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Note

Capillary column gas–liquid chromatographic analysis of cholesterol derivatives

Application to the autoxidation products of cholesterol

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An accurate and rapid method to detect and assay cholesterol autoxidation products in biological and organic specimens, *e.g.*, in treated human food, and to determine their biological activities, *e.g.*, angiotoxic, carcinogenic, cytotoxic and mutagenic[1], is required. A minimum of 50 sterols has been identified in cholesterol autoxidation products[2–4]. They have been analyzed by liquid chromatography on silica and Sephadex LH-20 columns[5], thin-layer chromatography (TLC)[6], high-performance liquid chromatography[4,7] and gas–liquid chromatography (GLC) on OV-101 or SE-30 columns[8].

The use of capillary columns, impregnated with OV-101, achieves better resolution and enables the detection of quantities of sterols in the range of 10 ng. The method was applied to the analysis of the products of cholesterol autoxidation at 100°C in the presence of air for 30 days. Oxidation of cholesterol occurs both in the rings and in the side chain.

MATERIALS AND METHODS

Reagents

Solvents for extraction and chromatography were double-distilled before use. Pyridine, hexane, hexamethyldisilazane and trichloromethylsilane were Merck reagents. The methoxyamine hydrochloride was supplied by Pierce (Rockford, IL, U.S.A.).

Steroids

Cholestane, dihydrocholesterol, 7α-, 7β-, 25-, (20 *S*)-20-hydroxycholesterol and 3β-acetoxy-5,7-cholestadiene were obtained from Steraloids (Pawling, NJ, U.S.A.). The (22*S*)-22-, (24*R*)-24- and (24*S*)-24-hydroxy-; the (25*R*)-26-hydroxy- and 22ξ,23ξ-dihydroxycholesterol were generously provided by Professor Van Lier (Sherbrooke, Quebec, Canada). 5α,6α-Epoxycholestan-3β-ol, 5α-cholestane-3β,5α,6β-triol and 3,5-cholestadien-7-one[9] were prepared in our laboratory.

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Apparatus

For GLC, a Carlo Erba Fractovap 2300 chromatograph, equipped with a capillary column (25 m × 0.25 mm) impregnated with OV-101, was used and helium was the carrier gas at 285°C and a flow-rate of 1 ml/min. For injection, a 1-μl Pyrex stream splitter, Type ROS (Carlo Erba, Milan, Italy) was used. Other conditions: column temperature, 285°C; injection temperature, 350°C; flame ionization detector; recorder speed, 30 cm/min.

Mass spectra were recorded on an LKB 2091 gas chromatograph–mass spectrometer at 70 eV voltage. The chromatograph was equipped with a column packed with SE-30 (25 m × 0.25 mm). The helium flow-rate was 4 ml/min, and the column temperature was programmed from 250 to 310°C at 10°C/min.

TLC

Silica gel 60 F_{254} plates, 20 × 20 cm, 0.2 mm thick (E. Merck, Darmstadt, G.F.R.) were developed twice with diethyl ether–cyclohexane (9:1). The sterols were revealed by their UV absorption at 254 nm or by spraying with a 50% aqueous solution of H_2SO_4, followed by heating. Preparative TLC was performed in the same manner, but with 0.25-mm thick plates. After scraping of the zones outlined by UV spectroscopy at 254 nm, they were eluted with 50 ml of chloroform–methanol (2:1).

Derivatization of sterols

The residue to be derivatized (generally obtained by preparative TLC) was deposited in a screw-cap culture tube by evaporating the solvents under nitrogen. Then 0.5 ml methoxyamine hydrochloride solution in pyridine (20 mg/ml) were added. The tube was plugged with a PTFE stopper and heated in a water-bath for 4 h at 60°C. After evaporation of the excess of pyridine under nitrogen, 150 μl of pyridine–hexamethyldisilazane–trichloromethylsilane (1:1:1) were added and the tube was allowed to stand at ambient temperature for 12 h. Excess of solvent was evaporated under nitrogen. The residue was taken up in hexane (1 ml per 100 μl) and injected into the gas chromatograph.

Cholesterol autoxidation

A 500-mg amount of Merck cholesterol, recrystallized twice in ethanol, was placed in a tube and heated in an oven at 100°C in the presence of air for 30 days. The crude product was chromatographed on preparative silica plates. After double development with diethyl ether–cyclohexane (9:1), cholesterol and the products having $R_F > 0.8$ were scraped from the plate and collected for subsequent analysis by capillary GLC. The remainder of the products was recovered by elution with chloroform–methanol (2:1) and derivatized by the method indicated.

RESULTS AND DISCUSSION

Twenty-two authentic cholesterol derivatives were chromatographed separately or as mixtures. Their retention times (RRT) relative to cholestane are given in Table I. The accuracy of these values, ± 0.02, corresponds to the maximum shift observable during successive injections of the same compound.

The chromatogram of a mixture of these 22 sterols is shown in Fig. 1. Sixteen

TABLE I

RELATIVE RETENTION TIMES OF SOME OXIDIZED CHOLESTEROL DERIVATIVES (ACCURACY ±0.02)

	Compound	*RRT*
1	5α-Cholestane	1.00
2	5-Cholestene-3β,7α-diol	1.74
3	5-Cholesten-3β-ol (cholesterol)	1.80
4	5α-Cholestan-3β-ol	1.84
5	3,5-Cholestadien-7-one	1.91
6	5,24-Cholestadien-3β-ol (desmosterol)	
7	5α-Cholestan-3-one	
8	5,7-Cholestadien-3β-ol	2.01
9	5α-Cholest-7-en-3β-ol	
10	4-Cholesten-3-one	2.14
11	5-Cholestene-3β,7β-diol	2.25
12	5α,6α-Epoxycholestan-3β-ol	2.32
13	5-Cholestene-3β,(22*S*)-22-diol	2.41
14	5-Cholestene-3β,(22*S*)-20-diol	2.46
15	3β-Hydroxy-5-cholesten-7-one	2.95
16	5α-Cholestane-3β,5α,6β-triol	
17	5-Cholestene-3β,(24*R*)-24-diol	3.14
18	5-Cholestene-3β,(24*S*)-24-diol	
19	5-Cholestene-3β,25-diol	3.26
20	5-Cholestene-3β,(23*R*)-23-diol	3.53
21	5-Cholestene-3β,22ξ,23ξ-triol	3.53
22	5-Cholestene-3β,(25*R*)-26-diol	3.67

peaks can be seen in this figure. Dihydrocholesterol (4), having a retention time closely similar to that of cholesterol (3), was chromatographed separately (Fig. 2). The difference in RRT between compounds 3 and 4, 0.04, is the minimum value for effective separation of two sterols.

Peaks 5, 8, 15 and 17 correspond, respectively, to mixtures of compounds 5–7, 8 and 9, 15 and 16, and 17 and 18, which have similar retention times in the proposed system. In the analysis of samples of biological or chemical origin, these overlapping RRT values would be no bar to the identification of compounds eluted together.

Such sterols can easily be separated by preparative TLC prior to GLC. With diethyl ether–cyclohexane (9:1), the following R_F values are obtained: 3,5-cholestadien-7-one (5), 0.53; desmosterol (6), 0.44; 5α-cholestan-3-one (7), 0.66. 3β-Hydroxy-5-cholesten-7-one (15) and 5α-cholestane-3β,5α,6β-triol (16) are also easily separated. Only the pairs 8 and 9 and 17 and 18 are difficult to separate. 5,7-Cholestadien-3β-ol (8) is quite unstable. It changes spontaneously or after heating to an apolar compound, which has the RRT of a triene (probably $\Delta^{3,5,7}$ derived from the dehydration of 8). Consequently, the presence of compound 8 does not preclude the identification of 5α-cholest-7-en-3β-ol, which is stable. The resolution of the pair 17 and 18 by paper chromatography is described by Van Lier and Smith[10].

Oxidation of crystallized cholesterol at 100°C

Determination of cholesterol in the crude autoxidation mixture on an SE-30 column shows that 80% of the cholesterol remains unchanged after 30 days; only 6%

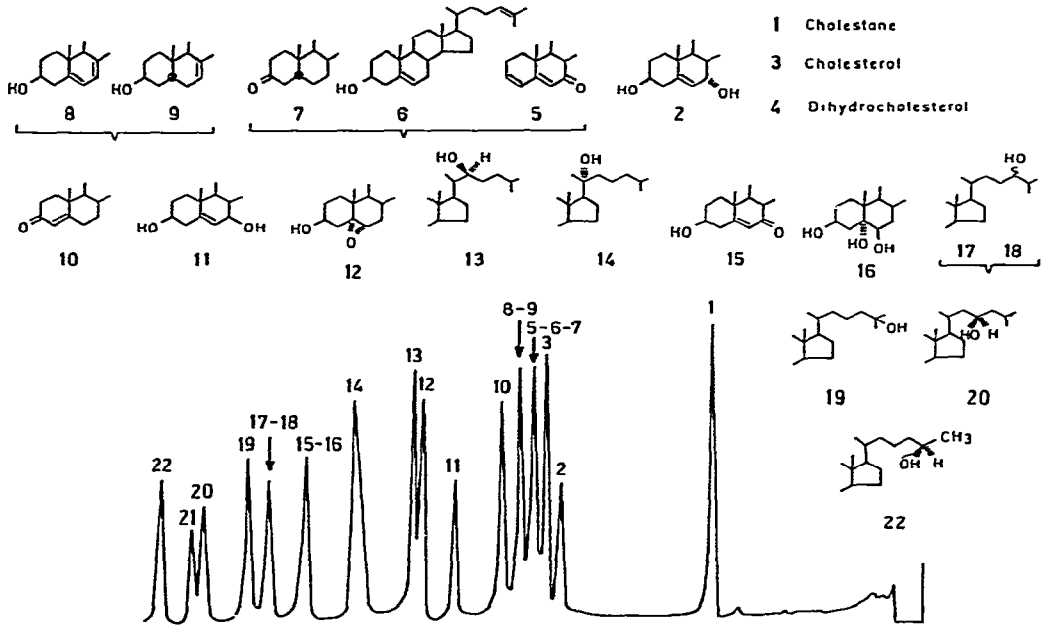

Fig. 1. Chromatogram of oxygenated sterols on a capillary column coated with OV-101.

was detected as oxidation products. This mixture (100 mg) was developed twice with diethyl ether–cyclohexane (9:1) on a preparative plate. The cholesterol was eluted, as previously described, and the remainder was eluted, derivatized and resolved by capillary GLC on OV-101 (Fig. 3). The different constituents are identified by their RRT in comparison with cholestane and by addition of an authentic sample. This identification was confirmed by gas chromatography–mass spectrometry. The proportions of oxidation products were as follows: cholesterol (3), 80%; 25-hydroxycholesterol (19), 1.5%; 7-oxocholesterol (15), 0.75%; 20-hydroxycholesterol (14),

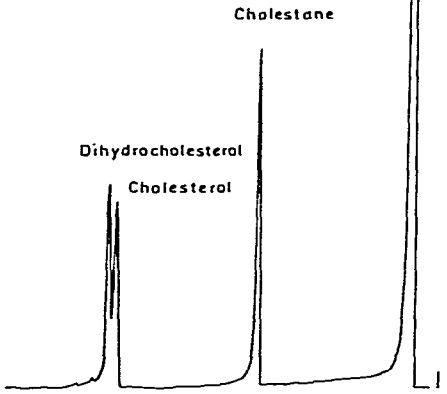

Fig. 2. Separation of cholesterol and dihydrocholesterol on a capillary column coated with OV-101.

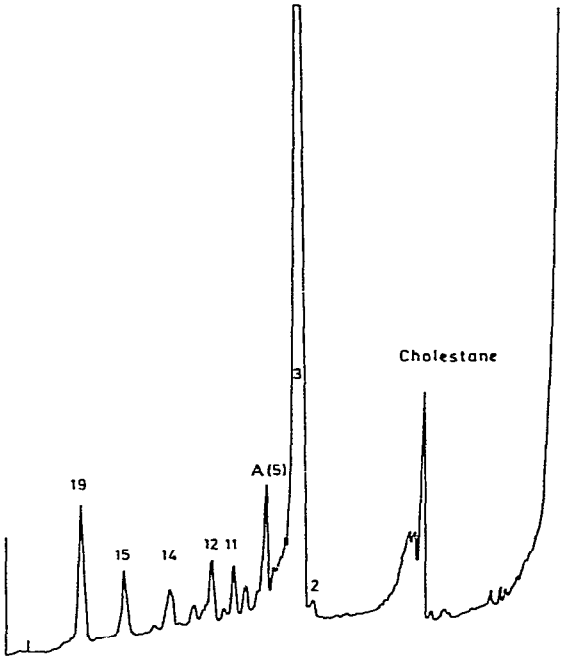

Fig. 3. Chromatogram of crude autoxidation products of cholesterol. A = Unidentified peak eluted together with the dienone (5).

0.75%; 5α,6α-epoxycholesterol (12), 1.1%; 7β-hydroxycholesterol (11), 0.6%; 7α-hydroxycholesterol (2), 0.1%; 3,5-cholestadien-7-one (5) mixed with an unknown compound, 1.1%.

Traces of peroxides were observed at the end of the autoxidation reaction, implying that hydroperoxides were first formed, as in liquid-phase autoxidation[3], although the temperature was 100°C. This view appears to be supported by the fact that mixtures containing a polyethylenic compound [able to provide hydroperoxy radicals, *e.g.*, squalane–cholesterol (1:1)] in addition to cholesterol, enhance the autoxidation reaction and give rise to the formation of large amounts of epoxide (12).

The various products identified are those usually formed in liquid-phase autoxidation[3]. Also observed are the 20- and 25-hydroxycholesterols which are specific to solid-phase autoxidation.

25-Hydroxycholesterol (19) is highly stable at 100°C. It undergoes no change if heated for 6 months in the presence of air. 20-Hydroxycholesterol, on the other hand, is unstable. It decomposes slowly on heating to give compounds of lower molecular weight than the sterols identified here.

The stabilities of the ring B oxidation products are analogous to those described for liquid-phase autoxidation[11]. 7-Hydroperoxycholesterols give rise successively to 7-hydroxy, 7-oxo and 7-oxo-$\Delta^{3,5}$ derivatives. The 7α-hydroperoxy- and hydroxycholesterols epimerize partly to 7β-derivatives as follows:

Mass spectrometry confirms most of the characterizations made for the different sterols. The secondary diols (7-hydroxycholesterols) and secondary/tertiary thiols (20- and 25-hydroxycholesterol) are disilylated. Their mass spectra are characterized by molecular ions at *m/e* 546.5 and fragment ions at *m/e* 531.5 ($M^+ - 15$), 457.5 ($M^+ - 89$) and 368 ($M^+ - 178$). 7-Oxocholesterol is characterized by molecular ions at *m/e* 501 and fragment ions at *m/e* 470 ($M^+ - 31$) and 456 ($M^+ - 45$). The epoxide (12) sometimes appears as a diol, according to its molecular and fragmentation ions.

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