^{5,22}$-campestadienol (epibrassicasterol) preceded $\Delta^{5,22}$-stigmastadienol (stigmasterol). Another peculiar phenomenon was the relatively small effect of addition

of a 24β-methyl substituent on retention; e.g., Δ^7- and Δ^5-ergostenols emerged close to lathosterol and cholesterol, respectively.

The TSK column was also useful in resolving cholesterol, lathosterol, 7-dehydrocholesterol, and desmosterol, four major metabolites of phytosterols in some insects (16) and nematodes (7,8), and three of which are metabolically important mammalian sterols (1). Although fractionation of the free sterols with the TSK column is not as sensitive as the recently reported fractionation of the enone derivatives of cholesterol, desmosterol, and 7-dehydrocholesterol (17), the direct analysis of free sterols is quicker.

Similarly, the method is not as sensitive as the previously reported fractionation of steryl benzoates (6) and does not resolve sitosterol and clionasterol. The latter pair was the most difficult-to separate pair of epimers as benzoates (6); fortunately, clionasterol rarely occurs in higher plants (1). Our method, with its emphasis upon subambient temperature, does not involve additional derivatizations, is well suited to preparative work, involves reasonable chromatographic analysis times, and involves compounds with widely available mass spectral data.

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