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# HYDROGENATION OF CHOLESTERYL ESTERS DURING GAS CHROMATOGRAPHY ON A POLAR FUSED-SILICA CAPILLARY COLUMN

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## SUMMARY

Hydrogenation and isomerization of the fatty acid moiety of cholesteryl esters occur during gas chromatography on fused-silica SP2330 wall-coated open tubular columns at 250°C when hydrogen is used as the carrier gas  These reactions do not occur when fatty acid methyl esters are chromatographed on the same column at 130°C. Therefore it is concluded that if a new class of unsaturated compounds is to be analyzed or a new set of gas chromatographic analysis conditions to be used, the possibility of the occurrence of hydrogenation must be ruled out whenever hydrogen is to be employed as the carrier gas  This can be done by performing parallel analyses using helium as the carrier gas and comparing the results with those obtained using hydrogen.

## INTRODUCTION

The popularity of capillary gas chromatography (GC) with wall-coated open tubular (WCOT) columns has increased dramatically in the past few years, especially with the recent availability of fused-silica columns  The choice of carrier gas to be used in a given GC system has major effects on the optimal working conditions for a given set of components[1-4]  Hydrogen increasingly is becoming the carrier gas of choice because its low viscosity allows for shorter analysis times, lower column operating temperatures, and likely extended column life[1-4]. Furthermore hydrogen is inexpensive. Thus with its flammability being its only draw-back up to now[3,4], hydrogen has appeared to be the ideal carrier gas to use with WCOT columns in capillary GC.

As part of efforts in this laboratory to analyze cholesteryl esters from small atherosclerotic arterial lesions[5], capillary GC of these esters has been investigated in non-polar and polar columns using both the splitless[6] and cool on-column[7] injection techniques. In most of this work hydrogen was used as the carrier gas. However, with a new polar fused-silica WCOT column, hydrogenation of the cholesteryl esters has become a serious problem.

EXPERIMENTAL

All solvents (reagent grade) were purchased from Fisher Scientific (Toronto, Canada) The lipids were obtained from Sigma (St. Louis, MO, U.S.A.), Serdary Research Labs. (London, Canada), or Applied Science (State College, PA, U S A.)

Cholesteryl elaidate and cholestanyl palmitate were prepared in the laboratory from their respective sterols and fatty acids with N,N-carbonyldiimidazole (Aldrich) as the acylation reagent by a modification of the method of Boss *et al.*[8]. An amount of 1–4 mg of sterol was mixed with a ten-fold excess of the appropriate fatty acylimidazole intermediate in a few hundred $\mu$l of dichloromethane and heated in a tightly sealed, nitrogen-flushed vial at 110°C for 2 h. The mixture was then quenched with water, and Folch extracted[9]. Following concentration to dryness, the lipids were dissolved in chloroform and the cholesteryl ester isolated by thin-layer chromatography on silica gel H with light petroleum (b p. 35–52°C)–diethyl ether–acetic acid (90:10:1) as the solvent system. The cholesteryl ester was extracted from the silica gel with chloroform–methanol–acetic acid–water (50·39:1:10) and partitioned with 4 $M$ $NH_4OH$[10]. The extract was dried with anhydrous sodium sulphate, evaporated to dryness, and redissolved in heptane for GC analysis.

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 WCOT column system consisted of a custom-made Supelco (Bellefonte, PA, U.S.A.) 10 m fused-silica column (0.25 mm I.D.) coated with SP2330 (0.2 $\mu$m film thickness) which was connected by a Supelco capillary butt connector[11] to a 60-cm length of empty wide-bore (0 3 mm I D ) fused-silica tubing, a kind gift from Hewlett-Packard. 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. The inside walls of the empty tubing had been silylated with *tert* -butyldimethylchlorosilane–imidazole reagent (Applied Science), 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. The injector port was air-cooled as described elsewhere[7] and the detector temperature was 300°C The make-up gas and hydrogen flow-rates through the detector were each 38 ml/min and the air flow-rate was 400 ml/min.

Analysis of the cholesteryl esters (CE) was performed as follows. 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 two min after the start of the program. About 5 ng of cholesteryl ester in 0 5 $\mu$l heptane was injected. 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

Analysis of the fatty acid methyl esters (FAME) was performed following on-column injection. The instrument splitless mode was maintained for 0 6 min. The column temperature was held at 100°C for 0.5 min, then increased at 30°C/min to 130°C and kept at that temperature thereafter.

In all analyses of CE and FAME with hydrogen as the carrier gas, the inlet pressure was 2 p.s.i. (linear velocity 30 cm/sec at 100°C) and nitrogen was the make-up gas. With helium as the carrier gas, the inlet pressure was 5 p s.i. (linear velocity 40 cm/sec) and helium was the make-up gas.

RESULTS

The CE were analyzed both individually and as a mixture The left panel of Fig. 1 shows the individual analyses of steryl esters containing $C_{16}$ fatty acids, with helium used as the carrier gas Each ester migrated as a single band, with cholesteryl palmitoleate (CE 16 $1_c$) having a longer retention time (Fig 1, Table I) than cholesteryl palmitate (CE 16 0) Cholestanyl palmitate (CA 16 0) is the analogue of cholesteryl palmitate which is completely saturated in the sterol moiety, and was used to test for the possibility of hydrogenation of the sterol portion of the ester molecule With helium as the carrier gas, CA 16 0 chromatographed with a retention time identical to that of cholesteryl palmitate Thus it appears that with the SP2330 fused-silica WCOT column used under the present conditions, the location of a carbon–carbon double bond greatly affects the GC properties of the steryl ester molecule

The right panel of Fig 1 shows the chromatograms obtained with these esters when hydrogen was used as the carrier gas CE 16 0 and CA 16 0 again appeared as single peaks with identical retention times CE 16 $1_c$ chromatographed in this example with a major peak which had the same retention time as the other two esters, and a small shoulder with a retention time which probably corresponds to that of cholesteryl palmitelaidate (CE 16 $1_t$) (cf Table I) This peak pattern suggests that in the presence of hydrogen CE 16 $1_c$ was almost completely hydrogenated to CE 16 0 Whether or not the sterol part of the ester molecule was also hydrogenated cannot be determined under these GC conditions, since CE 16 0 and CA 16 0 chromatograph in an identical manner regardless of the carrier gas used Indeed because CE 16 0 and CA 16 0 have the same retention time, sterol hydrogenation for all practical purposes in this study can be ignored.

In Fig 2 the effect of carrier gas on CE containing $C_{18}$ fatty acids of various degrees of unsaturation is shown. The left panel presents the analysis of each CE with helium as the carrier gas Each of these esters, containing stearic acid (18 0), elaidic acid (18 $1_t$), oleic acid (18 $1_c$), linoleic acid (18 2), and linolenic acid (18 3), respectively, migrated as a single peak with the retention time increasing with the degree of unsaturation In contrast, when hydrogen was used as the carrier gas (Fig 2, right panel) only cholesteryl stearate, the saturated CE, chromatographed with a single peak All chromatograms of the unsaturated CE contained a cluster of peaks, the largest two of which corresponded in retention time to those of cholesteryl stearate and cholesteryl elaidate, respectively. The small peaks and peak shoulders were probably cholesteryl oleate and various trans isomers derived from the corresponding parent fatty acids The exact proportions of the various peaks varied somewhat from one run to another, but were similar to those illustrated Similarly the extent of hydrogenation of CE 16·$1_c$ varied from one analysis to another Clearly, however, hydrogenation and isomerization of unsaturated CE were extensive in all analyses in which hydrogen was used as the carrier gas.

The effect of hydrogenation and isomerization on the GC analysis of a mixture of CE is illustrated in Fig 3 The normal chromatogram of well resolved peaks obtained when helium was used as the carrier gas is in sharp contrast to the completely distorted and unusable chromatogram obtained when hydrogen was used With hydrogen, the chromatogram is reduced to five recognizable peaks corresponding in retention time (Table I) to the saturated and monounsaturated (mainly the trans isomer) analogues of the original CE species.
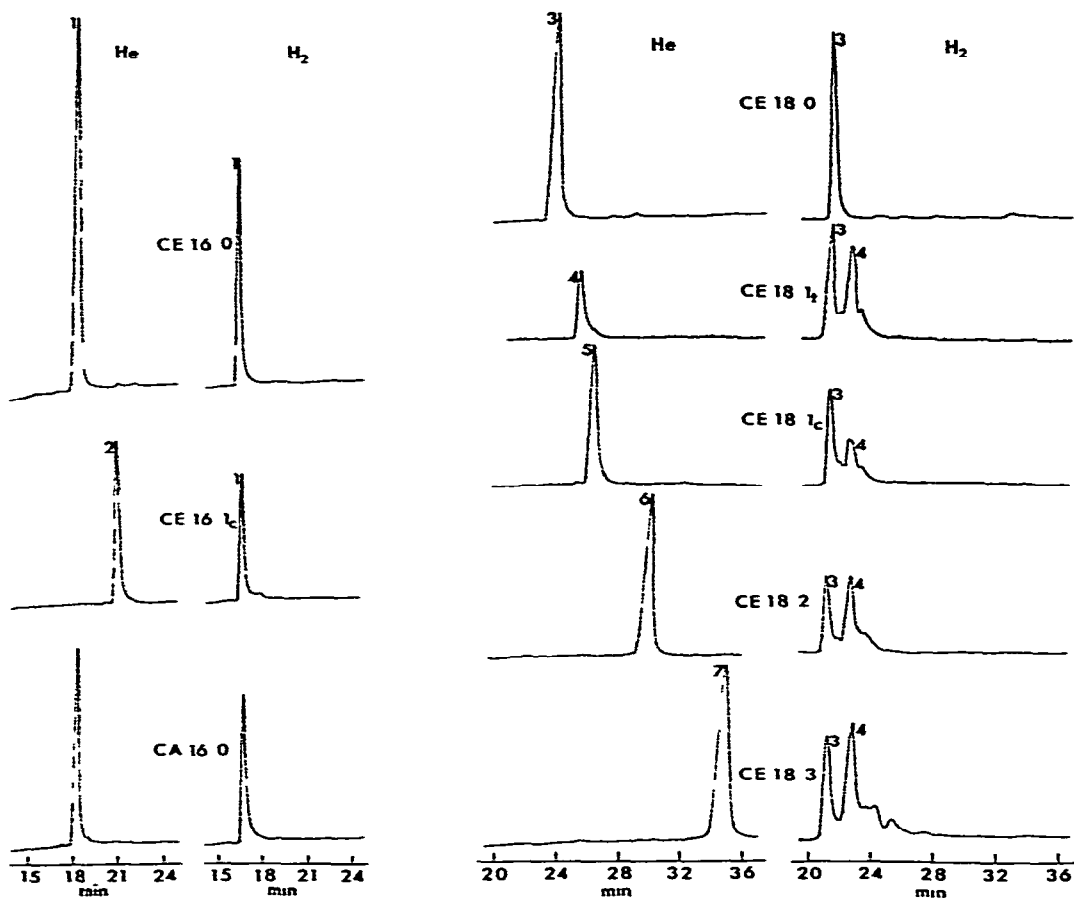
Fig. 1 Chromatograms of individual steryl esters containing $C_{16}$ fatty acids, left panel with helium and right panel with hydrogen as carrier gas. Molecular species notation. CE $m.n$, cholesteryl ester with a fatty acid chain containing $m$ carbon atoms and $n$ carbon–carbon double bonds, subscript c indicates *cis* double bond; CA 16:0, cholestanyl palmitate. Arabic numeral beside each CE peak is the peak number, which indicates that the numbered peak has a retention time which corresponds to a known normal (unreacted) CE; 1 = CE 16:0, 2 = CE 16:1$_c$. GC conditions: 10-m SP2330 fused-silica WCOT column, 0.25 mm I.D., 0.2 $\mu$m film thickness; cool on-column injection, 0.5 $\mu$l 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 5 p.s.i. (average linear velocity, 40 cm/sec at 100°C) or hydrogen at 2 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

Fig. 2. Chromatograms of individual CE containing $C_{18}$ fatty acids, left panel with helium and right panel with hydrogen as carrier gas. Molecular species notation as in Fig. 1. Subscripts c and t indicate *cis* and *trans* isomers respectively of monoenoic esters. Polyenoic CE are all-*cis* isomers. Arabic numerals at each major peak indicate peak number; 3 = CE 18:0, 4 = CE 18:1$_t$; 5 = CE 18:1$_c$; 6 = CE 18:2; 7 = CE 18:3; see Fig. 1 for further explanation and GC conditions.

When FAME were analyzed on the same SP2330 column as that used in the CE analyses, the chromatograms obtained with either helium or hydrogen as the carrier gas were essentially identical (Fig. 4), and showed little or no evidence of hydrogenation or isomerization. The only significant difference between the analysis conditions for the CE and FAME was the column temperature (250°C *vs.* 130°C,
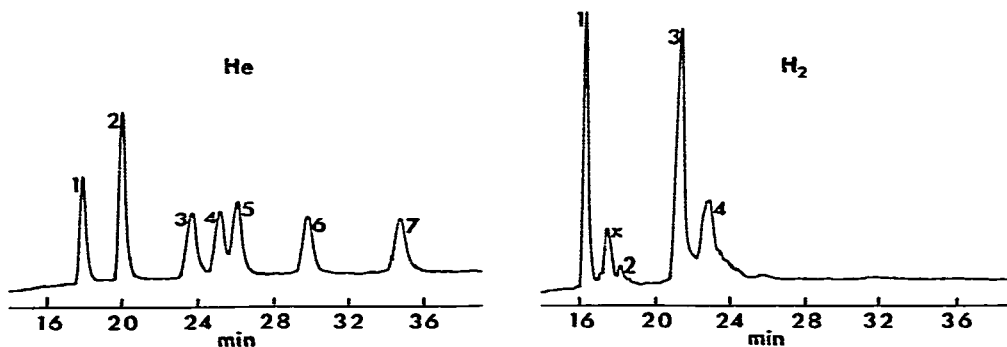
Fig 3 Chromatograms of a mixture of CE, left panel with helium, and right panel with hydrogen as carrier gas See Figs 1 and 2 for explanation of peak numbers See Fig. 1 for GC conditions

respectively). Another FAME mixture (not shown) identical to that in Fig. 4 except that methyl elaidate was not included, also chromatographed identically in the presence of hydrogen or helium.

TABLE I

GC RETENTION AND IDENTITY DATA OF CE MIXTURE IN FIG 3

| Peak number | Carrier gas | Retention time relative to saturated CE | Peak identity |
|---|---|---|---|
| 1 | He | 1 0 | CE 16 0 |
| 2 | He | 1 12 | CE 16 1$_c$ |
| 3 | He | 1 0 | CE 18 0 |
| 4 | He | 1 07 | CE 18 1$_t$ |
| 5 | He | 1 11 | CE 18 1$_c$ |
| 6 | He | 1 27 | CE 18.2 |
| 7 | He | 1 48 | CE 18 3 |
| 1 | H$_2$ | 1 0 | CE 16 0 |
| × | H$_2$ | 1 07 | CE 16 1$_t$* |
| 2 | H$_2$ | 1 12 | CE 16 1$_c$ |
| 3 | H$_2$ | 1 0 | CE 18-0 |
| 4 | H$_2$ | 1 06 | CE 18 1$_t$ |

* Identity of 16 1$_t$ is based on similarity of the value for the retention time relative to saturated CE, to corresponding values for CE 18 1$_t$ (with both He and H$_2$ as carrier gas)

DISCUSSION

The current opinion in the literature is that as a carrier gas hydrogen is superior to other gases such as helium and nitrogen[1-4]. The optimum linear carrier gas velocity for hydrogen is higher than that of the other gases, and the non-optimal velocity range at which hydrogen can be used without serious loss of efficiency is much broader than that of the other gases[1-4]. Hydrogen is less viscous than helium and nitrogen and therefore changes in flow-rate during temperature programming are less
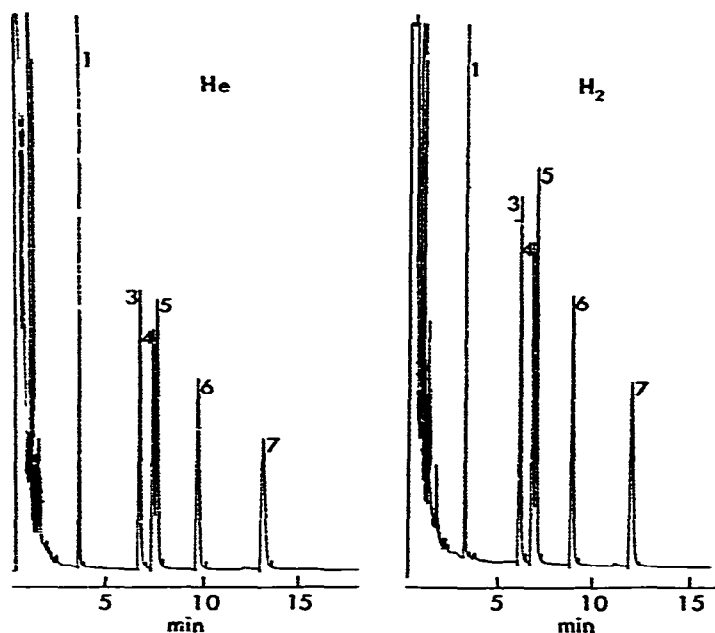
Fig. 4. Chromatograms of mixture of FAME, left panel with helium, and right panel with hydrogen as carrier gas Peak numbers are the same as those of the corresponding CE molecular species (i e peak No 1 is 16 0, No. 3 is 18 0, etc ). GC conditions as in Fig 1 except that final temperature was 130°C

drastic[4]. Thus with hydrogen, relatively rapid analyses at high carrier gas flow-rates but low inlet head pressures have become practical. Consequently many laboratories now use hydrogen. Hydrogen has been satisfactorily used in the analysis of triacylglycerols[12-14], diacyl glycerols[15 16] cholesteryl esters[6], and coal tar compounds[17], among others. Hydrogenation has not been a problem in any of these studies, one of which was from this laboratory[6]. Nevertheless, with the observation now that hydrogenation can occur, hydrogen should now be used with caution.

The mechanism by which hydrogenation occurs is quite unclear. The fact that hydrogenation occurred with the CE at 250°C, but not with FAME at 130°C might suggest that high temperature alone was responsible for the effect. However, hydrogenation of CE was not observed previously during GC analysis at 255–320°C on OV-1 coated fused-silica columns[6 7]. Similarly, triacylglycerols[12-14] and diacyl glycerols[15] have been separated on non-polar columns above 250°C without hydrogenation occurring. Myher and Kuksis[16] recently described the separation of the diacylglycerol moieties of natural glycerophospholipids on a 10-m glass SP2330 column at 250°C. Their column was coated with the same liquid phase and operated at the same temperature as that used in the present study, but the column material (i e., glass) was different from that described here (fused silica). No hydrogenation apparently occurred. What role the nature of the tubing material may play in the hydrogenation process is not clear.

It seems likely that the hydrogenation reaction proceeds catalytically at the inner surface of the capillary wall, and that elevated temperatures may accelerate the process. However, the necessary initiating conditions remain unknown. Until these con-

ditions are understood, the analyst must test for and rule out the possibility of hydrogenation occurring in each new GC analysis situation This would mean testing for each class of compound, column temperature, liquid phase, column material, and perhaps even column age. When hydrogenation is not a problem, hydrogen is still the carrier gas of choice; otherwise, helium is recommended

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