SHORT COMMUNICATION

Derivatization of 5α-Cholestane-3β,5,6β-Triol into Trimethylsilyl Ether Sterol for GC Analysis

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Chemical identity of 5α-cholestane-3β,5,6β-triol (C-Triol) as a trimethylsilyl (TMS) ether derivative was studied using gas chromatography (GC), mass spectrometry (MS), and proton nuclear magnetic resonance (NMR) spectroscopy. The derivatization mixture, held at 23°C for 30 and 300 min, showed only a single peak (B) by GC. When the mixture was heated at 70°C for a few hours, another peak (A) emerged ahead of peak (B). GC-MS analysis revealed that the GC peaks (A) and (B) are C-Triol as *tris*- and *bis*-TMS ether derivatives, respectively. NMR analysis suggested that the hydroxyl groups at C_3 and C_6 of C-Triol were involved in the formation of the *bis*-TMS ether.

We documented that 5α-cholestane-3β,5,6β-triol (C-Triol) was derivatized into tris-trimethylsilyl (TMS) ether sterol when we reported separation of some common cholesterol oxidation products (COPS) for their gas chromatographic quantitation (1). However, when C-Triol, known to be among the most toxic COPS (2,3), was found in freeze-dried pork stored under abusive conditions (4), the mass spectrum of C-Triol as TMS ether (4) did not agree with that of C-Triol as *tris*-TMS ether (1). The later study (4) suggested that only two hydroxyl groups of C-Triol were derivatized into the TMS ether, giving rise to *bis*- rather than *tris*-TMS ether. Such a conflict in mass spectra for C-Triol as TMS ether between those two reports (1,4) prompted the present reinvestigation. In this communication, the unambiguous assignment has been made for the structural identity of C-Triol as *bis*-, not *tris*-TMS ether when C-Triol is to be derivatized following procedures used by the authors (1, 4–7).

EXPERIMENTAL

Derivatization of C-Triol into TMS ether was carried out under two different conditions: (i) at room temperature (about 23°C) as reported previously (1) for 30 and 300 min, and (ii) at 70°C for up to four days. Gas chromatography (GC) conditions were the same as used previously (1,4), except for higher GC oven temperatures (210–250°C at 3°C/min rise) with a Varian gas chromatograph (Model 3400).

Mass spectrometry (MS) was performed with a combined GC-MS in a manner similar to previous studies (1,4), except that a V.G. Analytical mass spectrometer (Model 70-S, Manchester, England) equipped with an 11-250J data system was used. C-Triol derivatized at room temperature for 30 min was analyzed by proton (^1H) nuclear magnetic resonance (NMR) spectral studies. C-Triol TMS ether, derivatized at 23°C for 30 min, was extracted with hexane and freed of hexane under a stream of nitrogen. After redissolving the extract into deuterated chloroform containing no tetramethylsilane, ^1H NMR spectrum was obtained with a Varian XL-200 spectrometer operating at a frequency of 200 MHz.

RESULTS AND DISCUSSION

The upper chromatogram (Fig. 1) is obtained with the reaction mixture held at room temperature for 30 min, a condition routinely used by authors to quantify COPS

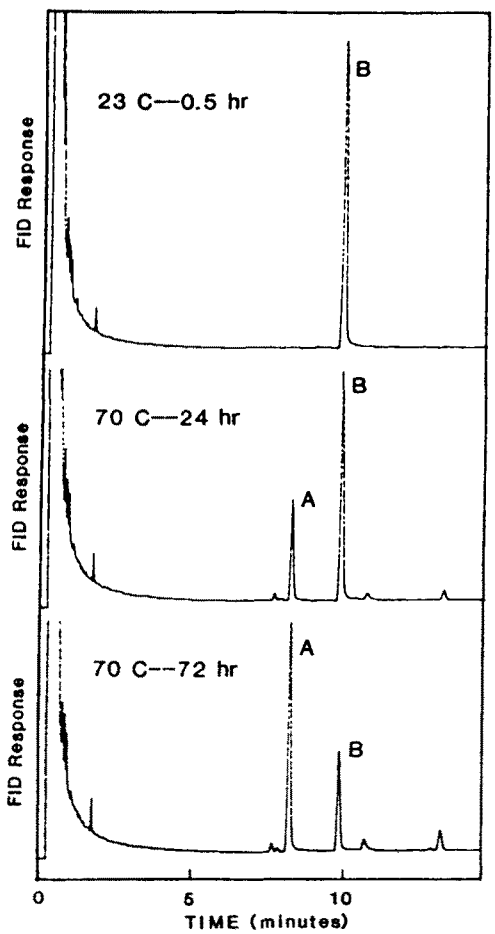

FIG. 1. Gas chromatograms of trimethylsilyl ethers of 5α-cholestane-3β,5,6β-triol derivatized under different heating temperature and time conditions.

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in foods (1,4-7). Only a single peak (B) resulted without any noticeable peaks other than solvents background, which agreed with the previous report (1,4). After 300 min at room temperature only the same single peak (B) was observed with the approximately same peak area. When the derivatization proceeded at 70°C, another peak (A) emerged (middle and lower chromatograms in Fig. 1). With prolonged reaction for derivatization, the area ratio of peaks A/B grew. Since C-Triol as a *tris*-TMS ether derivative would be less polar and more volatile than that as a *bis*-TMS ether, the former is expected to elute ahead of the latter under GC conditions of the present study. Therefore, peaks (A) and (B) were inferred to be C-Triol as *tris*- and *bis*-TMS ethers, respectively.

Supposing that the three hydroxyl groups at positions C_3, C_5 and C_6 of C-Triol are converted to TMS ethers, the molecular weight of the resultant derivative amounts to 636, which is the molecular ion (M^+) of C-Triol as *tris*-TMS ether. The peak at m/z = 636 is clearly shown in the mass spectrum (A) of Figure 2 for the peak (A) of Figure 1. Similarly, if only two hydroxyl groups are involved in the TMS derivatization, the resultant molecular weight becomes 564. The peak at m/z = 564, the molecular ion of C-Triol as *bis*-TMS ether, is displayed clearly as the ion with the highest m/z in the spectrum (B) of Figure 2 for the peak (B) of Figure 1. Apart from peaks at m/z = 636 (and thus 621, $M^+ - 15$) and m/z = 564 in spectra (A) and (B), respectively, both spectra (A) and (B) look very much alike in terms of the same major ions as well as their relative intensities. The peak at m/z = 456 appeared as the base peak in both spectra followed by m/z = 546, 483, 321 and 367. Therefore, based on the observation of molecular ions, (i.e., m/z = 636 and 564 in the spectra (A) and (B), respectively) *tris*- and *bis*-TMS ether structures are assigned for the peaks (A) and (B) of Figure 1, respectively. Worthy of mention is that the mass spectrum (B), now representing C-Triol as *bis*-TMS ether, differed in the relative intensity of major ions from the mass spectrum tentatively identified as *bis*-TMS ether in our previous report (4) on the basis of its excellent agreement with the report for bis-TMS ether (8). This difficulty in reproducing a mass spectrum with regard to the order of relative intensities of major ions when different instruments were used, whether or not it accounts for the observed difference, stresses the importance of running standards to establish the reference mass spectra prior to sample runs.

^{1}H NMR analysis of the extracts from the derivatization mixture held at room temperature for 30 min, essentially the same procedure producing only peak (B) in Figure 1, displayed only two lines with almost equal intensity around zero ppm (Fig. 3). This result

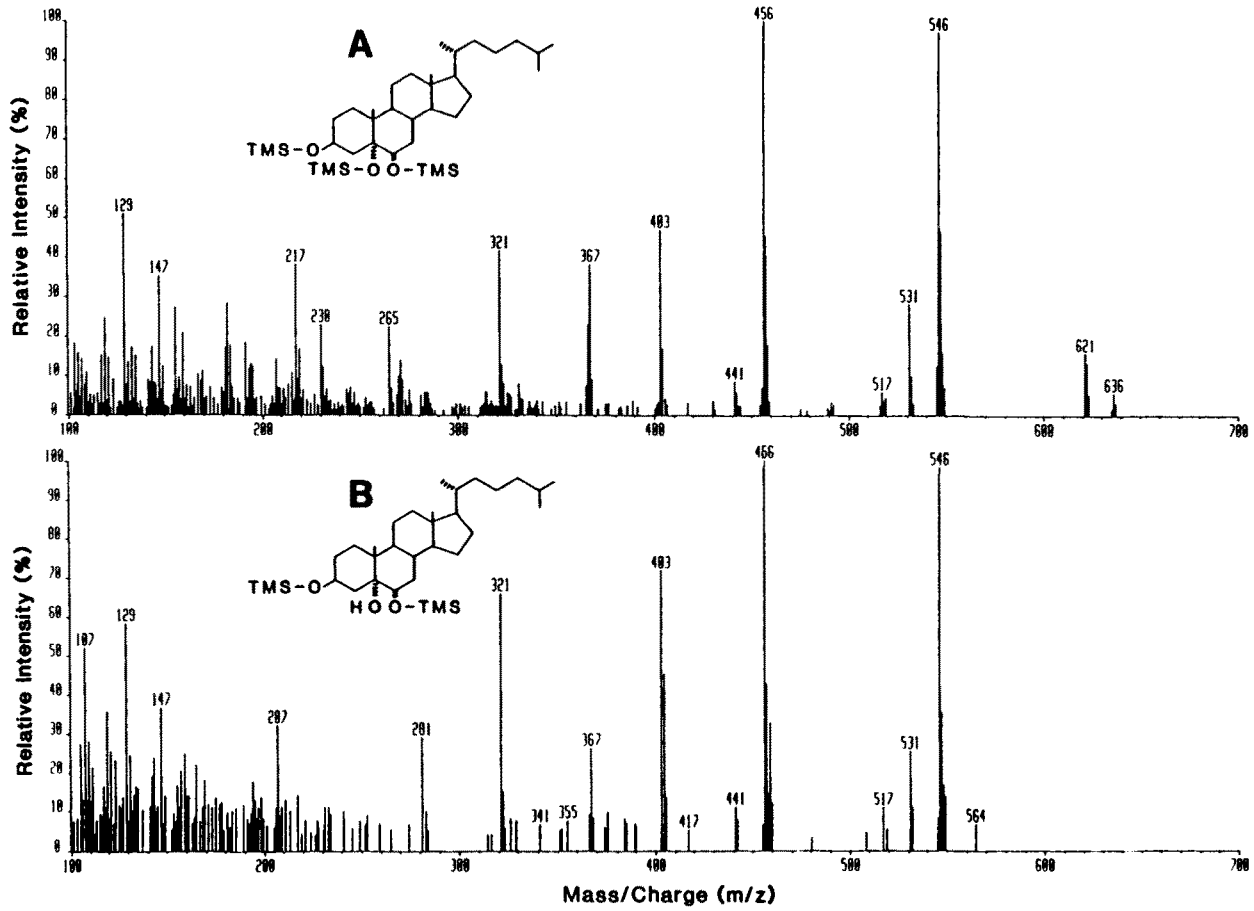

FIG. 2. Electron impact (70 eV) mass spectra of 5α-cholestane-3β,5,6β-triol trimethylsilyl ethers isolated by GC from the derivatization mixture heated at 70°C for 24 hr. Spectra A and B are *tris*- and *bis*-trimethylsilyl ethers, respectively, for the GC peaks A and B in Figure 1.

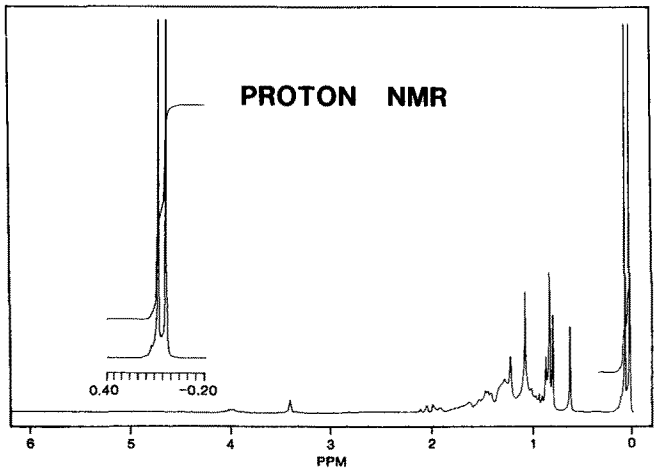

FIG. 3. Proton NMR spectrum of 5α-cholestane-3β,5,6β-triol after trimethylsilyl derivatization at 23°C for 30 min.

clearly supported the concept that hydroxyl groups at C_3 and C_6 position of C-Triol are derivatized into TMS ethers. When C-Triol was converted to p-nitrobenzoyl derivatives after being heated at 80°C for one hr, ^1H NMR studies also revealed reactions on hydroxyl groups at C_3 and C_6, but not C_5 due to steric hindrance (unpublished).

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