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**Note**

# Gas chromatographic analysis of bile acid methyl esters as partial trimethylsilyl ether derivatives using N,O-bis(trimethylsilyl)trifluoroacetamide as silylating reagent

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Trimethylsilyl (TMS) ethers of methyl esters are routinely used derivatives for the gas–liquid chromatographic (GLC) analysis of fecal bile acids. A variety of liquid phases such as SE-30, QF-1, or HiEFF 8BP have been reported to be useful to achieve separation[1,2]. A major problem, particularly in the analysis of rodent feces, is that no one liquid phase can effectively separate the wide spectrum of primary and secondary bile acids as their TMS derivatives. Additional purification of the fecal extracts by thin-layer chromatography and subsequent GLC analysis is often required to detect certain bile acids such as hyodeoxycholic acid (HDC)[3]. This bile acid is found in substantial quantities in the feces of several strains of rats but its detection is not always reported[2].

In this investigation we have used N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), a compound previously used in steroid analysis[4], as the silylating reagent to form partial TMS ethers of bile acids. The derivatives formed allow effective separation on 3% QF-1 of six important fecal bile acids including HDC found in rat feces. We have used this procedure in several studies relating fecal bile acids to intestinal carcinogenesis[5,6].

## MATERIALS

Cholic, deoxycholic, chenodeoxycholic, hyodeoxycholic and lithocholic acids were purchased as methyl esters from Applied Science Labs. (State College, Pa., U.S.A.) The β-muricholic acid sample purified from mouse bile by Dr. H. Eyssen, Rega Institute (Louvain, Belgium), was kindly provided by Dr. David Madsen, University of Notre Dame (Notre Dame, Ind., U.S.A.). The 12-ketolitholic acid was synthesized in this laboratory by the method of Bergstrom and Haslewood[7] and methylated with diazomethane.

The silylating reagents BSTFA and the mixture of hexamethyldisilazane (HMDS), chlorotrimethylsilane (CTMS) and pyridine (3:1:9) were obtained from Applied Science Labs. HMDS and N,N-dimethylformamide were from Sigma (St. Louis, Mo., U.S.A.).

## METHODS

Full TMS ethers of bile methyl esters were prepared by reacting at room temperature 100 $\mu$g of bile acid methyl ester and 25 $\mu$g of 5$\alpha$-cholestane (Applied Science Labs.) as internal standard with 0.2 ml of HMDS–CTMS–pyridine (3:1:9) for 30 min according to the method of Grundy *et al.*[1]. The partial TMS derivatives were formed by reacting the bile acid methyl esters and 5$\alpha$-cholestane in sealed tubes with either 0.2 ml of BSTFA as solvent and reagent at 37° overnight or by the method of Eneroth *et al.*[8] using 0.06 ml dry N,N-dimethylformamide and 0.03 ml HMDS at 50° for 3 h. Excess reagents were removed under a stream of dry nitrogen and the residue dissolved in 0.1 ml of carbon disulfide.

GLC analyses were performed on a Hewlett-Packard Model 5831A gas chromatograph equipped with a 6-ft. glass column (2 mm I.D. × 0.25 in. O.D.) packed with 3% QF-1 on Gas-Chrom Q (100–120 mesh; Applied Science Labs.). Temperatures were maintained at 230° for the column, 250° for the injector, and 275° for the detector. The nitrogen carrier gas flow-rate was maintained at 20 ml/min. The flame-ionization detector gases were held at 40 ml of hydrogen per min and 300 ml of air per min. Retention times and peak areas were reported directly by the instrument.

Bile acids from rat feces were extracted and purified as previously described[5] and partial TMS derivatives were prepared with BSTFA for comparison to standard bile acid chromatograms.

## RESULTS

Table I summarizes the relative retention time values for bile acid methyl ester TMS ethers prepared by the three methods described. A mixture of lithocholic,

TABLE I

RELATIVE RETENTION TIMES ON 3% OF-1 OF DIFFERENT BILE ACID METHYL ESTERS AS TRIMETHYLSILYL ETHER DERIVATIVES FORMED WITH DIFFERENT SILYLATING REAGENTS*

sh = shoulder on the peak.

| *Bile acid methyl ester* | *Hydroxyl positions* | *Relative retention times* | | |
|---|---|---|---|---|
| | | *HMDS–TMCS–pyridine*** (3:1:9)* | *HMDS**** | *BSTFA* |
| Lithocholic | 3$\alpha$ | 3.28 | 3.29 | 3.27 |
| Hyodeocholic | 3$\alpha$,6$\alpha$ | 3.94 | 4.00 | 3.95 |
| Deoxycholic | 3$\alpha$,12$\alpha$ | 3.54 | 6.33 | 6.29 |
| Chenodeoxycholic | 3$\alpha$,7$\alpha$ | 3.77 | 7.08 | 7.04 |
| 12-Ketolithocholic | 3$\alpha$ | 10.79 | 10.84 | 10.78 |
| Cholic | 3$\alpha$,7$\alpha$,12$\alpha$ | 3.72 | 13.95 | 13.87 |
| $\beta$-Muricholic | 3$\alpha$,6$\beta$,7$\beta$ | 4.89 | — | 7.90sh |

* Retention times relative to 5$\alpha$-cholestane at 230° at carrier gas flow-rate of 20 ml/min.

** Full TMS derivatives formed[1].

*** Hydroxyls derivatizing are 3$\alpha$, 6$\alpha$ and 7$\beta$ according to Eneroth *et al.*[8] and Briggs and Lipsky[9].

hyodeoxycholic, deoxycholic, chenodeoxycholic and cholic acids as full TMS ethers were poorly resolved and appeared as two merged peaks under the conditions of analysis described. As full TMS derivatives $\beta$-muricholic and 12-ketolithocholic acids were separated from the other bile acids. The relatively long retention time of the keto acid is characteristic of the QF-1 liquid phase. In contrast, bile acid TMS derivatives formed with BSTFA or HMDS were well separated. Fig. 1 shows a typical standard GLC chromatogram without $\beta$-muricholic acid for derivatives formed using BSTFA.

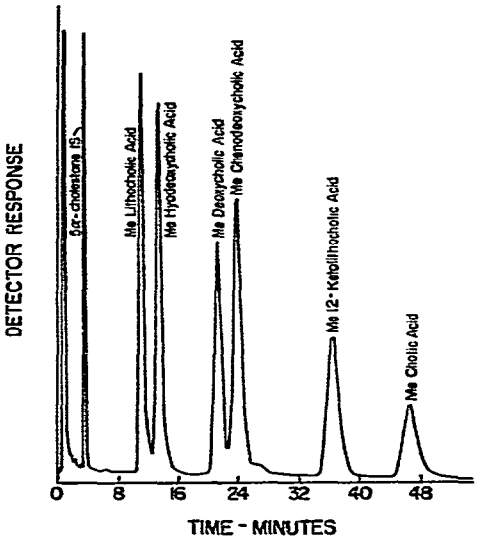


Fig. 1. Chromatogram for mixture of standard bile acid methyl esters derivatized with BSTFA as described under Methods. Amounts of bile acids were 1 $\mu$g with 0.25 $\mu$g of 5$\alpha$-cholestane as internal standard injected in a 1.0-$\mu$l volume of $CS_2$. The small shoulder after Me chenodeoxycholic acid is an unidentified contaminant.

TMS ethers only on the unhindered hydroxyls (3$\alpha$, 6$\alpha$ and 7$\beta$) are reportedly formed using HMDS[8]. The nearly identical relative times for derivatives formed with either HMDS or BSTFA suggest that partial derivatives are formed with each reagent. Such a result would seem logical since BSTFA is a large molecule and thus would be unable to react with sterically hindered hydroxyls. Further, the retention times for HDC derivatives prepared by all three reagents are the same while bile acids with 7$\alpha$ and/or 12$\alpha$ hydroxyls appear at longer retention times when HMDS or BSTFA is used as silylating reagent. The shoulder on the $\beta$-muricholic acid peak appearing at a longer retention time is most likely an incomplete ether formation at the 7$\beta$ hydroxyl with BSTFA. Thus $\beta$-muricholic acid, appearing in small amounts in the feces of most rat strains, is best detected as a full TMS derivative.

Fig. 2 shows a GLC chromatogram of bile acids derivatized with BSTFA that were extracted from the feces of Sprague–Dawley rats[5]. Tentative identification of the peaks was made by comparison to the relative retention times of the standard bile acid chromatogram. Only one peak of considerable size, at 32 min retention, remains unidentified.

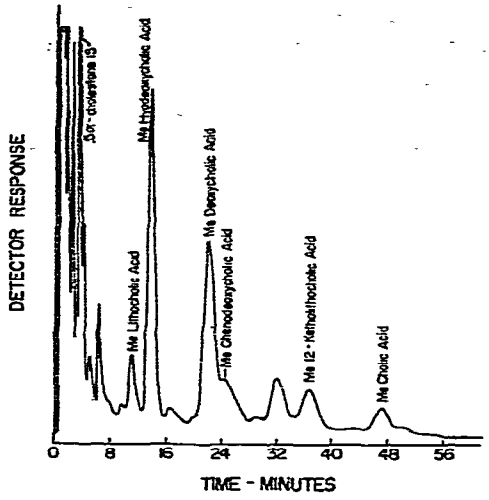


Fig. 2. Chromatogram of fecal bile acids excreted by Sprague–Dawley rats using BSTFA as silylating reagent, GLC conditions as described under Methods. Peaks as labeled were identified by comparison of relative retention times.

## DISCUSSION

The results of this study indicate that the GLC analysis of fecal bile acids from rats may be simplified by using QF-1 as a liquid phase and a combination of full and partial TMS derivatives. Lithocholic, hyodeoxycholic, deoxycholic, chenodeoxycholic, 12-ketolithocholic, and cholic acids which constitute a large part of the bile acids excreted by conventional rats can be conveniently determined as partial TMS derivatives by using BSTFA as silylating reagent. The resulting derivatives have retention times identical to those formed with HMDS by a method previously documented for the formation of partial derivatives[8]. The use of BSTFA, however, is advantageous since it can serve as reaction solvent, it is highly reactive, and its by-products appear in the solvent front. Further, if BSTFA is used as injection solvent the transient appearance of HF in the flame-ionization detector helps prevent excessive silicon dioxide deposits.

The method described may also be used quantitatively. Standard response curves have been found to be linear from less than 0.3 to 5 $\mu$g or more of each of the six bile acids examined. Reproducibility appears excellent if conditions and reagents are kept constant. However, we have found in further work (data not presented here) that if N,N-bis(trimethylsilyl)trifluoroacetamide is used in place of the usual reagent N,O-compound, hyodeoxycholic acid appears as two peaks, the new peak appearing between chenodeoxycholic and 12-ketolithocholic acids. The reason for this change is not known.

In conclusion, the use of BSTFA to form partial TMS derivatives allows the GLC separation on 3% QF-1 of six important bile acids found in rat feces. Subsequent conversion to full TMS derivatives allows the detection of $\beta$-muricholic acid as well as keto acids that are greatly retained by the QF-1 liquid phase. This method is useful for structural determinations by peak shift analysis and for quantitation.

Extension of this method to less common bile acids such as α-muricholic and ω-muricholic acids where available coupled with GLC and mass spectrometry studies will more clearly define the silylating specificity of this reagent toward bile acids.

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