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A New Method of Bile Acid Silylation for Their GLC–MS Analysis

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The trimethylsilyl (TMS) derivatives of a mixture of nine bile acids (six free and three conjugated), namely lithocholic, deoxycholic, chenocholic, cholic, hyodeoxycholic, ursodeoxycholic, glycodeoxycholic, glycocholic and glycochenodeoxycholic acids, have been prepared by a new, simple, efficient derivatization procedure, based on the use of a mixture of *N*-methyl-*N*-trimethylsilyl-1,1,1-trifluoroacetamide and 1-(trimethylsilyl)imidazole, as the silylating agent. The above-mentioned bile acids were completely trimethylsilylated on all hydroxyl and carboxyl groups whereas carbonyl and amino groups remained untouched.

Keywords: bile acids; conjugated bile acids; trimethylsilyl (TMS) derivatives; GLC–MS analysis

INTRODUCTION

Bile acids (CA) are a complex mixture of acidic steroids (either free or conjugated with the amino-acids glycine and taurine) which are produced in the liver and play a vital role in its function. Therefore, their determination in biological fluids is of great clinical importance.[1–4]

Due to the very low quantities of available CA, combined gas chromatography/mass spectrometry (GLC–MS) is a powerful technique for their analysis, providing high sensitivity, selectivity and accuracy.[5,6]

GLC–MS analysis of CA requires their extraction, hydrolysis and derivatization. In routine clinical analysis, minimization of the duration of the whole lengthy procedure of bile analysis is one of the main tasks.

CA are too polar to be analyzed by gas chromatography. Therefore, it becomes necessary to derivatize them. Alkylsilyl and especially trimethylsilyl derivatives are considered as the most suitable derivatives to increase the compounds' volatility. Common derivatives used in CA analysis are methyl ester trimethylsilyl ethers,[7] methyl ester acetates or methyl ethers,[8–12], methyl or ethyl ester dimethylethylsilyl ethers,[13,14] isobutyl ester trimethylsilyl ethers[15] (derivatization of carboxyl or hydroxyl groups, respectively), perhexafluorobutyrate[16] and pertrimethylsilyl[15,17,18] derivatives. However, all these derivatization procedures suffer from prolonged reaction times and complex reaction conditions.

We report here on a new and efficient derivatization procedure which acts selectively for the trimethylsilylation of all hydroxyl and carboxyl groups of bile acids, while amino and carbonyl groups remain unaffected, using a mixture of *N*-methyl-*N*-trimethylsilyl-1,1,1-trifluoroacetamide (MSTFA) and 1-(trimethylsilyl)imidazole (TMSIM) as the silylating agent and also the solvent, leading to sharp peaks formation in GLC analysis.

EXPERIMENTAL

Materials

Bile acids were obtained from Sigma and Calbiochem, USA. MSTFA and TMSIM were purchased from Macherey–Nagel, Germany.

Derivatization

A portion (50 μl) of a 1000 ppm stock solution of each bile acid in methanol was added to a screw-capped vial and dried under a stream of nitrogen. Then 100 μl of a solution of 2%

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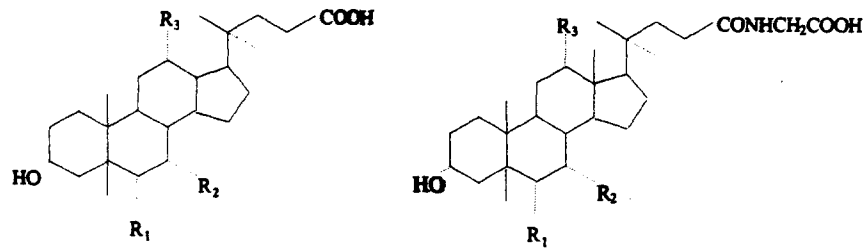

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No.	Bile acid	Abbreviation	Type	R_1	R_2	R_3
1	Lithocholic	LCA	I	α-H	α-H	α-H
2	Deoxycholic	DCA	I	α-H	α-H	α-OH
3	Chenodeoxycholic	CDCA	I	α-H	α-OH	α-H
4	Cholic	CA	I	α-H	α-OH	α-OH
5	Hyodeoxycholic	HDCA	I	α-OH	α-H	α-H
6	Ursodeoxycholic	UDCA	I	β-OH	α-H	α-H
7	Glycodeoxycholic	GDCA	II	α-H	α-H	α-OH
8	Glycocholic	GCA	II	α-H	α-OH	α-OH
9	Glycochenodeoxycholic	GCDCA	II	α-H	α-OH	α-H

Figure 1 Structures of the bile acids **1–9**.

TMSIM in MSTFA was added to the dried analyte and the mixture was heated at 80 °C for 30 min. Finally 1 μl of the derivatized mixture was injected into the gas chromatograph/mass spectrometer (GC–MS) without any prior work-up. Non-derivatized bile acids are not affected by the procedure described, due to their low volatility.

Gas chromatography/mass spectrometry

GLC–MS analysis was carried out on a Hewlett–Packard model HP 5970 gas chromatograph coupled with an MSD mass spectrometer. The GC system was fitted with a 25 m × 0.2 mm i.d. Ultra-1 capillary column with a dimethyl poly-

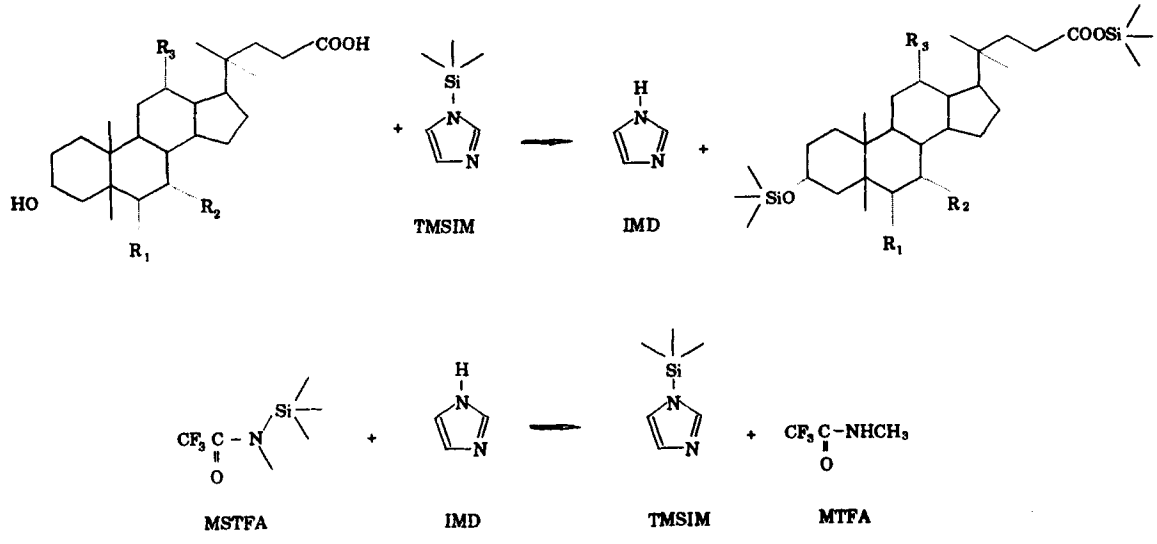

Figure 2 Proposed mechanism for the silylation reaction of **1–9**.

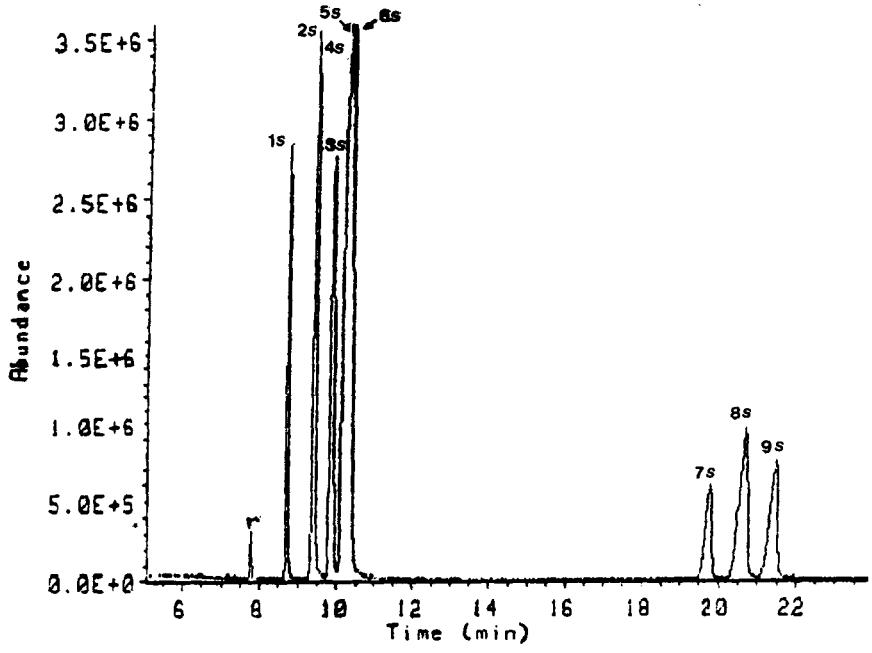

Figure 3 Total Ion Chromatogram (TIC) of per-TMS derivatives of bile acids **1–9**, viz. **r**, cholesterol, **1s**, LCA-TMS; **2s**, DCA-2-TMS; **3s**, CDCA-2-TMS; **4s**, CA-3-TMS; **5s**, HDCA-2-TMS, **6s**, UDCA-2-TMS; **7s**, GDCA-2-TMS; **8s**, GDCA-2-TMS; **9s**, GCA-3-TMS.

siloxane coating and 0.11 μm film thickness, and the end of the column was introduced directly into the mass spectrometer analyzer chamber.

The system was operated under the following conditions: helium pressure 5 psi; injector temperature 250 °C; and GC column temperature 180 °C for 2 min, increased at 10 °C min^{-1} from 180 to 300 °C, held at 300 °C for 1 min. The mass spectrometer was set to scan from 50 to 800 amu per nominal second with an ionizing voltage of 70 eV.

RESULTS AND DISCUSSION

The proposed method was applied to a synthetic mixture of nine CA (six free and three conjugated), the structural features of which are shown in Fig, 1.

Trimethylsilylation of hydroxyl and carboxyl groups of CA was rapidly and simply achieved by the proposed method in one step, as it is desirable in routine analysis. The presence of free and glycine-conjugated CA in the synthetic mixture showed that the method works equally well for both kinds of CA, thus making hydrolysis of conjugated acids unnecessary.

MSTFA and TMSIM are two of the most powerful silylating agents; the silylation mixture used in the proposed method is already used routinely for the derivatization of hindered hydroxyl groups in sugars and steroids, with a catalyst.[19,20] A particular advantage of MSTFA is that the reagent itself and its by-product, *N*-methyl-1,1,1-trifluoroacetamide (MTFA), are highly volatile[22] and are eluted in the solvent range in their GLC analysis, thus allowing direct injection of the derivatized mixture in GC. Furthermore, due to its polarity it can dissolve even highly polar substances[22] so it can be used without any other solvent. MSTFA has already been used for the selective protection of the hydroxyl groups in hydroxyquinones.[21]

The low concentration of TMSIM in the silylating mixture used protected the amino groups of CA from silylation, due to its selective silylation of hydroxyl and carboxyl groups but not basic amino ones,[23,24] thus making the silylating mixture very useful for routine analysis of both free and conjugated CA, simultaneously. In contrast, MSTFA silylates all kinds of functional groups with active proton.[22]

Table 1 GLC–MS data of TMS derivatives of bile acids **1–9**

No.[a]	Per-TMS derivative	$M^{+\cdot}$ (*m/z*, RRT[b] rel.int.,[c] %)	Main ions[d] (*m/z*, rel. int.,[c] %)										
			$[M-15]^+$	$[M-90]^{+\cdot}$	$[M-2\times90]^{+\cdot}$	$[M-3\times90]^{+\cdot}$	$[M-90-173]^+$	$[M-2\times90-173]^+$	$[M-3\times90-173]^+$	$[M-90-230]^+$	$[M-2\times90-230]^+$	$[M-3\times90-230]^+$
1s	LCA-TMS	1.122 520 (7)	505 (11)	430 (41)	—	—	257 (41)	—	—	—	—	—
2s	DCA-2-TMS	1.213 608 (—)	593 (15)	518 (5)	428 (25)	—	345 (9)	255 (94)	—	—	—	—
3s	CDCA-2-TMS	1.264 608 (—)	593 (—)	518 (2)	428 (64)	—	345 (5)	255 (34)	—	—	—	—
4s	CA-3-TMS	1.316 696 (—)	682[e] (8)	606 (2)	516 (22)	426 (19)	—	343 (25)	253 (28)	—	—	—
5s	HDCA-2-TMS	1.329 608 (—)	593 (3)	518 (4)	428 (18)	—	345 (10)	255 (51)	—	—	—	—
6s	UDCA-2-TMS	1.342 608 (—)	593 (7)	518 (33)	428 (17)	—	345 (2)	255 (25)	—	—	—	—
7s	GDCA-2-TMS	2.542 665 (—)	651[e] (9)	575 (10)	485 (7)	—	—	—	—	345 (4)	255 (98)	—
8s	GCDCA-2-TMS	2.645 665 (—)	651[e] (4)	575 (4)	485 (10)	—	—	—	—	345 (6)	255 (45)	—
9s	GCA-3-TMS	2.761 753 (—)	739[e] (7)	663 (—)	573 (13)	483 (10)	—	—	—	—	343 (42)	253 (80)

[a] The *silyl ether corresponding to substrate n is denoted as ns*

[b] Relative to the retention time of cholesterol

[c] Relative to the base peak at *m/z* 73 (except for GCDA where the base peak is attributed to the ion at *m/z* 189).

[d] $[M-15]^+ \equiv [M-Me]^+$, $[M-90]^{+\cdot} \equiv [M - Me_3SiOH]^{+\cdot}$, $[M-90-173]^+ \equiv [M-Me_3SiOH-(CH_2)_4COOSiMe_3]^+$. $[M-90-230]^+ \equiv [M-Me_3SiOH-(CH_2)_4CONHCH_2COOSiMe_3]^+$.

[e] $[M-Me+1]^+$.

TMSIM is more active than MSTFA for the silylation of hydroxyl groups.[25] Hence, TMSIM is likely to be responsible for the silylation of the hydroxyl and carboxyl groups of **1s–9s**. MSTFA interacts with the imidazole by-product to regenerate consumed TMSIM. Therefore, a catalytic amount of TMSIM in the silylation mixture is necessary for the initiation of the silylation reaction (Fig, 2).

As can be seen in the Total Ion Chromatogram (TIC) of **1s–9s** (Fig. 3), all free CA (**1s–6s**) elute first, giving excellent sharp peaks, while glycine-conjugated ones (**7s–9s**) follow later, giving slightly broader peaks, thus raising the detection limits.

Table 1 shows the retention times relative to cholesterol (RRT) of **1s–9s** and the characteristic fragment ions in their mass specta. RRT of **1s–6s** are quite different from those of TMS derivatives of conjugated CA (**7s–9s**), resulting in their elution as two different groups of peaks in the GLC analysis.

Mass spectra of **1s–9s** reveal characteristic fragmentation patterns, which are dominated by two main expulsions, that of all trimethylsilanol molecules [Me_3SiOH] and that of the side chain ([$C_8H_{17}O_2Si$] or [$C_{10}H_{20}O_3NSi$]) (Table 1).

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