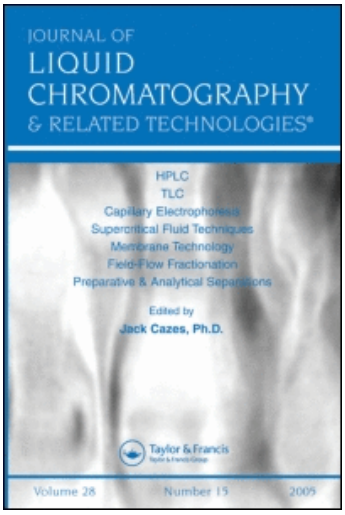

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Tert-Butyldiphenylsilyl Derivatization for Liquid Chromatography and Mass Spectrometry

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TERT-BUTYLDIPHENYLSILYL DERIVATIZATION FOR LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY

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ABSTRACT

Tert-butyldiphenylsilyl (TBDPS) derivatization has been investigated for the purposes of improving the HPLC analysis of polar compounds such as fatty acids, cholesterol and bile acids with ultraviolet absorption and/or mass spectral detection. Very mild reaction conditions (room temperature, 30 min) gave quantitative conversion of carboxyls and sterically accessible hydroxyls. The sterically hindered 7α and 12α hydroxyls of bile acids were not silylated to any extent under these conditions. The TBDPS ethers and esters proved stable under HPLC conditions and could be separated on an octylsilyl (RP-8) reverse phase column using acetonitrile as the mobile phase. Detection with an ultraviolet absorption detector at 254 nm was possible but 220 nm provided maximum sensitivity. The derivatives proved to be sufficiently volatile for electron-impact mass spectrometry and the apolar nature of the mobile phase facilitated combined LC/MS with a moving belt interface. The spectra were characterized by abundant ions at $[M-57]^+$ due to loss of a tert-butyl radical and m/z 199 due to Ph_2SiOH^+.

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INTRODUCTION

Chemical derivatization has found wide application in gas chromatography (GC) and mass spectrometry (MS) for improving the volatility of polar compounds, as well as their chromatographic resolution, detectability and mass spectral fragmentation behavior. Of the many methods used, trimethylsilylation is the most common because of the ease of reaction with a wide variety of functional groups (-OH, -COOH, -SH, and -NH), the excellent volatility of the derivatives, and the useful nature of their mass spectra (1,2).

Derivatization in liquid chromatography (HPLC) has been used primarily for improving detectability through the attachment of chromophoric or fluorescent groups to compounds that are normally not easily detected with UV absorption or fluorescence detectors. Alkylation and acylation reactions are most commonly used (3).

The technique of combined liquid chromatography/mass spectrometry (LC/MS) has attracted a great deal of interest in that it affords both high sensitivity detection and structural information for many compounds that cannot be analyzed by GC/MS (4). Unfortunately, electron-impact mass spectrometry, which is the most useful technique for structural information, is still a gas phase analytical method and requires the analytes to have some volatility. It seems reasonable that, as in GC/MS, derivatization should be useful in extending the range of compounds that can be analyzed by LC/MS. In addition to improving the volatility of polar compounds for the mass spectrometer, derivatization should also lead to an improved compatibility of LC and MS by allowing the use of apolar, volatile mobile phases. Of course, it would also be desirable if the derivatization group used also imparted good mass spectral behavior to the derivative such as an abundant molecular ion and structurally significant fragmentations. Unfortunately, the derivatives most commmonly used for HPLC give rather poor mass spectra. For example, the phenacyl esters of

fatty acids (5) give spectra with very weak molecular ions that are dominated by fragment ions at low mass associated mainly with the phenacyl group (e.g., $PhCO^+$, m/z 105).

Silylations are potentially attractive for LC/MS for the same reasons they are favored for GC/MS. However, trimethylsilyl derivatives are very easily hydrolyzed and therefore are unstable to most LC conditions. In recent years, sterically crowded trialkylsilyl groups such as tert-butyldimethylsilyl (TBDMS) have been used extensively in synthetic organic chemistry as protecting groups due to their ease of formation and high stablility (6). They have also proved useful for mass spectrometry and GC/MS due to their good volatility and the abundance of an $[M-57]^+$ ion that is useful for the determination of molecular weight and for selected ion monitoring (7). In addition, TBDMS derivatives exhibit structurally informative fragmentations and rearrangements (8). The excellent stability of TBDMS derivatives during liquid chromatography led us to investigate their application to the combined LC/MS of nucleosides (9). We have had excellent results with this derivatization method , but have found that for compounds such as bile acids that do not possess a chromophore for UV detection, it was difficult to develop chromatographic conditions using an optical detector prior to LC/MS. This prompted us to examine other sterically crowded silyl groups with a chromophore as a substituent. One such group, tert-butyldiphenylsilyl (TBDPS), has proved useful in organic synthesis because of its stability and the visualization of protected compounds in chromatography (10,11). In this preliminary study we have investigated the utility of TBDPS derivatization for HPLC and HPLC/MS for fatty acids, cholesterol and bile acids.

MATERIALS

The fatty acids were purchased from Sigma Chemical Co., St. Louis, Missouri. Bile acids and cholesterol were supplied by

Steraloids Inc., Wilton, NH. Tert-butyldiphenylchlorosilane, imidazole and dimethylformamide dimethylacetal were purchased from Aldrich Chemical Co., Milwaukee, Wis.. Silylation grade DMF was purchased from Pierce Chemical Co., Rockford, Ill.. Sephadex LH-20 was purchased from Pharmacia, Uppsala, Sweden. HPLC-grade solvents were obtained from Caledon Laboratories, Georgetown, Ontario.

The human bile sample was prepared from duodenal bile aspirate (provided by Dr. M.M. Fisher, Dept. Pathology, Univ. Toronto). Conjugated bile acids were saponified by incubation in 2.5 N NaOH for 16 hr at 110°C and the free bile acids were extracted with ether after acidification to pH 1.

METHODS

Derivatizations were performed in dry, teflon-lined septum-capped vials. Approximately one hundred micrograms of substrate was weighed into the vials and reagents added so that the resultant substrate concentration was about 0.1 mg/ml with a 50-fold molar excess of reagent. Reaction with the silylation reagent, composed of 1 M tert-butyldimethylchlorosilane and 2 M imidazole in DMF (9), proceeded to completion in 30 min at room temperature. Excess reagent was then removed by passing this reaction mixture through a short column (6 x 0.5 cm) packed with Sephadex LH-20 swollen in hexane/ethyl acetate (3:1, v/v) with elution by the same. The first 3 ml was collected and evaporated to dryness under a nitrogen stream. The sample was dissolved in acetonitrile for analysis.

HPLC separations were performed on a Spectra-Physics Model 8000 instrument with detection provided by a Schoeffel Model 770 variable wavelength UV absorption detector. A 10 um Spectra-Physics RP-8 (octylsilane bonded phase, 25 cm x 4.6 mm i.d.) column was used.

Combined LC/MS was performed with a VG7070F mass spectrometer (VG Analytical, Altrincham, UK) and a VG2035 data system. The instrument was equipped with a VG moving belt interface with a Kapton belt. The entire effluent from the HPLC was run onto the belt and an infrared evaporator was used to evaporate the solvent prior to the vacuum lock system. Electron impact ionization was performed with an electron energy of 70 eV.

RESULTS AND DISCUSSION

Fatty Acids

TBDPS derivatives of fatty acids were found to be formed rapidly and quantitatively by room temperature reaction for 30 min with tert-butyldiphenylchlorosilane in dimethylformamide with imidazole as a catalyst (10). Analyses could be performed by direct injection of the reaction mixture but UV absorption chromatograms were simplified by a preliminary removal of excess reagent by passage through a short column of Sephadex LH-20 (12). Figure 1a shows the reverse phase HPLC separation of three fatty acid TBDPS derivatives and their detection with a UV detector at 254 nm. Due to their very low polarity, an RP-8 column and a mobile phase of 100% acetonitrile was used. Excellent hydrolytic stability was observed for the derivatives. There was no apparent decomposition during chromatography and storage of the esters as a dry residue was possible for a few days. The sensitivity for detection of the derivatives by UV absorption at 254 nm is only modest compared to phenacyl (5) or p-bromophenacyl (13) derivatives due to the low extinction coefficient for TBDPS group at that wavelength (ε = 750). However, improved sensitivity is possible by monitoring at the absorption maximum of 220 nm (ε = 33,000).

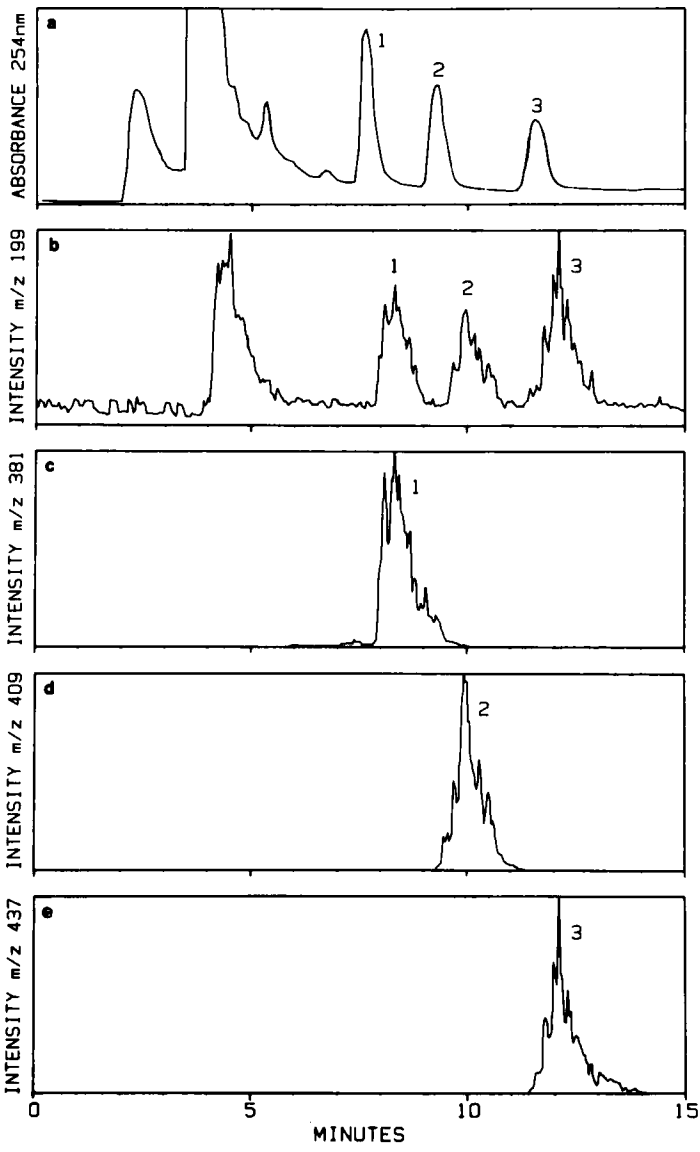


FIGURE 1: Combined LC/MS of the TBDPS derivatives of three fatty acids (1 = myristic acid, n-$C_{13}H_{27}COOH$; 2 = palmitic acid, n-$C_{15}H_{31}COOH$; 3= stearic acid, n-$C_{17}H_{35}COOH$). The five traces are due to absorption at 254 nm (a) and computer reconstructed mass chromatograms of ions at m/z 199 (b), m/z 381 (c), m/z 409 (d) and m/z 437 (e). Chromatographic conditions: RP-8 column with 1 ml/min of 100% acetonitrile.

The TBDPS derivatives proved to be excellent for combined LC/MS. A mobile phase of 100% acetonitrile allowed the entire 1 ml/min of effluent to be sent to the LC/MS interface, whereas a split has to be used when appreciable percentages of water are present in the mobile phase. As shown in Figure 2, the mass spectra of the TBDPS fatty acid derivatives are very simple with abundant ions at $[M-57]^+$ which allow the unambiguous determination of the molecular weights of the original compounds. In addition, all the spectra contain common ions at m/z 199 and m/z 253. Possible mechanisms leading to the formation of these ions by rearrangement of the $[M-57]^+$ siliconium ion are presented in Scheme 1. The computer reconstructed mass chromatograms in Figures 1b to 1e illustrate the ease with which the mass spectrometer can be used to detect either all or individual TBDPS derivatives through the common ion at m/z 199 or through $[M-57]^+$ ions, respectively. Selected ion monitoring of these ions should provide excellent sensitivity. (It should be noted that most of the noise in the mass chromatograms is due to poor spreading of the effluent on the LC/MS interface belt. Recent experiments have shown that chromatograms can be improved through use the spray deposition method reported by Smith (15).)

Cholesterol

Cholesterol was studied next to determine if a steroid hydroxyl could be silylated easily with the TBDPS reagent. Reaction at room temperature for 30 min was found to give quantitative conversion to the TBDPS ether which proved to be very stable to HPLC and during storage as a dry residue or in solution for several weeks. Combined LC/MS gave a mass spectrum with a base peak at m/z 567 corresponding to $[M-tBu]^+$ and peaks at m/z 199 (Ph_2SiOH^+) and m/z 367 ($[M-tBu-Ph_2HSiOH]^+$).

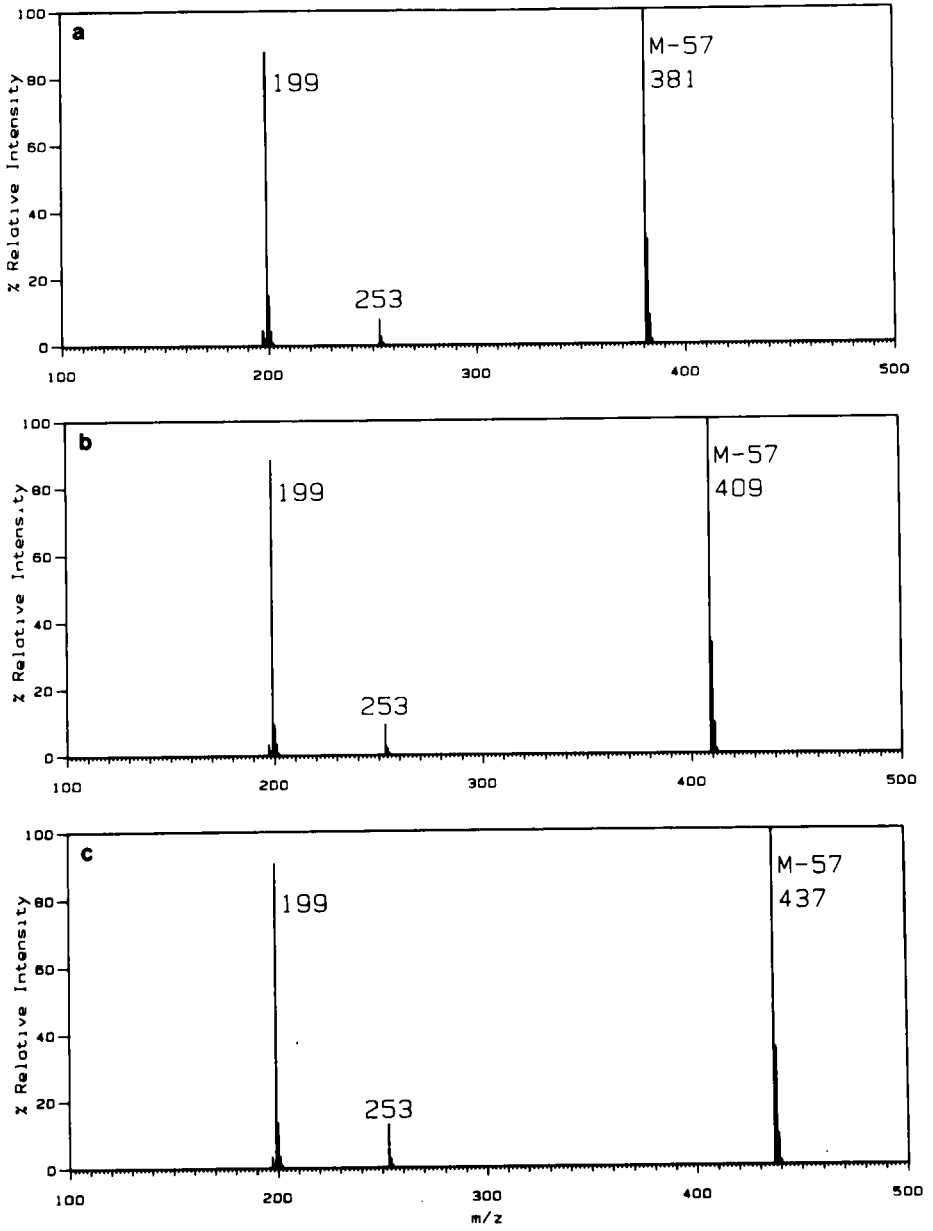
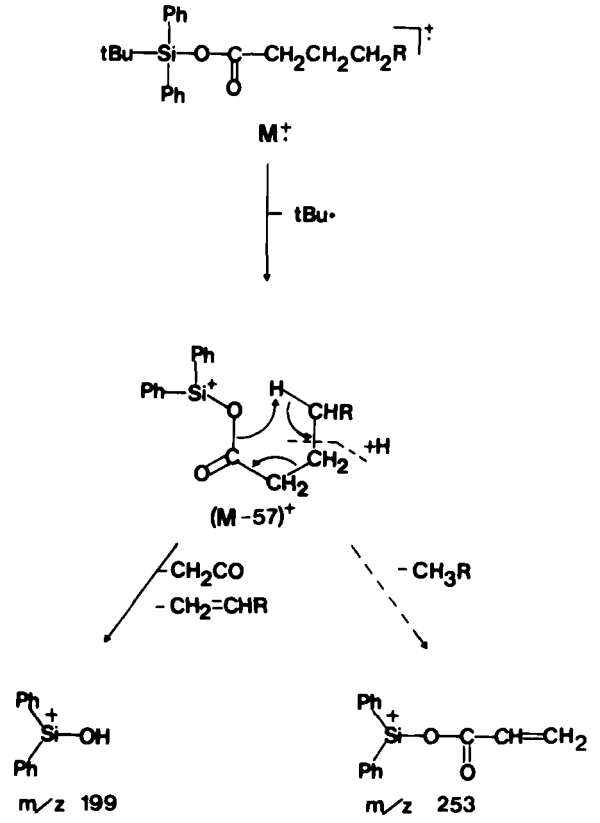


FIGURE 2: Electron impact mass spectra of the TBDPS derivatives of three fatty acids acquired from the LC/MS experiment in Figure 1: (a) myristic acid (1), (b) palmitic acid (2), and (c) stearic acid (3).



Scheme 1

Bile Acids

Once it had been established that both TBDPS esters and ethers could be prepared and were stable to HPLC, the more complicated bile acids were examined. Bile acids presented an interesting challenge in that they possess both carboxyl and hydroxyl functions. Some of the compounds also have sterically hindered hydroxyls in the 7α and 12α positions. It was anticipated that the latter hydroxyls would not silylate readily as has been

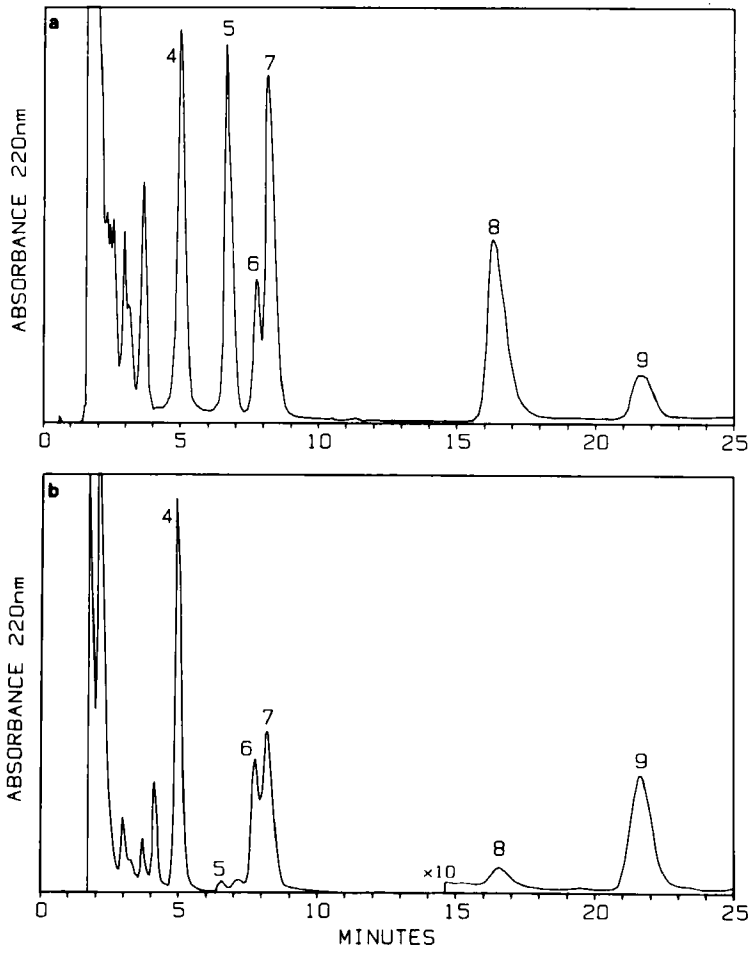


FIGURE 3: HPLC separation of TBDPS derivatives of bile acids and cholesterol: (a) standard compounds (4 = cholic acid, 5 = ursodeoxycholic acid, 6 = chenodeoxycholic acid, 7 = deoxycholic acid, and 8 = lithocholic acid and 9 = cholesterol); (b) human bile hydrolyzate. Conditions: RP-8 column with 2 ml/min of 100% acetonitrile and detection at 220 nm.

reported for the derivatization of steroids (8). Nevertheless, it was of concern that a mixture of derivatives may result. Studies with individual bile acids showed, however, that single derivatives were formed quantitatively by reaction with the TBDPS reagent at room temperature for 30 min. Treatment of the compounds under more forcing conditions (100°C overnight) showed significant further reaction only for ursodeoxycholic acid, 5 , with a second peak appearing in the chromatogram at long retention time, presumably due to reaction of the 7α-hydroxyl. Mass spectrometry via the solid probe inlet system proved that all the bile acids were converted to bis-TBDPS derivatives. Abundant $[M-57]^+$ ions were observed at m/z 827 for cholic acid, m/z 811 for ursodeoxycholic, chenodeoxycholic and deoxycholic acids, and m/z 795 for lithocholic acid. These ions could be useful for selected ion monitoring LC/MS analyses, but it is likely that the lower molecular weight TBDMS derivatives would prove more useful.

Figure 3a shows the HPLC analysis of the TBDPS derivatives of a mixture of bile acid standards as well as cholesterol, and Figure 3b shows the detection of the same compounds in a hydrolyzed human bile sample. Excellent separation was provided for all of the compound except the isomers 6 (chenodeoxycholic) and 7 (deoxycholic) which were only partially resolved. The general order of elution is inversely proportional to the number of hydroxyls at the 7α and 12α positions. The UV detector was adjusted to 220 nm for these separations for better sensitivity. The degree of separation compares favorably with published methods using phenacyl derivatives (14).

CONCLUSIONS

From these results, it is possible to conclude that TBDPS derivatives can be formed quantitatively from compounds with carboxyls and sterically unhindered hydroxyls under very mild

reaction conditions and that the resulting TBDPS esters and ethers are stable under reverse phase HPLC conditions. Complex mixtures of analytes such as fatty acids and bile acids proved separable by HPLC as the TBDPS derivatives and could be detected with a UV absorption detector. Maximum sensitivity is achieved by monitoring at 220 nm. TBDPS derivatives appear to be useful for combined LC/MS due to their volatility and the use of apolar mobile phases for their chromatography. The abundant $[M-57]^+$ and m/z 199 ions provide molecular weight information and should allow high sensitivity detection with selected ion monitoring. We are currently developing other silyl reagents with better chromophores for higher sensitivity detection with UV absorption detectors and will be extending silylation methods for HPLC and HPLC/MS to other classes of compounds.

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