Isolation and identification of cholesteryl alkyl ethers from bovine cardiac muscle

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ABSTRACT Cholesteryl alkyl ethers have been isolated from bovine cardiac muscle and characterized by thin-layer and gas–liquid chromatography. The fraction contained at least three homologues. Cholesteryl hexadecyl ether, which accounted for over 90% of the total components observed on gas chromatography, was identified by mass spectrometry.

SUPPLEMENTARY KEY WORDS thin-layer · gas–liquid · silicic acid column · chromatography · mass spectrometry

CHOLESTERYL ESTERS and triglycerides represent the major classes of naturally occurring neutral lipids. Diacyl glycerol monoethers are well-recognized minor components of the lipid fraction that consists mainly of triglycerides (1–6); the purpose of this study was to determine whether, by analogy, cholesteryl ethers occur in a natural lipid mixture.

EXPERIMENTAL PROCEDURE AND RESULTS

Materials

All solvents were reagent grade and, with the exception of methanol, were redistilled before use. The column packing for GLC was obtained from Applied Science Laboratories Inc., State College, Pa. Mallinckrodt silicic acid, 100 mesh, "suitable for chromatography by the method of Ramsey and Patterson," was used in the column chromatography procedures. Silica Gel G was purchased from Applied Science Laboratories, and Aluminum Oxide H was obtained from Brinkmann Instruments, Inc., Westbury, L.I., N.Y.

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

Synthetic cholesteryl alkyl ethers were prepared by an adaptation of a method previously used to synthesize glycerol alkyl ethers (7). The authenticity of the synthetic cholesteryl alkyl ethers was established by IR and mass spectroscopy. These synthetic ethers were then used to establish the chromatographic procedures employed in the isolation of naturally occurring cholesteryl ethers.

Chromatography of Cholesteryl Hexadecyl Ether

A model system containing 50 mg each of cholesteryl hexadecyl ether, octadecanal, cholesteryl stearate, cholesterol, and squalene was applied to a chromatographic column, 2 cm I.D. containing 18 g of silicic acid in *n*-heptane. The lipids were eluted with 1% diethyl ether in *n*-heptane, and 15-ml fractions were collected, concentrated, and analyzed by TLC on 20 × 20 cm glass plates coated with a 0.5 mm thick layer of activated Silica Gel G containing Na_2CO_3 (8). The chromatograms were developed with hexane–chloroform 3:1 and fractions were detected in UV light after the plates had been sprayed with Rhodamine 6G in 80% methanol. Components were identified by comparison of their mobilities with those of standards on the same plate.

Cholesteryl hexadecyl ether was eluted in fractions 3–12 along with squalene, cholesteryl stearate, and octadecanal; it could be separated by the TLC system described from squalene and octadecanal, as well as from other lipid components, but not from cholesteryl esters (Fig. 1). The final separation from cholesteryl esters could be achieved (Fig. 2) by TLC on layers of Aluminum Oxide H, 0.25 mm thick, with hexane–benzene 9:1 as solvent (9).

Isolation of Cholesteryl Alkyl Ethers from Bovine Heart

Bovine hearts were obtained from a slaughterhouse and cooled in ice during transit. Pericardial fat was removed

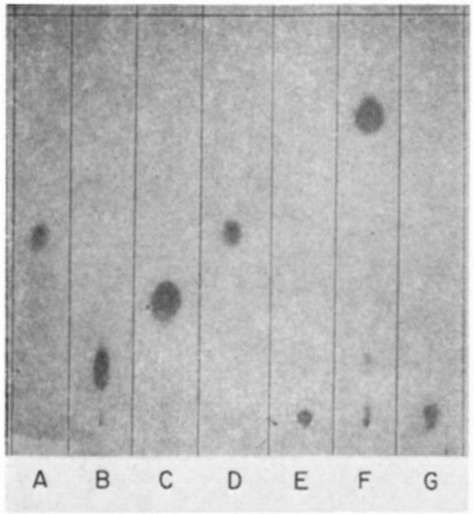

FIG. 1. Thin-layer chromatogram of pure compounds on Silica Gel G in hexane–chloroform 75:25. Indicator, iodine vapor. *A*, cholesteryl hexadecyl ether; *B*, α-tocopherol; *C*, octadecanal; *D*, cholesteryl palmitate; *E*, cholesterol; *F*, squalene; *G*, Coenzyme Q.

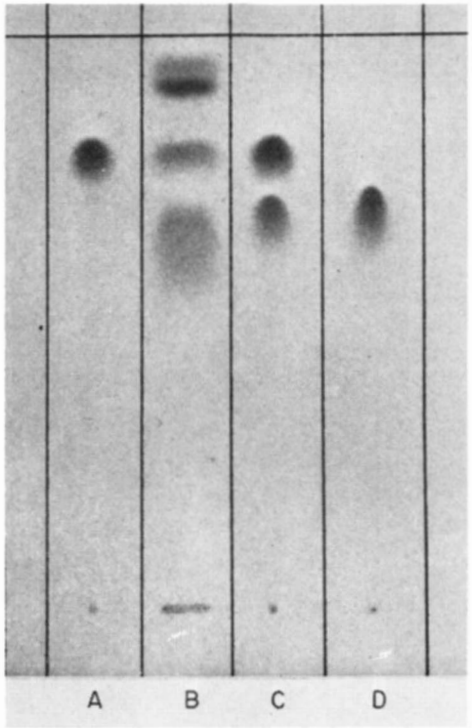

FIG. 2. Thin-layer chromatogram on Aluminum Oxide H of the nonsaponifiable lipids isolated from Silica Gel G layers. Solvent, hexane–benzene 9:1; indicator, iodine vapor. *A*, cholesteryl hexadecyl ether; *B*, isolated nonsaponifiable lipid; *C*, cholesteryl hexadecyl ether and cholesteryl palmitate; *D*, cholesteryl palmitate.

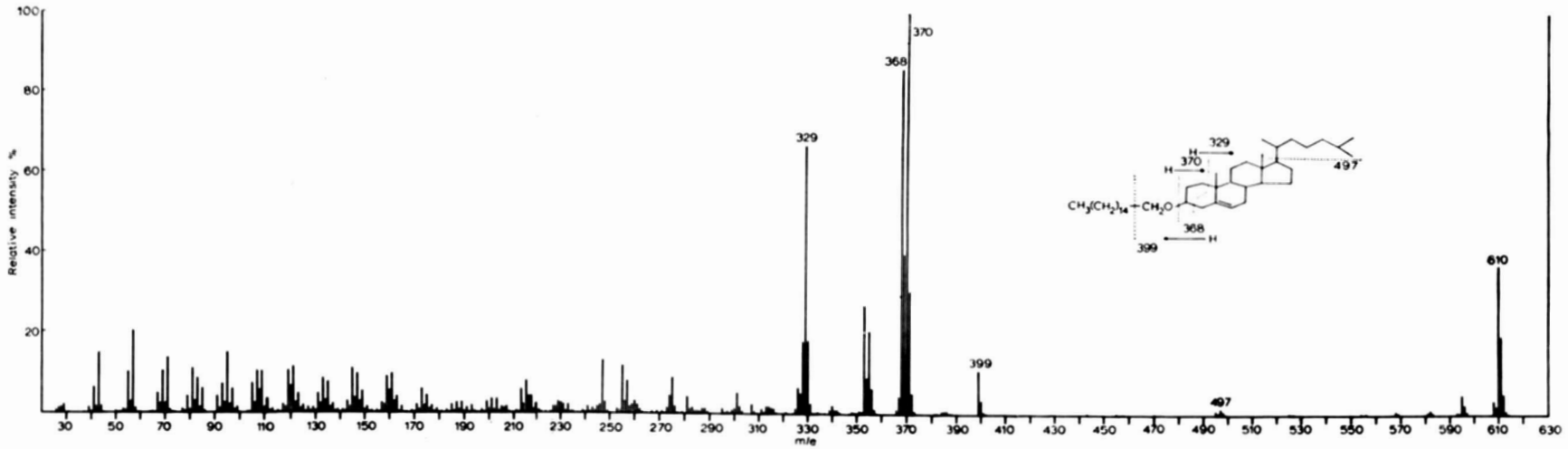

FIG. 3. Mass spectrum of cholesteryl hexadecyl ether.

in the cold, the tissue was homogenized in a Waring Blendor with chloroform–methanol 2:1 (10), and the extract was filtered through Whatman No. 1 paper. The filtrate was shaken with a volume of 1% NaCl equal to 0.25 of the filtrate volume. The phases were allowed to separate and the lower phase was dried over anhydrous Na_2SO_4. The lipid extract was concentrated under reduced pressure in a rotary evaporator at 33°C and stored in chloroform under nitrogen at 4°C.

Phospholipids were removed from the total lipid extract by flocculation with acetone and $MgCl_2$ (11) until less than 1 μg of P was detectable in 235 mg of neutral lipid (12). 20 g of this neutral lipid was saponified by refluxing for 2 hr with an excess of 0.5 N ethanolic sodium hydroxide to eliminate cholesteryl esters and glycerides and thus minimize contamination by these in the later chromatographic separations. After the addition of water, the nonsaponifiable lipids were extracted with diethyl ether; the solution was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The nonsaponifiable lipids were dissolved in *n*-heptane and subjected to silicic acid column chromatography as above. The fraction eluted in the first 180 ml was further purified by TLC on Silica Gel G and then on Aluminum Oxide H, each time with synthetic cholesteryl hexadecyl ether as marker, and with chloroform being used to elute the fraction from the adsorbent.

GLC and Mass Spectrometry

A borosilicate glass column containing 1% SE-30 (methylpolysiloxy gum) on alkali-washed silanized Gas-Chrom S, 100–120 mesh, was kept at 270°C; the flash evaporator and detector were at 285°C. Argon was the carrier gas, at a flow rate of 112 ml/min and an inlet pressure of 40 psi. GLC of the fraction isolated from the thin layers of Aluminum Oxide H indicated the presence of three components with retention times similar to those of synthetic cholesteryl alkyl ethers (Table 1). One of these components accounted for 90% of the total area of all peaks obtained; it had the same retention time as synthetic cholesteryl hexadecyl ether and cochromatographed with it.

Effluent from the GLC column corresponding to this major peak was collected in a cool glass tube and purified from bled stationary phase by TLC. This fraction was then subjected to direct probe analysis with an LKB 9000 single focusing spectrometer. The spectrum of the natural ether was superimposable on that of the synthetic cholesteryl hexadecyl ether (Fig. 3). The parent peak corresponding to the unfragmented molecule indicated a molecular weight of 610. The ions which result in high intensity peaks at m/e 329 and 368 have been reported before in the spectrum of cholesteryl trimethylsilyl ether (13, 14). The peak at m/e 370 probably represents cholest-5-ene, which can result from the transfer of a hydrogen atom from the α-carbon of the aliphatic chain to the steroid moiety.

TABLE 1 RELATIVE RETENTION TIMES OF CHOLESTERYL ALKYL ETHERS ON SE-30 AT 270°C

Alkyl Chain Length	Natural Lipid	Synthetic Cholesteryl Alkyl Ether
15		0.491
16	0.623*	0.623
17	0.780	0.787
18	1.000	(1.000)

* Absolute retention time, 55.6 min; relative to cholesteryl palmitate, 0.786.

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