Liquid–gel chromatography on lipophilic–hydrophobic Sephadex derivatives

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ABSTRACT Hydrophobic long-chain alkyl ethers of Sephadex have been synthesized and tested for use in liquid chromatography. With columns prepared in such polar solvents as methanol, reversed-phase systems are obtained where compounds separate in order of decreasing polarity. In such nonpolar solvents as heptane, straight-phase systems are formed, and separations occur in order of increasing polarity. The retention volume of a compound is determined mainly by its polarity, the type and degree of substitution of Sephadex, and the nature of the solvent system. Examples are given of straight- and reversed-phase separations of waxes, fatty acids, glycerides, glycerol ethers, sterols, bile acids, and hormonal steroids. The Sephadex derivatives are simple to make, the columns are easy to prepare, and they can be used over long periods of time. Liquid–gel chromatography on alkyl ethers of Sephadex is a mild separation technique and should be a useful complement to other chromatographic methods.

SUPPLEMENTARY KEY WORDS alkyl ether · column chromatography · reversed- and straight-phase partition · wax · fatty acid · glyceride · glycerol ether · sterol · bile acid · hormonal steroid

Progress in liquid chromatographic methods has been slow compared with the rapid development of gas chromatography. This is partially due to lack of column packings, which are simple to prepare and use in permanent column systems with automatic detection, and which are suitable for work both on a preparative and an analytical scale. Sephadex has many of the properties required of such a column material. It does not swell in organic solvents, but its hydroxyl groups can be converted into less polar derivatives. Thus, a methyl ether of Sephadex has been prepared and used in the chromatography of lipids and steroids (1, 2). Sephadex LH-20 is a hydroxypropyl ether of Sephadex G-25 and is available commercially (3). These derivatives have been successfully used in liquid–gel chromatography with solvents of medium polarity, but they are too polar to be used in nonpolar solvent mixtures (see review by Sjövall, Haahti, and Nyström [4]). Attempts have, therefore, been made to prepare derivatives which swell in aliphatic hydrocarbons. This paper describes the synthesis and chromatographic applications of hydrophobic Sephadex derivatives containing long-chain hydroxyalkyl substituents. A preliminary report has been published (5).

EXPERIMENTAL METHODS

All solvents were of analytical grade. When necessary, e.g. when a flame ionization detector was used, they were redistilled before use. Steroids were gifts from Dr. J. Babcock; glycerides and phenylthiohydantoins were kindly supplied by Professors B. Borgström and J. Sjöqvist, respectively. Other reference compounds were obtained from commercial sources. All compounds were checked for purity with gas–liquid chromatography and(or) thin-layer chromatography.

Sephadex G-25 and G-50, superfine, and Sephadex LH-20 were kindly supplied by Dr. B. Gelotte, Pharmacia, Uppsala, Sweden. When small beads with narrow, well-defined size distribution were needed, the particles were fractionated as described by Hamilton (6). Nedox 1114 (C_{11}–C_{14} chain length) and Nedox 1518 (C_{15}–C_{18} chain length) olefin oxides were purchased from Ashland Chemical Company, Columbus, Ohio.

Hydroxypropyl Sephadex

Sephadex LH-20 is a hydroxypropyl derivative of Sephadex G-25, fine. When material with smaller bead

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size is needed, it is necessary to prepare a hydroxypropyl derivative of Sephadex G-25, in the following manner. Fractionated Sephadex G-25, superfine (bead size 30–40 μ in water), 32.0 g, is soaked for 2 hr in 4% aqueous sodium hydroxide. Excess aqueous phase is then removed by filtration with suction. The wet Sephadex, 188 g, is suspended in 600 ml of propylene oxide (technical grade). The volume of the propylene oxide should be four to five times that of the water trapped in the gel. The mixture is refluxed with stirring overnight. The stirring was achieved with a stirring rod which passed through a bulb condenser. The mixture is then filtered on a Büchner funnel with suction. The product is washed exhaustively with ethanol (96% w/w) and chloroform, and is then dried at 60°C to a constant weight of 64.3 g. If cross-linkages are neglected, this corresponds to 100% substitution of the hydroxyl groups in Sephadex. The same degree of substitution is obtained with Sephadex G-50.

Hydroxyalkoxypropyl Sephadex[1]

Hydroxypropyl Sephadex (prepared as described above) or Sephadex LH-20 are used as starting materials for the preparation of the hydrophobic derivatives. 10 g are soaked in 100 ml of methylene chloride in a 1000 ml Erlenmeyer flask. Boron trifluoride ethyletherate (48% BF_3), 2.5 ml, is added and thorough mixing is achieved by shaking (the gel particles will be destroyed if a magnetic stirrer is used). After 15 min, 15-ml portions of a 20% (v/v) solution of Nedox 1114 in methylene chloride are added every 10 min with shaking and cooling of the reaction flask in ice water. When 150 ml of the Nedox solution have been added, the reaction is allowed to proceed for another 20 min. The product is then filtered on a Büchner funnel and is washed with successive portions of chloroform, ethanol (96% w/w), and chloroform–methanol 1:1 (v/v). The product is then refluxed with stirring in chloroform–methanol 1:1 for 4 hr, washed with chloroform–methanol 1:1 and benzene, and refluxed in benzene for 4 hr. The product is carefully washed with benzene and refluxed for a second 4 hr period in benzene. After a final washing with benzene, the product (22.1 g) is dried at room temperature, preferably in vacuo. The weight increase corresponds to a hydroxyalkyl group content of 55% (w/w).

Solvent regain values, i.e. amount of solvent imbibed in the gel beads after removal of interstitial fluid by centrifugation (g of solvent per g of dry Sephadex derivative), were determined as previously described (7).

Chromatography Columns

The following three types of columns were used: Teflon spaghetti columns, 1–2 m × 1.5 mm; jacketed glass columns, 600 × 25 mm, suitable for recycling chromatography (LKB Produkter, Stockholm, Sweden); and glass columns, 200–500 × 4 mm. The first two column types were used as previously described for chromatography with methyl Sephadex (4, 5). The small-diameter glass columns had a bottom endpiece of Teflon which was pushed into the lower end of the glass tube. This endpiece had a central hole for a stainless steel capillary tubing ($^1/_{16}$ in. O.D., 0.01 in. I.D.), and an inserted filter of porous Teflon on which the column bed rested. Circular ridges on the surface of the endpiece provided a leak-free seal between the glass and the Teflon surfaces.

Columns were prepared as described for previous Sephadex derivatives, using an equilibrated slurry of the material in the solvent to be used (4). Agitation for a few minutes in an ultrasonic bath gave a homogenous slurry. The column bed was covered with a tight-fitting filter of porous Teflon. This was necessary when solvents having a density higher than that of the Sephadex derivative were used.

Samples, up to 50 mg/cm² column area, were dissolved in a small amount of mobile phase and were applied on the columns through an injection port (Teflon spaghetti columns), through a selector valve (recycling column), or with a capillary pipette (small-diameter glass columns). When the solubility of the sample in the mobile phase was too low, the Teflon columns were charged by injection of a chloroform solution through the injection port (4).

Solvent flow rates in capillary and small-diameter glass columns were measured with a calibrated, narrow-diameter glass or Teflon tube attached to the column outlet.

The effluent from columns with 1.5–4 mm I.D. was monitored with a moving-chain flame ionization detector (4). A UV-detection system was used with the large diameter columns.

RESULTS

Synthesis of Hydroxyalkoxypropyl Sephadex

The optimal amount of boron trifluoride etherate is 0.1–0.3 ml/g of hydroxypropyl Sephadex (Fig. 1). The degree of hydroxyalkylation can be selected by varying the amount of alkyl olefin oxide in the reaction mixture (Fig. 1). Epoxides other than Nedox 1114 and 1518 have also been tried; phenyl ethylene oxide gives phenyl hydroxyethyl derivatives. Sephadex derivatives carrying both hydroxyalkyl and phenyl hydroxyethyl groups in different proportions can also be prepared. With a derivative synthesized and purified as described above, there is virtually no column bleed. Thus, 100 ml of the effluent from a 25 g column contains no detectable ma-

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[1] This material will be manufactured by Terra-Marine Bioresearch, P. O. Box 725, Poway, Calif. 92064.

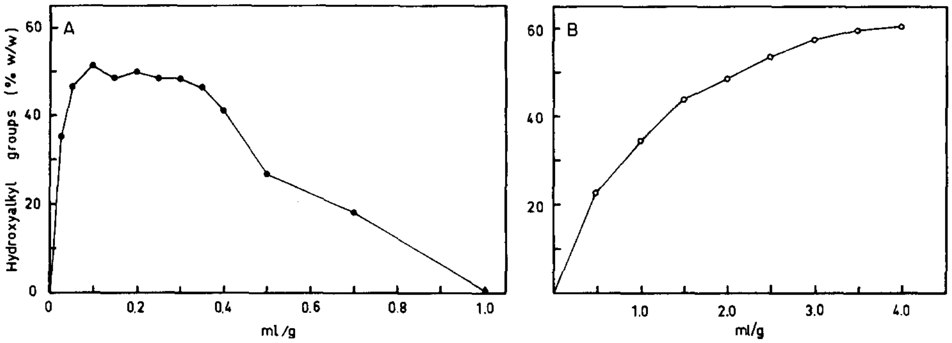

Fig. 1. Effect of the amount of (*A*) boron trifluoride etherate (●), or (*B*) Nedox 1114 (○), on the degree of substitution of Sephadex LH-20. The optimum amount of BF_3 etherate was determined using 2.5 ml of Nedox 1114 per g of Sephadex LH-20. When the volume of Nedox was varied, 0.25 ml of BF_3 etherate per g of Sephadex LH-20 was used.

terial (<0.1 mg). No interferring peaks have been found in gas–liquid chromatographic analyses of column fractions containing sterols, hormonal steroids, and fatty acid methyl esters.

Even moderately substituted hydroxyalkyl derivatives are not wetted by water. The highly substituted derivatives swell best in solvents like hexane, heptane, benzene, toluene, and chlorinated hydrocarbons, whereas swelling in methanol, ethanol, and acetone is limited. As shown by the solvent regain values given in Table 1, the incorporation of solvent into the gel beads depends on the nature of the solvent and on the type and degree of substitution.

Chromatographic Properties

The chromatographic properties of hydrophobic Sephadex derivatives were tested by chromatography of cholesterol in different solvents. As shown in Table 2, the most pronounced retardation was obtained in highly polar (methanol) and highly nonpolar (aliphatic hydrocarbons) solvents. This was thought to be caused by the presence in cholesterol of a nonpolar hydrocarbon structure and a polar hydroxyl group, respectively. When heptane was added to methanol, cholesterol was eluted more rapidly than with methanol alone (Table 3). To investigate this further, a mixture of sterols with one, two, and three hydroxyl groups was chromatographed in a series of solvent mixtures with different polarities. The results are shown in Fig. 2. The three compounds are eluted in order of decreasing polarity in methanol and in methanol containing ethylene chloride. Large separation factors between tri- and dihydroxysterols are obtained in reversed-phase separations with methanol–water mixtures. Straight-phase separations are obtained with heptane and with mixtures of heptane and ethylene chloride. When ethylene chloride is used, the three sterols have essentially the same elution volume. This indicates that the polarities of the stationary gel and the mobile solvent phases are similar and that separations based on molecular sieving may take place (4).

The polarity of the gel phase also depends on the type and degree of substitution of Sephadex. Thus, increasing hydroxyalkyl group content gives an increased retardation of cholesterol in methanol (Table 4).

As in other chromatographic systems, the efficiency of the columns is influenced by the particle size of the stationary phase and the solvent flow rate. An effect of temperature on the efficiency of the columns has been

TABLE 1 Solvent Regain Values for Different Hydrophobic Derivatives Prepared from Sephadex LH-20, 170–325 Mesh

		Solvent Regain Value† In:					
Substituent*	Degree of Substitution	Water	Methanol	Propan-2-ol	Ethylene Chloride	Benzene	Heptane
	%w/w						
C_{11}–C_{14}	30.5	0.5	1.1	1.4	2.0	1.3	0.3
C_{11}–C_{14}	41.3	0.4	0.7	1.1	1.9	1.4	0.5
C_{11}–C_{14}	62.9	0.3	0.3	0.8	1.7	1.5	0.8
C_{15}–C_{18}	66.2	0.2	0.2	0.4	1.1	1.2	0.8
C_6H_5	55.0	0.2	0.4	0.4	1.2	0.7	0.05

* The derivatives were prepared by reaction with Nedox 1114 (C_{11}–C_{14}), Nedox 1518 (C_{15}–C_{18}), or phenyl ethylene oxide (C_6H_5).

† g of solvent imbibed in the gel beads per g of dry Sephadex derivative.

TABLE 2 Effect of Solvent on the Elution Volume* of Cholesterol in Columns Containing Hydrophobic Sephadex†

Solvent	Bed Volume	V_e
	ml/g	
Methanol	2.2	430
Ethanol	2.2	140
Propan-1-ol	2.9	85
Propan-2-ol	2.5	110
Butan-1-ol	2.9	115
Butan-2-ol	2.9	90
t-Butanol	2.9	85
Acetone	2.2	180
Dioxane	2.7	85
Tetrahydrofuran	3.6	60
Ethyl acetate	2.5	120
Methyl ethyl ketone	2.8	110
Methylene chloride	3.4	75
Chloroform	3.9	50
Ethylene chloride	2.9	100
Benzene	3.5	100
Toluene	3.5	120
Carbon tetrachloride	4.2	120
Cyclohexane	3.0	260
2,2,4-Trimethyl pentane	2.9	590
Hexane	2.9	390
Heptane	3.1	390

* Elution volume (V_e) expressed in percentage of total column volume.

† Hydroxyalkyl Sephadex LH-20, containing 55% (w/w) of C_{11}–C_{14} hydroxyalkyl groups. The column, 200–300 mm × 4.3 mm, contained 1–2 g of derivative. Flow rate 0.3–3 ml/h

TABLE 3 Retention Volumes of Cholesterol on Hydroxyalkyl Sephadex LH-20 in Heptane–Methanol Mixtures*

Heptane–Methanol	Bed Volume	Retention Volume
v/v	*ml/g of derivative*	*% of total column volume*
0:100	2.2	430
5:95	2.2	380
10:90	2.3	290
15:85	2.5	240
20:80	2.8	220
25:75	2.9	150

* Chromatographic conditions as described in Table 2.

noted in the reversed-phase chromatography of phenylthiohydantoins on hydroxyalkyl Sephadex LH-20 using the solvent system heptane–chloroform–isopropanol–water 1:9:70:70 (by vol). Fig. 3 shows that there is a decrease in the height equivalent to a theoretical plate with increasing temperature. However, in this system an increased temperature also resulted in the decrease of the elution volume (about 25% from 11°C to 39°C) and of the separation factors between the phenylthiohydantoins. Most analyses have, therefore, been run at room temperature. Problems with column packing and chromatographic reproducibility are avoided if all steps are carried out in a room with constant temperature.

TABLE 4 Retention Volumes in Methanol of Cholesterol on Different Hydrophobic Derivatives Prepared from Sephadex LH-20, 170–325 Mesh

Substituent*	Degree of Substitution	Retention Volume†
	% w/w	*% of total column volume*
C_{11}–C_{14}	30.5	150
C_{11}–C_{14}	41.3	250
C_{11}–C_{14}	55.0	430
C_{15}–C_{18}	22.4	180
C_{15}–C_{18}	30.3	330
C_{15}–C_{18}	66.3	510
C_6H_5	41.6	90

* The derivatives were prepared by reaction with Nedox 1114 (C_{11}–C_{14}), Nedox 1518 (C_{15}–C_{18}), or phenyl ethylene oxide (C_6H_5).

† The columns were prepared as described in Table 2.

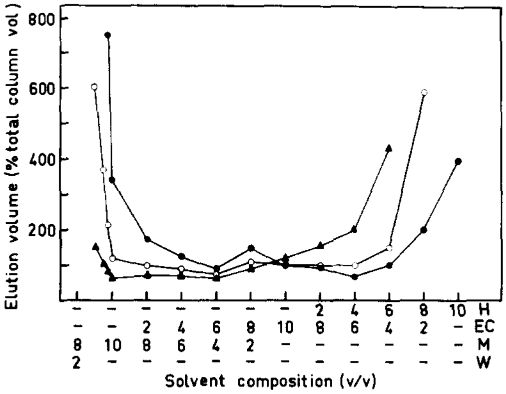

Fig. 2. Effect of the polarity of the solvent on the elution volume of cholesterol (●), 5β-cholestan-3α,7α-diol (○), and 5β-cholestan-3α,7α,12α-triol (▲). Column: 200–300 mm × 4.3 mm, containing 1–2 g of hydroxyalkyl (C_{11}–C_{14}, 55% by weight) Sephadex LH-20, 170–325 mesh. *H*, heptane; *EC*, ethylene chloride; *M*, methanol; *W*, water.

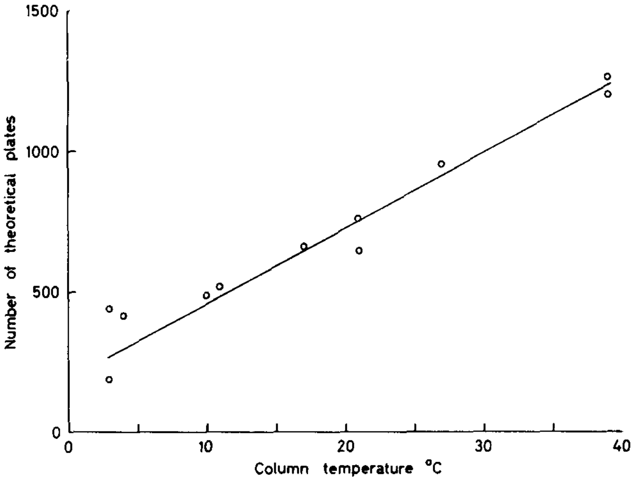

Fig. 3. Effect of the temperature on the number of theoretical plates calculated for the phenylthiohydantoin of alanine. Column: 570 × 25 mm, containing hydroxyalkyl (C_{11}–C_{14}, 50% by weight) Sephadex LH-20. Solvent: heptane–chloroform–isopropanol–water 1:9:70:70 (by vol). Flow rates about 60 ml/hr.

Applications

When the differences in the compounds to be separated are in the hydrocarbon part of the molecules, a reversed-phase system should be chosen. The sterols of cholesterol, campesterol, (24α-methylcholesterol), and β-sitosterol (24α-ethylcholesterol) differ by one methylene group in the side chain, and their separation is achieved using heptane–methanol 5:95 (v/v), as the solvent (Fig. 4).

The limited solubility in methanol of compounds such as cholesteryl esters and straight-chain waxes, makes the use of other solvents necessary when these compounds are to be separated. The separation of cholesteryl esters on hydroxyalkoxypropyl (C_{11}–C_{14}, 34% by weight) Sephadex G-25 in heptane–acetone–water 4:15:1 (by vol), was shown in a preliminary report (5). As shown in Fig. 5 straight-chain waxes are separated in the similar solvent, heptane–acetone–methanol 1:2:4 (by vol). The true retention volumes (8) of the waxes are a function of the chain length, and a plot of the logarithm of the true retention time vs. the number of carbons gives a straight line.

Triglycerides can be separated in the solvent system heptane–acetone–water 4:15:1. This system gives unfavorable partition coefficients for mono- and diglycerides which are eluted early. By choosing the more polar solvent system, heptane–chloroform–isopropanol–water 1:9:70:70 (by vol), the reversed-phase separation of 1-monoglycerides is accomplished on a short column as shown in Fig. 6.

Solvent mixtures containing a chlorinated hydrocarbon, methanol, and water have been found to give efficient reversed-phase systems. This is exemplified by the chromatography of bile acids using the solvent ethylene chloride–methanol–water 10:70:30 (by vol), (Fig. 7). In such systems unsaturated sterols and fatty acid esters are eluted more rapidly than the corresponding saturated compounds (Fig. 8).

When aliphatic hydrocarbons are used as solvents, the stationary gel phase becomes more polar than the mobile phase and a straight-phase partition system is obtained. Addition of a chlorinated hydrocarbon then leads to a more rapid elution of compounds with polar groups. Efficient straight-phase systems are obtained with mix-

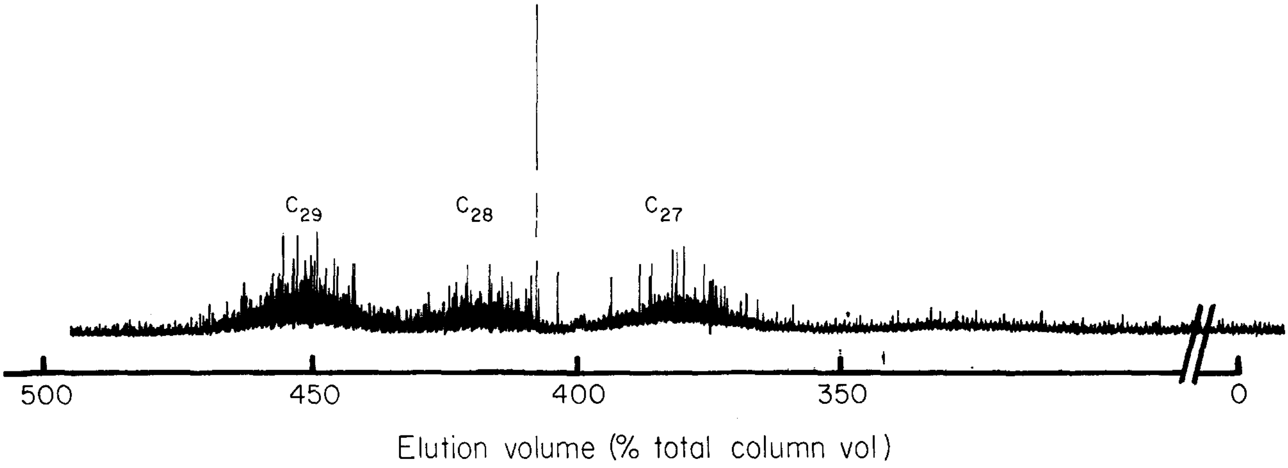

FIG. 4. Separation of cholesterol (C_{27}), campesterol (C_{28}), and β-sitosterol (C_{29}). Column: 578 × 4.4 mm containing hydroxyalkoxypropyl Sephadex (made from Sephadex G-25, 35 μ ± 5μ in water) with hydroxyalkyl (C_{15}–C_{18}) group content of 71% (by weight). Solvent: heptane–methanol 5:95 (v/v). Flow rate about 1.5 ml/hr.

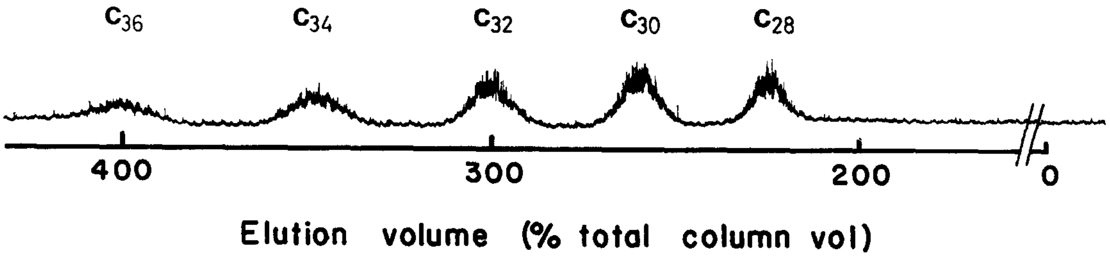

FIG. 5. Separation of straight-chain waxes with 28–36 carbon atoms. The waxes were synthesized from octadecanol and the appropriate fatty acid chlorides. Column: 285 × 4.4 mm containing hydroxyalkoxypropyl Sephadex (made from Sephadex G-25, 27.5 μ ± 5 μ) with hydroxyalkyl (C_{11}–C_{14})) group content of 34% (by weight). Solvent: heptane–acetone–methanol 1:2:4. Flow rate 0.65 ml/hr.

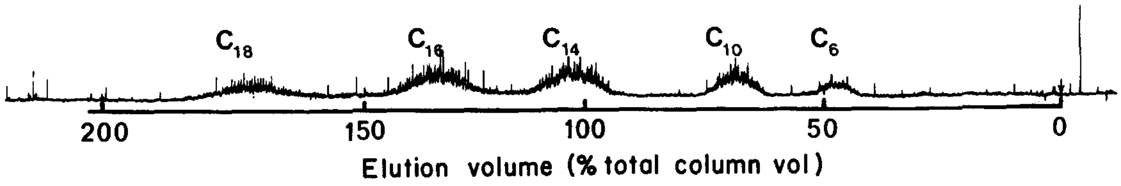

FIG. 6. Separation of 1-monoglycerides of hexanoic (C_6), decanoic (C_{10}), myristic (C_{14}), palmitic (C_{16}), and stearic (C_{18}) acids. Column: 275 × 4 mm, containing hydroxyalkyl (C_{15}–C_{18}, 58% by weight) Sephadex LH-20, 170–325 mesh. Solvent: heptane–chloroform–isopropanol–water 1:9:70:70 (by vol). Flow rate 0.39 ml/hr.

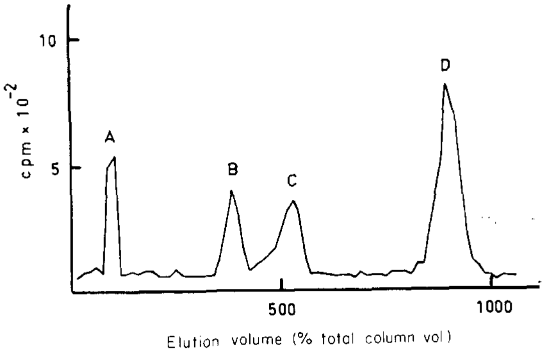

FIG. 7. Separation of (*A*) 3α,7α-dihydroxy-5β-cholanoic, (*B*) 3α-hydroxy-5β-cholanoic, (*C*) 3β-hydroxy-5β-cholanoic, and (*D*) 3-keto-5β-cholanoic acids. Trace amounts of ^{14}C-labeled reference compounds were added to a bile extract. Radioactivity was measured with a gas flow counter. Column: 240 × 10 mm, containing hydroxyalkyl (C_{11}–C_{14}, 55% by weight) Sephadex LH-20. Solvent: ethylene chloride–methanol–water 10:70:30 (by vol). Flow rate 3 ml/hr. Chromatography was kindly carried out by Dr. I. Makino.

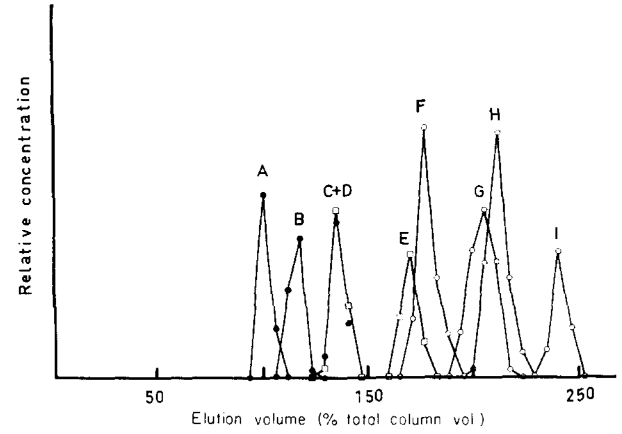

FIG. 8. Chromatography of a mixture of methyl esters of (*A*) linolenic, (*B*) linoleic, (*C*) oleic, (*D*) palmitic, and (*E*) stearic acids, and (*F*) 5,24-cholestadien-3β-ol, (*G*) cholesterol, (*H*) 24α-ethyl-5,22-cholestadien-3β-ol, and (*I*) 24α-ethyl-5-cholesten-3β-ol. The appearance of unsaturated (●—●, 0.1 mg of each) and saturated (□—□, 0.1 mg of each) fatty acid esters, and sterols (○—○, 0.1–0.2 mg) was determined by gas–liquid chromatography. Column: 461 × 9.6 mm containing hydroxyalkyl (C_{11}–C_{14}, 55% by weight) Sephadex LH-20, 200–325 mesh. Solvent: ethylene chloride–methanol–water 25:95:5 (by vol). Flow rate 4 ml/hr.

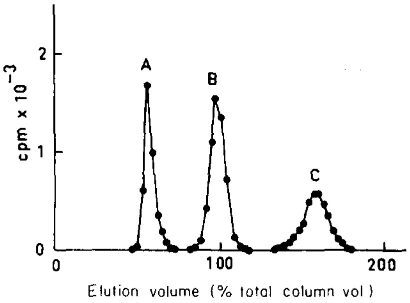

FIG. 9. Separation of 100 mg of a mixture of synthetic (*A*) chimyl dipalmitate, (*B*) chimyl α-monopalmitate, and (*C*) chimyl β-monopalmitate labeled with palmitic-^{14}C acid. Column: 415 × 15 mm, containing hydroxyalkyl (C_{11}–C_{14}, 49% by weight) Sephadex LH-20. Solvent: heptane–chloroform 100:5 (v/v). Flow rate 66 ml/hr.

tures of heptane and chloroform. Thus, the α- and β-palmityl esters of chimyl alcohol have been separated using heptane–chloroform 100:5 (v/v) (Fig. 9).

As a comparison of straight vs. reversed-phase separation, Figs. 10 and 11 illustrate the chromatography of a series of steroids with different numbers of hydroxyl and keto groups and a different configuration of the hydroxyl groups. The effect of the number of hydroxyl groups and their orientation on the elution volume in the two systems are clearly seen.

DISCUSSION

The lipophilic–hydrophobic Sephadex derivatives described in this paper offer several advantages in column chromatography. They are simple to prepare, and Sephadex has a well-defined degree of cross-linking, which is important for reproducibility of separations partly dependent on molecular size and shape of the solutes (molecular sieving). The bead form of the particles gives good solvent flow characteristics. Problems with column "bleed" are eliminated since the alkyl groups which give the material its lipophilic properties are covalently bound to the polysaccharide matrix. It should be pointed out, however, that the derivatives seem to decompose at temperatures above 80°C. They are also sensitive to hydrochloric acid and should not be stored for extended periods of time in chlorinated hydrocarbon solvents. A careful washing with benzene should precede the drying of the material.

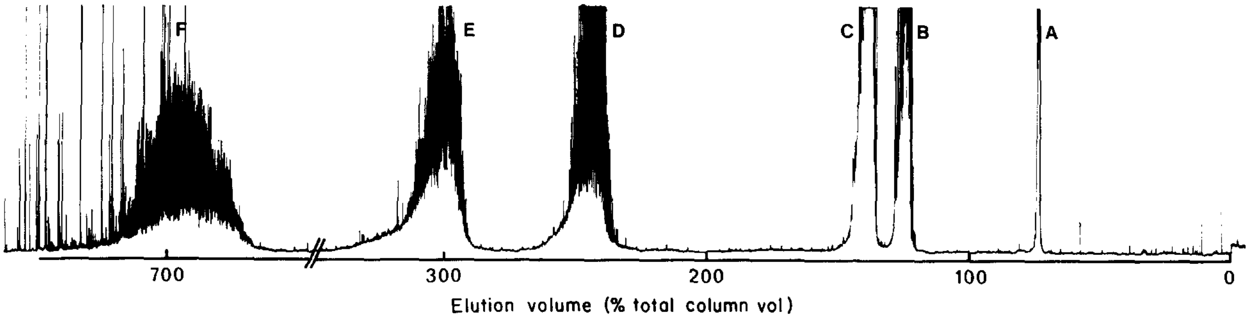

Fig. 10. Separation of (*A*) pregn-4-ene-3,20-dione, (*B*) 20β-hydroxypregn-4-en-3-one, (*C*) 20α-hydroxypregn-4-en-3-one, (*D*) 11β-hydroxypregn-4-ene-3,20-dione, (*E*) 11α-hydroxypregn-4-ene-3,20-dione, and (*F*) 11β,21-dihydroxypregn-4-ene-3,20-dione by straight-phase chromatography. Column: 1770 × 1.5 mm, containing hydroxyalkyl (C_{15}–C_{18}, 58% by weight) Sephadex LH-20. Solvent: heptane–chloroform 8:2 (v/v). Flow rate 0.47 ml/hr.

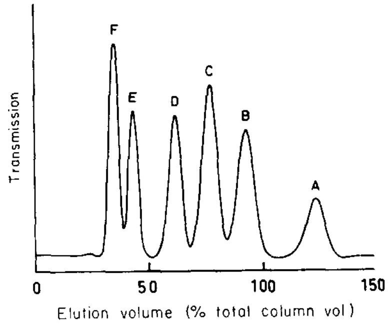

Fig. 11. Reversed-phase chromatographic separation of the steroid mixture (*A*–*F*) listed in Fig. 12. Column, solvent, and flow rate: see Fig. 3. Temperature: 20°C.

Hydroxyalkyl Sephadex can be used both for straight- and reversed-phase chromatography. This is due to the presence of both hydroxyl groups and long alkyl chains in the gel. The derivatives are solvated in solvents ranging in polarity from aqueous methanol to aliphatic hydrocarbons. The symmetrical shape of the solute bands indicates that the distribution (partition) coefficients are linear over a wide range of solvent polarities. This is not the case with the more polar Sephadex derivatives such as Sephadex LH-20, where exclusion and tailing even of nonpolar sterols are seen in chloroform–benzene 1:1 (v/v), and in less polar solvent systems (9).

Both straight-phase and reversed-phase chromatography is performed with miscible solvent systems. This makes it possible to prepare columns for repeated use over long periods of time. Problems with equilibration of conventional two-phase solvent systems are avoided. This is analogous to the reversed-phase liquid–gel chromatography system described by Hirsch (10) in which acetone–water mixtures were used with factice (polymerized and granulated soybean oil).

The factors governing the separation of compounds in liquid–gel chromatography have been discussed in a previous paper (4). The solvent systems in the present study have been designed to create a large difference in polarity between the stationary gel phase and the mobile phase. Straight-phase systems are obtained with solvents consisting of an aliphatic and a chlorinated hydrocarbon. With solvents containing both polar e.g. water, methanol, isopropanol, and acetone and nonpolar e.g. chloroform, ethylene chloride and heptane components, the latter are preferred by the gel phase and a reversed-phase system is obtained. It has been found that the volume of the combined polar components should be about 5–10 times that of the nonpolar components.

The liquid–gel chromatography systems presented in this paper can in most cases be regarded as typical liquid–liquid partition systems with high efficiency. Thus, in the chromatography of waxes shown in Fig. 5, a plot of the true retention time (8) vs. the number of carbon atoms will give a straight line. Furthermore, methyl oleate and palmitate have about the same mobility, indicating primarily a liquid–liquid or liquid–gel partitioning process.

Molecular sieving effects are less evident in this study; however, the amount of stationary phase available for a solute will depend on the size of the solvated molecules, and this will influence the separations. An extensive study of separations based on differences in molecular size has recently been made by Brooks and Keates (11). They used solvent systems in which the difference in polarity between the gel and the solvent was kept to a minimum, and their results show that group separations based on molecular sieving on hydrophobic Sephadex derivatives should be of considerable value in work with naturally occurring lipid mixtures.

The inert nature of the hydrophobic Sephadex derivatives make them suitable for separations of sensitive compounds. Preliminary results indicate that trimethylsilyl ethers of sugars and steroids can be subjected to reversed-phase chromatography on these materials. It is possible, however, that hydrophobic Sephadex contains acidic groups. This has been shown for other Sephadex derivatives which may act as cation exchangers with a capacity of 10 μeq/g (4). To assure that acid- or base-sensitive compounds will remain intact and also that no tailing will occur with charged compounds, it may sometimes be advisable to include in the eluting solvent very small amounts of pyridine or acetic acid, as the case may warrant, or occasionally to run a small amount of a basic or acidic solvent through the column.

The nature of a liquid–gel partition system is determined both by the solvent composition and the degree and type of substitution of Sephadex. The method for substitution of hydroxypropyl Sephadex can be applied to the synthesis of derivatives other than those described in this paper. Phenyl hydroxyethyl Sephadex LH-20 is an example of such a derivative which might give specific gel–solute interactions. A further development towards specifically substituted Sephadex derivatives for use in liquid–gel chromatography in organic solvents is the synthesis of a chlorohydroxypropyl derivative (12). This may serve as a starting material for introduction of a variety of groups.

Liquid–gel chromatography as described in this paper can be applied to both analytical and preparative problems. When used in the work-up of biological extracts containing radioactive steroid and bile acid metabolites of varying polarities, losses of activity have not been observed. Previous results (4) as well as the studies by Brooks et al. (11) also indicate quantitative recoveries in chromatographic separations on lipophilic Sephadex derivatives. In their investigation, Brooks and Keates also evaluated the effect of sample loading, and found that a practical limit is about 50 mg/cm^2 of column area; this agrees with our observations. In preparative separations of compounds with a high mobility, these derivatives permit the use of the recycling technique to achieve separations of compounds with a separation factor of about 1.02 (13). It is reasonable to believe that chromatography on hydrophobic Sephadex will be a useful complement to chromatographic techniques currently in use. We have tried to indicate some general features of the method to enable the individual investigator to devise liquid–gel chromatography systems for his particular separation problems.

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