THE GAS–LIQUID CHROMATOGRAPHIC BEHAVIOR OF METHANESULFONATES AND MIXED SILYL ETHERS OF BILE ACIDS

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INTRODUCTION

It has been demonstrated recently that the methanesulfonates of sterols undergo an elimination reaction when applied to a gas–liquid chromatography (GLC) column[1,2]. The structures of the resulting olefins were observed to depend upon the nature of the hydroxyl group of the parent steroid and its immediate molecular environment; alcohols of a given type exhibited characteristic olefin peak patterns. It was found that correlation of the GLC behavior of these "reactive derivatives" and the structure of their parent sterols could be facilitated by employing the values obtained when the retention time of the olefins are divided by the retention time of the parent sterol.

Trimethylsilyl (TMSi) ethers are recognized as especially suitable derivatives for the GLC of hydroxy-substituted compounds, and recently chloromethyldimethylsilyl (CMDMSi) ethers were also found to be excellent derivatives[3,4]. It was shown that when mixtures of reagents capable of forming both types of silyl ethers were allowed to react with polyhydroxy compounds the formation of the various possible derivatives permitted multiple characterization of the parent compound in a single chromatographic analysis[4]. The retention behavior of bile acid methyl esters and several types of derivatives has been studied extensively, especially by SJÖVALL *et al.* and MAKITA AND WELLS[5–13]. Two new and complimentary methods of structural characterization and identification—mixed silyl ether patterns and reactive derivative olefin patterns—have now been applied to the bile acids, and the results of the study are reported in this paper.

EXPERIMENTAL

Retention data were obtained with a Barber-Colman Model 15 gas chromatograph (Lovelock argon ionization detection system; 500 V). The column packing was 2 % SE-30 (a methylpolysiloxane; General Electric Company) coated on acid-washed and silanized[14] Gas-Chrom P. Column conditions: 6 ft. × 4 mm glass U-tube; 16 p.s.i.; 225° (methanesulfonates), 238° (silyl ethers). Methylation of the bile acids was accomplished by reaction with diazomethane in ether–methanol. Methanesulfonates were prepared using methanesulfonyl chloride in pyridine; silyl ether derivatives were prepared using hexamethyldisilazane and trimethylchlorosilane (TMSi ethers) and 1,3-bis(chloromethyldimethyl)-1,1,3,3-tetramethyldisilazane and chloro-

methyldimethylchlorosilane (CMDMSi ethers) in pyridine. The methanesulfonate of methyl lithocholate, the dimethanesulfonate of methyl deoxycholate, the CMDMSi ether of methyl 7-ketolithocholate, and the diCMDMSi ether of methyl deoxycholate were isolated and possessed satisfactory elemental analyses. Other derivatives were prepared from submilligram quantities of bile acid methyl esters by reaction with the appropriate reagents; excess reagent was removed in a stream of nitrogen, and the residue taken up in ethyl acetate (methanesulfonates) or carbon disulfide (silyl ethers) and used directly for chromatography. Combined GLC–mass spectrometry was carried out with the LKB Model 9000 instrument equipped with the molecular separator of RYHAGE[15] using an SE-30 column and chromatographic conditions comparable to those employed for gathering the retention data.

RESULTS AND DISCUSSION

The GLC retention data obtained with SE-30 for a number of bile acid methyl esters and their methanesulfonyl derivatives are presented in Table I. The methanesulfonates of methyl 3α-hydroxycholanate (methyl lithocholate) and methyl 12α-hydroxycholanate both give two major olefin peaks (see Fig. 1), those from the latter exhibiting considerably greater volatility, paralleling the relative retention behavior of the parent methyl esters. Only one olefin peak is observed for the sulfonate of methyl 7α-hydroxycholanate, however. The introduction of a second hydroxyl

TABLE I

RETENTION BEHAVIOR OF BILE ACID METHYL ESTERS AND METHANESULFONATES WITH SE-30

Compound	*Relative retention time*[a]
Methyl 3α-hydroxycholanate	2.06
Methyl 3α-hydroxycholanate MS[b]	1.02, 1.09
Methyl 7α-hydroxycholanate	1.84
Methyl 7α-hydroxycholanate MS	1.09
Methyl 12α-hydroxycholanate	1.68
Methyl 12α-hydroxycholanate MS	0.68, 0.74
Methyl 3β-hydroxy-5-cholenate	2.22
Methyl 3β-hydroxy-5-cholenate MS	0.95, 1.23, 1.30
Methyl 3α,6α-dihydroxycholanate	3.96
Methyl 3α,6α-dihydroxycholanate MS	0.96, 1.12, 1.22, 1.31[c]
Methyl 3α,7α-dihydroxycholanate	3.58
Methyl 3α,7α-dihydroxycholanate MS	0.98, 1.09
Methyl 3α,7β-dihydroxycholanate	3.53
Methyl 3α,7β-dihydroxycholanate MS	0.97, 1.08
Methyl 3α-hydroxy-7-ketocholanate	3.38
Methyl 3α-hydroxy-7-ketocholanate MS	1.75
Methyl 3α,12α-dihydroxycholanate	3.25
Methyl 3α,12α-dihydroxycholanate MS	0.63, 0.69, 0.72 (sh), 0.96
Methyl 3α-hydroxy-12-ketocholanate	3.37
Methyl 3α-hydroxy-12-ketocholanate MS	1.62, 1.80
Methyl 3α,7α,12α-trihydroxycholanate	5.60
Methyl 3α,7α,12α-trihydroxycholanate MS	0.62, 0.67, 0.73, 0.88, 0.92, 1.04

[a] Relative to cholestane, 1.00 (absolute retention time 12.0 min). Column conditions given in the Experimental section.

[b] Methanesulfonyl derivative, which undergoes elimination of the functional group to yield olefin.

[c] Underlining denotes major component.

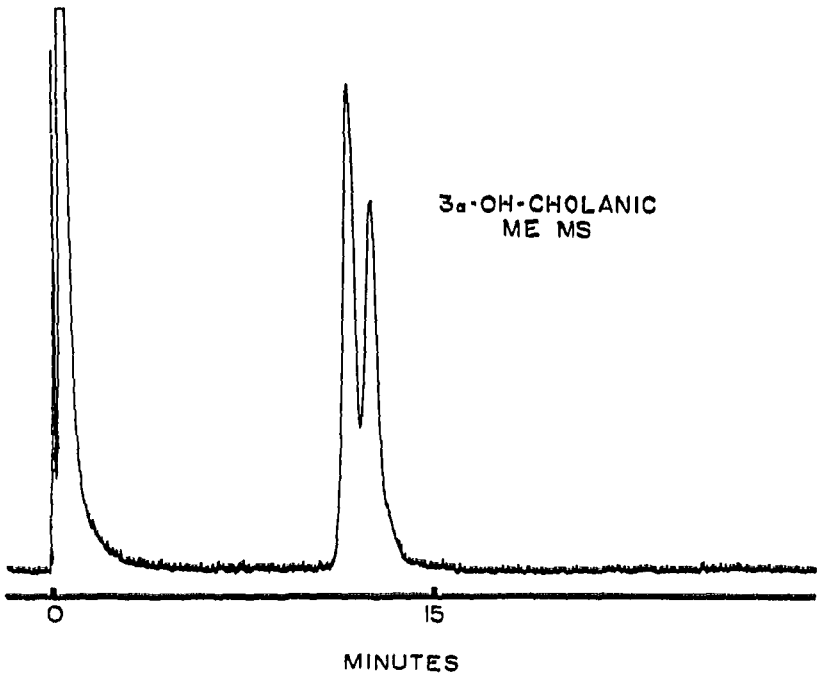

Fig. 1. Gas–liquid chromatographic behavior of the methanesulfonate of methyl lithocholate. Column conditions are given in the Experimental section.

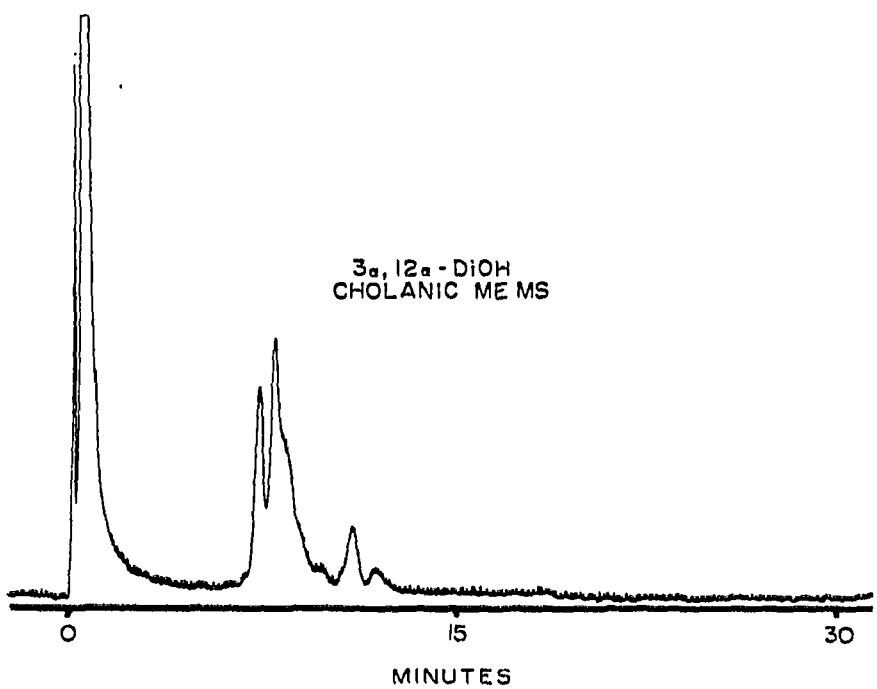

Fig. 2. Gas–liquid chromatographic behavior of the dimethanesulfonate of methyl deoxycholate. Column conditions are given in the Experimental section.

group into the bile acid nucleus (at the 6, 7 or 12-position) results in an approximate doubling of the retention time of the methyl ester. The olefins from the corresponding dimethanesulfonates, on the other hand, do not show any significant change in retention time from those of the monosulfonates, but the olefin peak patterns for the 3α,6α-dihydroxy and 3α,12α-dihydroxy (hyodeoxycholate and deoxycholate, respectively) compounds are considerably more complex (see Fig. 2). It is relatively easy to distinguish between the 3α,6α, the 3α,7α or 3α,7β, and the 3α,12α-substituted compounds with SE-30 on the basis of their olefin patterns (the use of polar stationary phases might lead to improved separation between olefins), but the methanesulfonates of the 3α,7α and 3α,7β-substituted methyl esters yield the same pattern (see Fig. 3)*. Keto and hydroxy groups at the 7 and 12-positions contribute approximately equally to volatility (compare retention times for methyl 3α,7α-dihydroxycholanate (chenodeoxycholate) and the corresponding 7-keto compound, and for

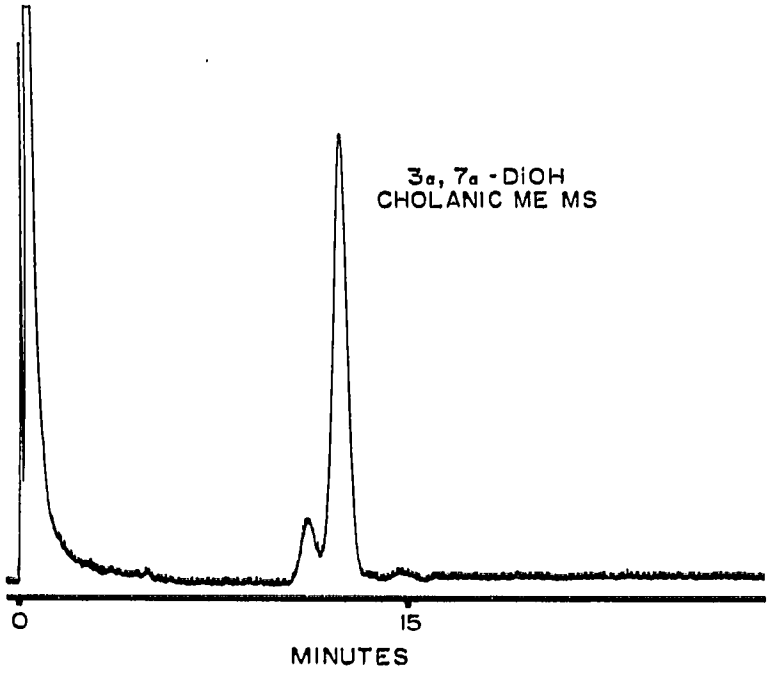

Fig. 3. Gas–liquid chromatographic behavior of the dimethanesulfonate of methyl chenodeoxycholate. Column conditions are given in the Experimental section.

methyl deoxycholate and its 12-keto analog), but chromatography of the methanesulfonyl derivatives leads to a clear differentiation. The methanesulfonate of methyl 12-ketolithocholate gives two olefin peaks (see Fig. 4), paralleling the results observed with the methyl lithocholate derivative, but the "reactive derivative" of methyl-7-ketolithocholate yields only one (see Fig. 5), indicating that the course of the elimination reaction is sensitive to substituents relatively far removed from the ester group. Introduction of a third hydroxyl group to give methyl cholate leads to another large increase in retention time, but the olefin peaks from the derivatized methyl ester are still in the range of those seen for the mono and disulfonated methyl esters, although the complexity of the pattern has again increased.

* The mass spectra of the peaks possessing the same retention times are virtually identical.

The behavior of the bile acid methyl ester methanesulfonates under GLC conditions can be expressed in a most meaningful and useful fashion by calculating their "olefin/bile acid methyl ester factors", values obtained when the retention times of the olefins are divided by the retention time of the bile acid methyl ester (see Table II). This approach was found earlier to be very helpful in characterizing sterols[2]; for example, all 3β-ol-Δ^5 sterols gave the same set of "olefin/sterol factors". It is clear that the pattern also holds for bile acids containing such a homoallylic system, for the methanesulfonate of methyl 3β-hydroxy-5-cholenate yields the same factors (SE-30) as found for the sterol derivatives. The methanesulfonate of methyl lithocholate gives two values which are intermediate in magnitude to those for methyl 7α-hydroxycholanate and methyl 12α-hydroxycholanate. The values are all approximately 0.50; this is because with non-selective[14] stationary phases compounds con-

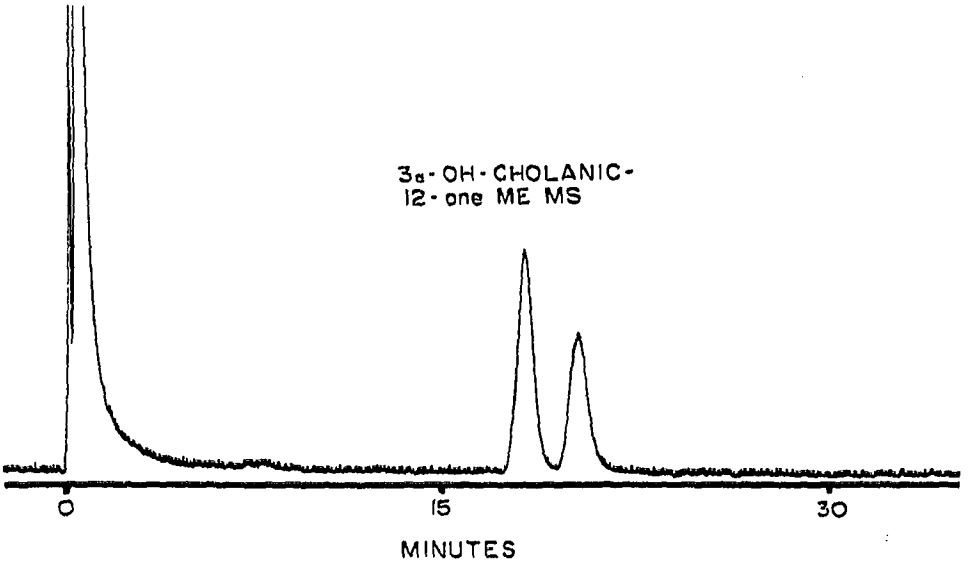

Fig. 4. Gas–liquid chromatographic behavior of the methanesulfonate of methyl 12-ketolithocholate. Column conditions are given in the Experimental section.

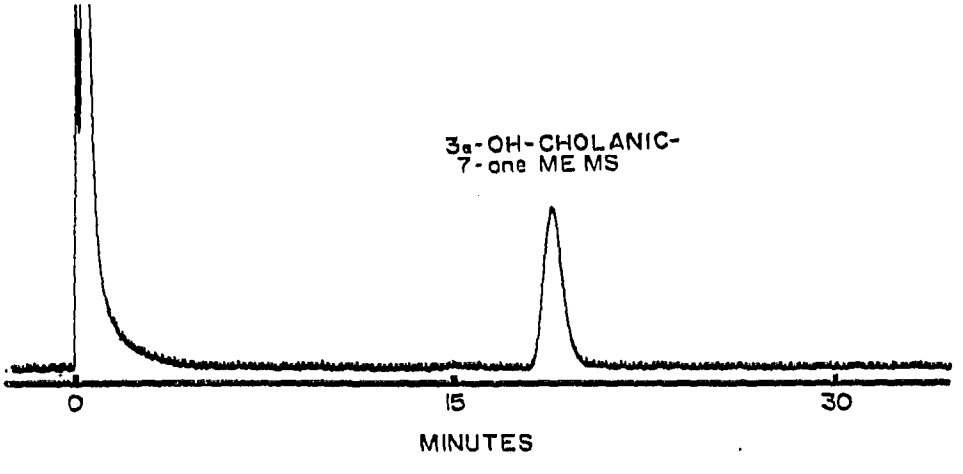

Fig. 5. Gas–liquid chromatographic behavior of the methanesulfonate of methyl 7-ketolithocholate. Column conditions are given in the Experimental section.

TABLE II

OLEFIN/BILE ACID METHYL ESTER FACTORS FOR SULFONATE ESTERS WITH SE-30

Compound	*Factors*[a]
Methyl 3α-hydroxycholanate	0.49, 0.52
Methyl 3β-hydroxy-5-cholenate	0.43, 0.55, 0.59
Methyl 7α-hydroxycholanate	0.59
Methyl 12α-hydroxycholanate	0.40, 0.44
Methyl 3α,6α-dihydroxycholanate	0.24, 0.28, 0.31, <u>0.33</u>[b]
Methyl 3α,7α-dihydroxycholanate	0.27, <u>0.30</u>
Methyl 3α,7β-dihydroxycholanate	0.27, <u>0.31</u>
Methyl 3α-hydroxy-7-ketocholanate	0.52
Methyl 3α,12α-dihydroxycholanate	0.19, 0.21, 0.22, 0.29
Methyl 3α-hydroxy-12-ketocholanate	0.48, 0.53
Methyl 3α,7α,12α-trihydroxycholanate	0.11, 0.12, 0.13, 0.16, 0.19

[a] Calculated from values found in Table I.
[b] Underlining denotes major component.

taining one hydroxyl group possess roughly twice the retention time of the corresponding unsubstituted compound. For example, methyl lithocholate has a retention time twice that of methyl cholanate.

The olefin factors observed for the dihydroxy-substituted bile acids sharply differentiate these compounds from the monohydroxy bile acids. This is a reflection of the fact that the "olefin/bile acid methyl ester factors" are dependent upon the retention times of both the olefin and the parent compound. Methyl 7-ketolithocholate and 12-ketolithocholate exhibit factors (0.52 and 0.48 and 0.53, respectively) completely compatible with their monohydroxy nature. The values for methyl cholate are significantly lower (about one-half) than those for the closely related methyl deoxycholate and methyl chenodeoxycholate. Indeed, it is possible to estimate with considerable accuracy the number of hydroxy groups present in a molecule from the values obtained when the retention time(s) of the olefin(s) derived from the methanesulfonation product is divided by the retention time of the parent compound*. In addition, the olefin peak pattern for a given compound and the corresponding set of olefin/parent compound factors can serve as characterization and identification data.

Derivatives such as TMSi ethers, which are stable to GLC conditions, are widely used in steroid work[7,13,17–20], and a comparison of the retention behavior observed for the TMSi and CMDMSi ethers of ten representative bile acid methyl esters is presented in Table III. It is clear from the data for the three monosubstituted bile acids that the CMDMSi ethers exhibit retention times nearly twice those for the corresponding TMSi ethers, the retention factors[21] for the changes in elution time accompanying this change in functionality being 2.07 at the 3α position (see Fig. 6), 2.00 at the 7α position, 2.14 at the 7β position, and 1.89 at the 12α position.

* This approach would only be valid with hydroxyl groups which form labile methanesulfonates. Since a highly hindered group such as the 12α-hydroxyl reacts under the conditions used in this work this approach does have a high degree of merit; however, certain methanesulfonates (of phenols and long chain primary alcohols[1,16]) appear to be stable toward GLC conditions.

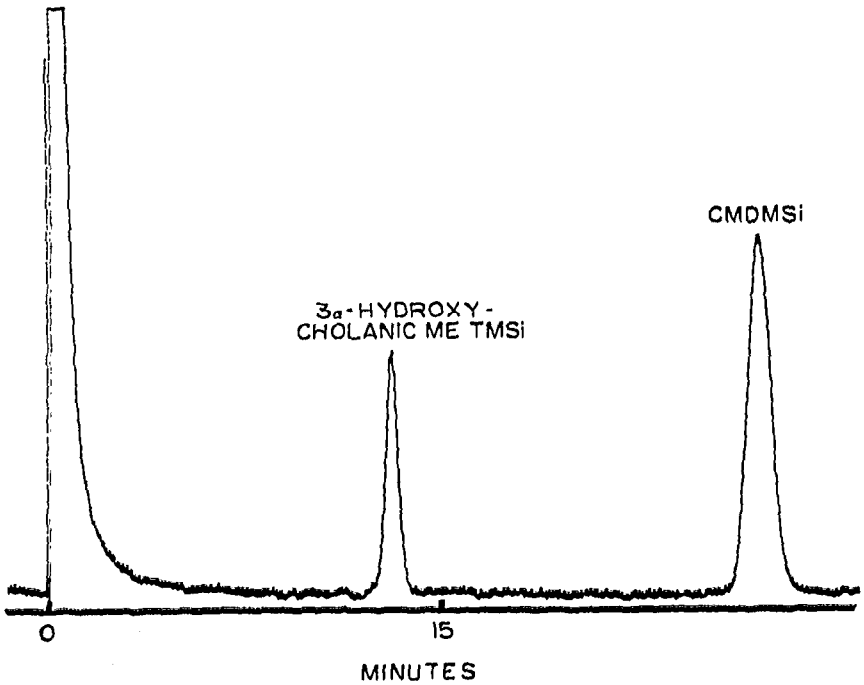

Fig. 6. Gas–liquid chromatographic separation of the trimethylsilyl (TMSi) amd chloromethyldimethylsilyl (CMDMSi) ethers of methyl lithocholate. Column conditions are given in the Experimental section.

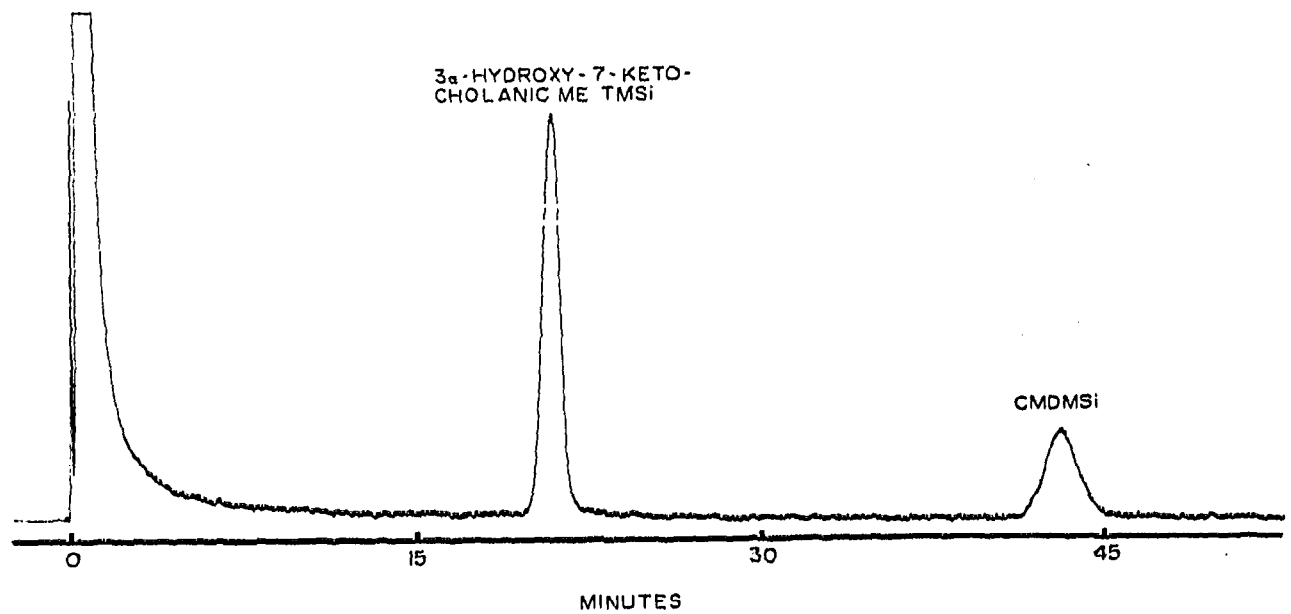

Fig. 7. Gas–liquid chromatographic separation of the trimethylsilyl (TMSi) and chloromethyldimethylsilyl (CMDMSi) ethers of methyl 7-ketolithocholate. Column conditions are given in the Experimental section.

The separation factor for the two silyl ethers of methyl 7-ketolithocholate is 2.06, comparing very favorably with the value observed for the 3α position with methyl lithocholate (see Figs. 6 and 7). The 7-keto group actually leads to a greater retention time than does a trimethylsiloxy group at this position, but with the chloromethyldimethylsilylated derivative the reverse is true (see Table III).

TABLE III

RETENTION BEHAVIOR OF BILE ACID METHYL ESTER SILYL ETHERS WITH SE-30

Compound	*Relative retention time*[a]		
	TMSi	*CMDMSi*	*Mixed silyls*
Methyl 3α-hydroxycholanate	2.14	4.43	—
Methyl 7α-hydroxycholanate	1.49	2.99	—
Methyl 7β-hydroxycholanate	1.71	3.66	—
Methyl 12α-hydroxycholanate	1.42	2.68	—
Methyl 3α-hydroxy-7-ketocholanate	3.28	6.78	—
Methyl 3α,6α-dihydroxycholanate	2.81	11.2	5.78
Methyl 3α,7α-dihydroxycholanate	2.64	10.4	5.09
Methyl 3α,7β-dihydroxycholanate	3.04	12.5	6.19
Methyl 3α,12α-dihydroxycholanate	2.25	8.40	4.07, 4.64
Methyl 3α,7α,12α-trihydroxycholanate	2.70	20.8	5.12, 5.61; 9.46, 10.5

[a] Relative to cholestane, 1.00 (absolute retention time 6.8 min). Column conditions given in Experimental section.

Although the epimeric compounds methyl 7α and 7β-hydroxycholanates, and methyl 3α,7α and 3α,7β-dihydroxycholanates, possess very similar volatilities, these isomers are well separated as the diTMSi and diCMDMSi ethers (see Table III). Improvement in the separation of epimeric hydroxysteroids by derivative formation is a not uncommon observation[7,18]. To a first approximation the greater the steric requirement of the substituent group the greater the separation, with the equatorial isomer possessing the longer retention time; both generalizations are true in the present example.

A comparison of the chromatographic behavior seen for the diTMSi and diCMDMSi ethers discloses that the latter possess retention times approximately four times greater than those for the former, confirming the earlier observation that each change from TMSi to CMDMSi leads to a doubling of retention time. It is even possible to predict the retention behavior of a diCMDMSi ether from the retention time of the corresponding diTMSi ether and the retention factors for the two positions of substitution. For example, the calculated retention time of diCMDMSi ether of methyl deoxycholate is 2.25 × 2.07 × 1.89, or 8.81, in satisfactory agreement with the observed value of 8.40.

Since the dihydroxy-substituted bile acids form disilyl ethers, one would expect the preparation of mixed disilyl ether derivatives (one TMSi group, one CMDMSi group) to be possible[4]. When a sample of methyl hyodeoxycholate (3α,6α-diol) is allowed to react with a mixture of reagents capable of forming both types of silyl ethers chromatography of the reaction product gives three peaks of theoretical shape (see Fig. 8), the most rapidly and most slowly eluted corresponding to the

diTMSi and diCMDMSi ethers, respectively. The retention time of the intermediate component is approximately twice that of the early peak, and half that of the late peak, and on this evidence is judged to be the mixed disilyl ether (confirmed by combined GLC–mass spectrometry). Methyl ursodeoxycholate (3α,7β-diol) and methyl chenodeoxycholate (3α,7α-diol) behave in a similar fashion. Methyl deoxycholate, however, exhibits two peaks of approximately equal size eluted between the diTMSi and diCMDMSi ethers (see Fig. 9). The two silyl ether groups in methyl deoxycholate (or in any of the diols) do not occupy equivalent positions and thus formation

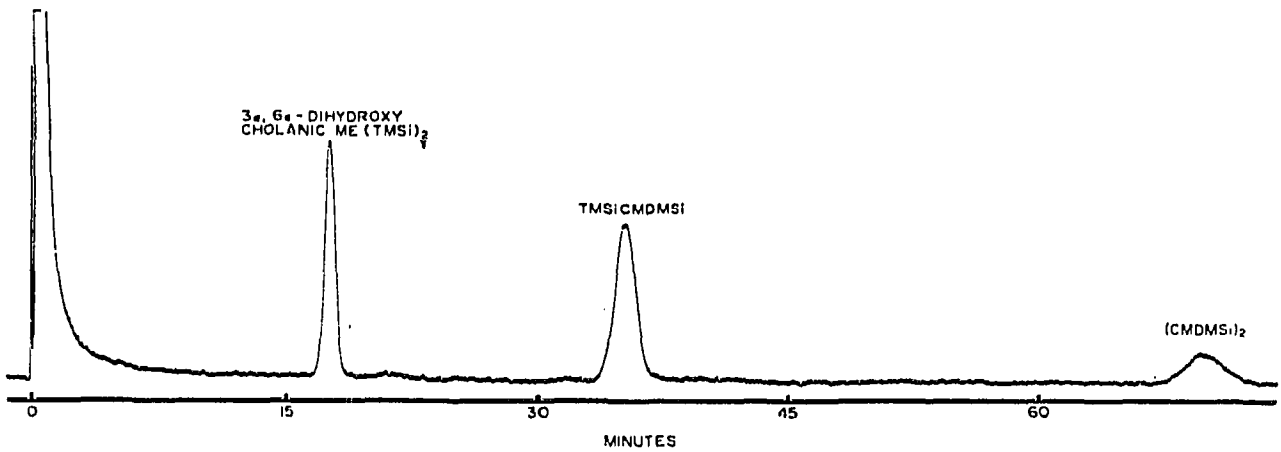

Fig. 8. Gas–liquid chromatographic separation of the mixture of products obtained when a sample of methyl hyodeoxycholate is treated with a combination of the four silylation reagents listed in the Experimental section. The derivatives are the di(trimethylsilyl) ether [3α,6α-dihydroxycholanic Me $(TMSi)_2$], the trimethylsilyl chloromethyldimethylsilyl mixed ether (TMSi CMDMSi) and the di(chloromethyldimethylsilyl) ether [$(CMDMSi)_2$]. Column conditions are given in the Experimental section.

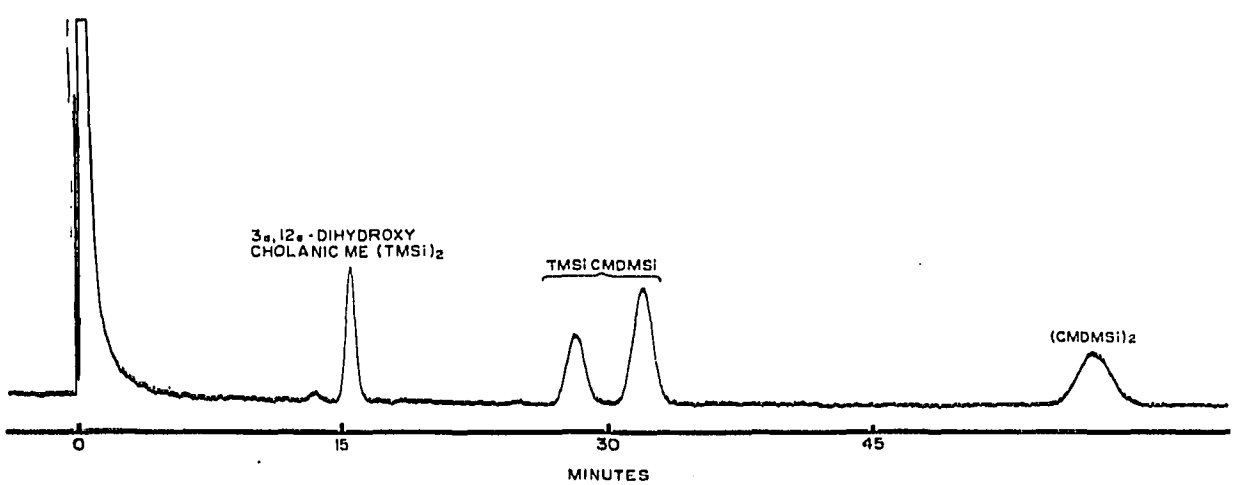

Fig. 9. Gas–liquid chromatographic separation of the mixture of products obtained when a sample of methyl deoxycholate is treated and analyzed as described in Fig. 8. The derivatives are the di(trimethylsilyl) ether [3α,12α-dihydroxycholanic Me $(TMSi)_2$], the trimethylsilyl chloromethyldimethylsilyl mixed ethers (TMSi CMDMSi) and the di(chloromethyldimethylsilyl) ether [$(CMDMSi)_2$].

of two isomeric mixed silyl ethers can occur. If formed these two isomers will, of course, not necessarily possess sufficient differences in volatility to be resolvable; peak broadening or the observation of a shoulder may be seen*. Through the use of the retention factors discussed earlier one can attempt to predict the retention behavior of the two theoretically possible isomers. The calculated relative retention time for 3-TMSi, 12-CMDMSi is 2.25 (the relative retention time of the diTMSi ether) × 1.89, or 4.25, whereas for the 3-CMDMSi, 12-TMSi it is 2.25 × 2.07, or 4.66. Correspondence between the calculated and observed values is excellent, and suggests that it is possible to assign structures to the two components. Characterization of these two compounds as mixed silyl ether derivatives was confirmed by combined GLC–mass spectrometry; further, the most rapidly and most slowly eluted components of the four observed for this reaction product were shown by this technique to be the diTMSi and diCMDMSi ether derivatives, respectively.

It is evident that the capability exists to "count" the number of hydroxyl groups in a bile acid and, presumably, other types of hydroxy-substituted compounds (the statement made on p. 14 concerning methanesulfonate formation holds equally well here for silyl ethers) with SE-30 by comparing the retention behavior of the trimethylsilylated and chloromethyldimethylsilylated derivatives, since each transformation of a TMSi group to a CMDMSi group results in an approximate doubling of retention time. Retention data for the various silyl ether derivatives of methyl cholate (3α,7α,12α-triol) are presented in Table III. As expected, the fully chloromethyldimethylsilylated derivative possesses a retention time roughly eight times that of methyl cholate tri-TMSi ether. Further, two pairs of peaks of intermediate retention times result from a mixed silylation reaction, one pair exhibiting retention times about twice that of the tri-TMSi ether and the other about four times the tri-TMSi value (or approximately one-half that of the tri-CMDMSi ether). Although six mixed silyl ethers are theoretically possible, only four peaks are observed. The first pair of peaks correspond to diTMSi monoCMDMSi derivatives, whereas the later pair is the pattern due to the monoTMSi diCMDMSi isomers.

The various techniques described in this paper—characterization and identification by GLC behavior through the use of non-classical derivatives and approaches such as mixed silyl ether patterns, olefin patterns from reactive derivatives, and combined GLC-mass spectrometry—are examples of methodology belonging to a new and developing field which HORNING[20] has christened "gas phase analytical chemistry". It is to be hoped that such techniques will find application in biochemistry, natural products chemistry, and related fields[20,22–24].

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* The broad, non-theoretical shape of the pregnanediol TMSi CMDMSi mixed silyl ether peak seen in Fig. 1 of Ref. 4 is a consequence of such isomerism.

SUMMARY

Methanesulfonates of bile acid methyl esters undergo an elimination reaction when applied to a gas–liquid chromatographic column. Although such behavior may appear to limit the usefulness of these derivatives, the characteristic olefin peak patterns observed reflect the nature of the hydroxyl groups of the parent compound. The values obtained when the retention times of the olefins are divided by the retention time of the parent bile acid methyl ester indicate the number of hydroxyl groups originally present in the molecule. It is also possible to deduce the number of hydroxyl groups by comparing the retention times of the TMSi and CMDMSi ethers observed with the stationary phase SE-30. Further, the peak patterns obtained when bile acid methyl esters are allowed to react with mixtures of reagents capable of forming both types of silyl ethers permit multiple characterization in a single chromatographic analysis.

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