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GAS–LIQUID CHROMATOGRAPHY OF CHOLESTERYL ESTERS ON NON-POLAR AND POLAR CAPILLARY COLUMNS FOLLOWING ON-COLUMN INJECTION

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SUMMARY

Capillary gas chromatography of cholesteryl esters following on-column injection was investigated in fused silica columns coated with a non-polar "bonded" liquid phase (DB-1) or a polar liquid phase (SP2330). The 15-m DB-1 column (0.3 mm I.D.) completely resolved the esters on the basis of carbon number, and further partially separated the saturated esters from unsaturated esters. The polar column system consisted of a 10-m SP2330 column (0.25 mm I.D.) butt-connected to a 60-cm empty deactivated wide-bore (0.3 mm I.D.) capillary column into which the sample was injected. This system completely separated the cholesteryl esters according to degree of unsaturation. The lower limits of sensitivity of the columns were about ten-fold lower than that previous obtainable with splitless injection. The lower limits for the DB-1 and SP2330 columns were about 500 and 1500 pg respectively. Thus these columns can be used for the analysis of cholesteryl esters from the smallest of biological or cell culture samples.

INTRODUCTION

As part of research in the pathogenesis of atherosclerosis, analyses of cholesteryl esters (CEs) from individual atherosclerotic lesions were performed recently in this laboratory[1]. Because of difficulties encountered in obtaining enough lipid from the smallest of the lesions for analysis by conventional packed-column gas chromatography (GC), capillary GC of the CE was investigated[2]. With the splitless injection technique, separation of CE on both non-polar (OV-1) and polar (Silar 10C) columns was studied. With the OV-1 wall-coated open tubular (WCOT) column, peak resolution far superior to that normally obtainable by GC on conventional packed OV-1 columns[3] was achieved. On the Silar 10C WCOT column separation of CE according to degree of unsaturation was comparable to that previously obtained on packed columns[4].

A problem encountered with the splitless injection technique, however, was the variability in the recovery of the CE, probably because of difficulties in effecting a

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reproducible transfer of the CE, especially those of the higher molecular weights (> 600 daltons), from the syringe needle to the column during injection. This problem was partially alleviated by frequent calibration runs with a mixture of synthetic CE, with fatty acids varying in length in even carbon numbers from 2 to 20 and by adding an internal standard mixture containing cholesteryl decanoate (CE 10:0) and cholesteryl eicosanoate (CE 20:0) to each unknown CE sample. These calibration analyses monitored current GC conditions and the internal standard mixture quantitated the CE in a given sample, by providing the slope of the CE recovery curve (peak area *vs.* molecular weight) in that particular analysis. The individual unknown peaks were then quantitated by interpolation from the slope and recent calibration runs.

Although the mathematical manipulations provided an adequate way of obtaining correct data from each chromatogram, the problem of CE losses during chromatography still remained unsolved. Grob[5] reported earlier that cool on-column injection is the most satisfactory technique for reducing injection losses of triglycerides due to inadequate sample transfer to the column during GC. Therefore with a view to maximizing the recovery of CE and thereby increasing the effective sensitivity of the capillary GC technique, analysis of CE after cool on-column injection was investigated.

EXPERIMENTAL

All solvents were reagent grade and were purchased from Fisher Scientific (Toronto, Canada). All lipids were obtained either from Sigma (St. Louis, MO, U.S.A.) or Serdary Research Laboratories (London, Canada).

GC analyses were performed with a Hewlett-Packard Model 5830A gas chromatograph equipped with a flame ionization detector, a Hewlett-Packard Model 18835B capillary inlet, and an on-column injection kit (Hewlett-Packard accessory No. 19320D). The capillary inlet was modified as recommended by Freeman *et al.*[6] to optimize injector conditions for cool on-column injection. The heating block and its surrounding insulation were removed from the injector assembly. Extra insulation was added to the area below the heating block location to ensure that the column oven was adequately insulated. The metal tube containing the injector port was then cooled with compressed air from a short length of 5 mm O.D. copper tubing attached with a length of rubber tubing to an in-house compressed air line. This modification reduced the turn-around time between temperature-programmed runs by minimizing the tendency for the temperature of the injector assembly to follow the oven temperature during a temperature programmed run.

CE analyses were performed both on non-polar and polar WCOT column systems. The non-polar column was a 15-m wide-bore (0.3 mm I.D.) DB-1 Durabond column (J & W Scientific, Rancho Cordova, CA, U.S.A.), a "bonded phase" fused-silica column which is coated with a "non-extractable" methyl silicone rubber equivalent in polarity to OV-1 and SE-30. The film thickness was 0.1 μm. The hydrogen inlet pressure was 2–3 p.s.i. (average linear velocity 40 cm/sec).

Prior to on-column injection, the gas chromatograph was placed in the splitless mode, in which purge of carrier gas through the inlet was stopped. This mode was maintained for 2 min after the start of the program. The column temperature was held at 100°C for 0.5 min, then increased at 30°C/min (maximum instrument rate) to

255°C and increased thereafter at 1.5°C/min to 320°C. The detector temperature was maintained at 340°C. Hydrogen, nitrogen (makeup gas), and air flow-rates through the detector were 40, 40 and 400 ml/min respectively.

The polar WCOT column system consisted of a custom-made Supelco 10-m fused silica column (0.25 mm I.D.) (Supelco, Bellefonte, PA, U.S.A.) coated with SP2330 (film thickness 0.2 μm) which was connected by a Supelco capillary butt connector[14] to a 60-cm length of wide-bore (0.3 mm I.D.) fused silica tubing, a kind gift from Hewlett-Packard Canada Ltd. The inside walls of the empty tubing had been silylated with *tert.*-butyldimethylchlorosilane–imidazole (2:5) reagent (Applied Science Labs., State College, PA, U.S.A.), flushed with heptane, and dried prior to connecting the tubing to the SP2330 WCOT column and the capillary inlet of the gas chromatograph. The other end of the SP2330 column was connected to the detector. This arrangement made easy the otherwise very difficult task of injecting samples directly on-column from a 32-gauge steel needle into the 0.25 mm I.D. SP2330 column. This column configuration also likely minimizes stripping of the liquid phase due to recondensation of the injected solvent onto the walls of the first 20–30 cm of the column, which can occur following on-column injection[7]. Another possible incidental benefit of this arrangement is that the empty tubing may provide a "retention gap" reported by Grob[8] which tends to reduce band broadening after on-column injection. Immediately before injection the gas chromatograph was placed in the splitless mode, which was maintained for 2 min. Column temperature was kept at 100°C for 0.5 min, then increased at 30°C/min to 250°C, and held there for the remainder of the analysis. Helium was used as the carrier gas, because of the problem of hydrogenation of the CE when hydrogen is used[9]. The helium inlet pressure was 3 p.s.i. (average linear velocity 30 cm/sec). The detector temperature was 300°C. The hydrogen, helium (makeup gas) and air flow-rates through the detector were 40, 40 and 400 ml/min respectively.

RESULTS AND DISCUSSION

The ability of the non-polar DB-1 column to separate intact CE following on-column injection is shown in Fig. 1 for two synthetic CE mixtures and CE from an atherosclerotic lesion of a rabbit aorta. As in a previous study with a 22-m narrow bore column and splitless injection[2], excellent resolution of the homologous series of saturated CE was achieved. The Trennzahl values (Table I) were lower than those obtained in the previous study[2], probably because of the larger diameter and shorter (15 m *vs.* 22 m) column used with the on-column injection technique. Close to baseline resolution of CE 18:0 and CE 18:1 (Fig. 1A) was achieved but a less adequate separation of CE 16:0 and CE 16:1 occurred. As before[2], the retention time of CE 18:1 and CE 18:2 were identical (not shown) and Fig. 1B shows that CE 18:3, which had a retention time between that of CE 18:1 and CE 18:2, and CE 18:0, tended to obscure the resolution of these peaks. In Fig. 1C the effect of the CE 18:3 together with the fact that CE 18:3 and CE 18:0 were present in relatively small amounts on the trailing side of the CE 18:1 and CE 18:2 peak, further reduced the resolution of the 18-carbon CE peaks.

An important advantage, however, with the wide-bore column and on-column injection is that the effective lower limit of sensitivity for a measurable peak is about

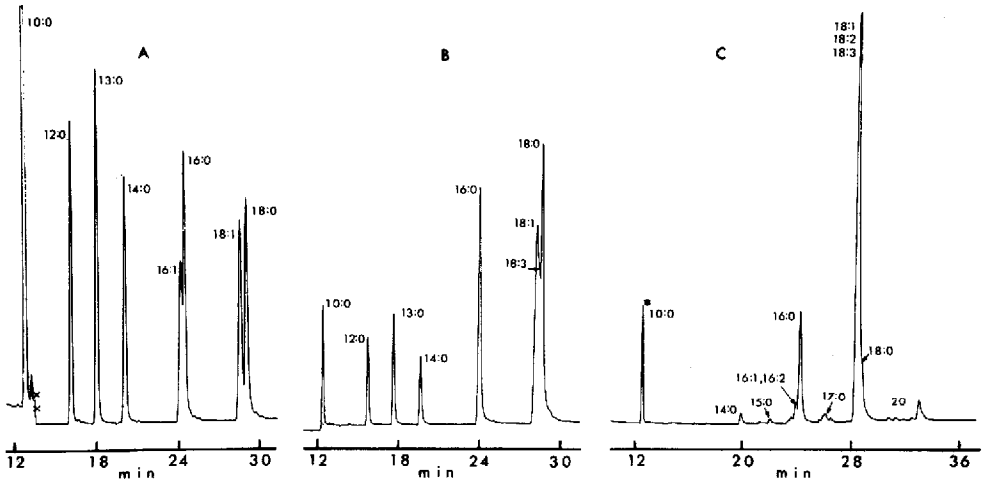

Fig. 1. Chromatograms of cholesteryl esters: A, B, synthetic CE mixtures; C, CE from a rabbit aortic atherosclerotic lesion. Molecular species notation: CE *m:n*, cholesteryl ester with a fatty acid chain containing *m* carbon atoms and *n* carbon–carbon double bonds. GC conditions: 15-m DB-1 bonded phase fused-silica WCOT column (0.3 mm I.D., film thickness 0.1 μm); cool on-column injection, 1 μl heptane solution; detector temperature, 340°C; carrier gas, hydrogen at 2–3 p.s.i. (average linear velocity, 40 cm/sec at 100°C); temperature program 100°C for 0.5 min, then 30°C/min to 255°C, then 1.5°C/min to 320°C.

500 pg, which is about ten-fold more sensitive than that previously obtained with the narrow-bore column and splitless injection[2]. Fig. 2 shows that except for the 22-carbon molecular species, recoveries of all CE were excellent, and all were greatly improved over those obtained before using splitless injection[2]. This is probably due to the fact that on-column injection technique ensures a quantitative transfer of the sample from the needle to the column. Because of its extremely low detection limits,

TABLE I

SEPARATION OF SATURATED CHOLESTERYL ESTERS ON FUSED-SILICA WCOT COLUMNS

Cholesteryl esters	*Trennzahl (TZ)**	
	*DB-1 column***	*SP2330 column****
20:0/18:0	10.7	5.2
18:0/16:0	11.0	5.7
18:0/17.0	4.9	3.0
17:0/16:0	5.0	1.5
16:0/14:0	12.4	5.9
14:0/12:0	12.3	6.7

$$* \; TZ = \frac{D}{b_{0.5}^{(1)} + b_{0.5}^{(2)}} - 1$$

where D is the distance between the peak maxima, and $b_{0.5}^{(1)}$ and $b_{0.5}^{(2)}$ are the widths of the peaks at half-height.

** GC conditions given in Fig. 1.

*** GC conditions given in Fig. 3.

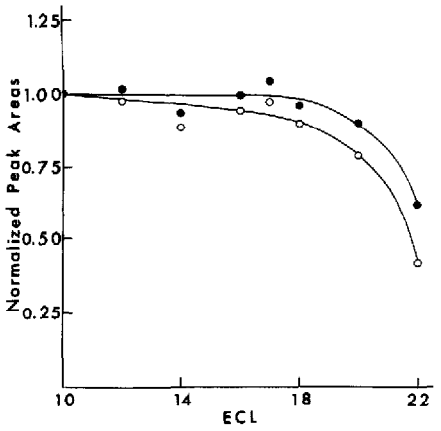

Fig. 2. Relationship between peak area and equivalent chain length (ECL) of cholesteryl esters chromatographed on: ●, DB-1 fused silica column, GC conditions in Fig. 1; ○, SP2330 column, GC conditions in Fig. 3.

the on-column injection technique in conjunction with the very efficient non-polar column is particularly useful for analyzing small CE samples.

With helium as the carrier gas, resolution of CE on the SP2330 column butt-connected at the injection end to the empty wide-bore column was excellent. Preliminary studies[9] showed that hydrogen cannot be used as a carrier gas because it hydrogenates the double bonds in the fatty acid moiety of the CE molecule. The separation of saturated CE (Fig. 3A, Table I) on this column was not quite as marked as on the DB-1 column but it was nevertheless more than enough to allow a more complete resolution of CE according to degree of unsaturation. The *cis* and *trans* isomers of the monoenoic CE were also resolved on this column[9]. These separations are a considerable improvement over those achieved earlier on a short Silar 10C glass WCOT column[2]. The equivalent chain lengths (ECL) of the CE were obtained by interpolation from a curve of retention time *vs.* fatty acid chain length from the saturated CE series. These ECL values are given in Table II. The distribution of the CE peaks was analogous to that observed previously for fatty acid methyl esters on 10-m SP2330 WCOT columns[2,10].

The recoveries of CE on the SP2330 WCOT column were similar to but slightly lower than that obtained on the DB-1 column (Fig. 2). The lower limit of sensitivity for the SP2330 column for CE with an ECL of 18:0 was about 1–2 ng, nearly as low as that of the DB-1 column (0.5 ng). Because the CE recoveries on both columns were quite reproducible, it was not necessary to have two CE (*i.e.*, CE 10:0 and CE 20:0) in the internal standard added to biological CE samples as it was previously using the splitless injection technique[2]. Therefore CE 10:0 alone was used as the internal standard.

Fig. 3C is a typical chromatogram of CE from an atherosclerotic lesion from a rabbit aorta. The major peaks are clearly resolved according to degree of unsaturation with any *trans* isomers being present only in negligible amounts. The monoenoic CE with a fatty acid chain *n* carbons in length had a tendency to co-elute

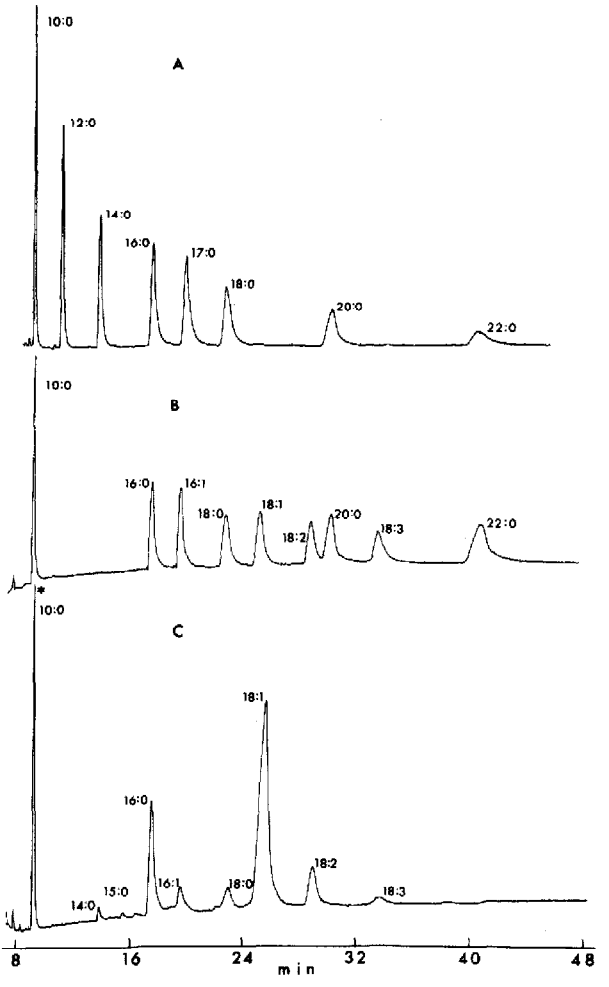

Fig. 3. Chromatograms of cholesteryl esters: A, B, synthetic CE mixtures; C, CE from a rabbit atherosclerotic lesion. See Fig. 1 for explanation of molecular species notation. GC conditions: 10-m SP2330 fused-silica WCOT column (0.25 mm I.D., film thickness 0.2 μm); cool on-column injection, 0.5 μl heptane solution into an empty 60-cm wide-bore fused-silica column butt-connected to the SP2330 column; detector temperature, 300°C; carrier gas, helium at 3 p.s.i. (average linear velocity, 30 cm/sec at 100°C); temperature program, 100°C for 0.5 min, then 30°C/min to 250°C, then isothermal.

with the corresponding odd chain $n + 1$ carbon CE. Normally the odd chain CE are present in very small amounts, and can usually be ignored. However if it is desired to know their concentration in a sample, this is easily done by analysis on the non-polar DB-1 column (Fig. 1). In biological samples, the very long chain CE (ECL > 21) are usually present in very small amounts, their peaks tend to be very broad because of the long retention times, and the recoveries are lower than those of the shorter chain CE. Therefore to obtain reliable data for these molecular species, one can inject a relatively large sample such that the major peaks are somewhat overloaded, but the small peaks of interest are present in measurable amounts. Although not shown in

TABLE II

CHROMATOGRAPHIC PROPERTIES OF CHOLESTERYL ESTERS ON AN SP2330 FUSED-SILICA WCOT COLUMN

*Cholesteryl ester**	*Relative retention time***		*Equivalent chain length (ECL)*
	RRT_{10}	RRT_{18}	
10:0	1.00	0.41	10.0
12:0	1.21	0.49	12.0
14:0	1.49	0.61	14.0
16:0	1.89	0.77	16.0
$16:1_c$	2.10	0.86	16.8
17:0	2.15	0.88	17.0
18:0	2.45	1.00	18.0
$18:1_t$	2.60	1.06	18.45
$18:1_c$	2.70	1.10	18.65
18:2	3.08	1.26	19.6
20:0	3.25	1.33	20.0
18:3	3.59	1.47	20.6
22:0	4.35	1.78	22.0
20:4	4.42	1.80	22.15
22:1	4.79	1.95	22.75

* Subscripts c and t indicate *cis* and *trans* isomers respectively. All unsaturated CE shown in Figs. 1 and 3 are all-*cis* isomers. Data for $18:1_t$ was obtained from a previous study (ref. 9).

** RRT_{10} = relative retention time with CE 10:0 as reference peak; RRT_{18} = relative retention time with CE 18:0 as reference peak.

TABLE III

QUANTITATION OF CHOLESTERYL ESTERS FROM A RABBIT ATHEROSCLEROTIC LESION

Cholesteryl ester	*Amount (mole%)**	
	*DB-1 column***	*SP2330 column****
14:0	0.8	1.0
15:0	0.4	0.6
16:0	18.4	16.0
16:1	2.3	2.5
17:0	0.6	
18:0	74.9	3.6
18:1		55.3
18:2		11.0
18:3		4.8
20:4	2.7	5.3

* Corrected for recoveries as in Fig. 2.

** GC conditions as in Fig. 1.

*** GC conditions as in Fig. 3.

Fig. 3C, this procedure was successfully followed for the analysis of the atherosclerotic lesion CE.

Table III gives the fatty acid composition of atherosclerotic lesions as analyzed by the two column types. It is evident that the data agree well both with each other and with the results of previous studies[11–13].

The capillary GC of CE following cool on-column injection provides a sensitive procedure for the analysis of CE at high resolution. Since only small nanogram amounts of lipid are required, the complete analysis of intact CE from small individual atherosclerotic lesions or from a very few dishes of cultured cells is now practical. The availability now of a leak-proof, low dead volume capillary butt connector by which narrow bore columns can be connected to a short length of empty wide-bore tubing makes it possible to use the on-column injection technique with WCOT columns of any diameter.

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