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Note

Flash heater derivatization of unconjugated bile acids using trimethylanilinium hydroxide

Rapid analysis by capillary gas—liquid chromatography*

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Bile acid methyl ester methyl ethers (BAMME) are stable derivatives which are highly suited to capillary gas—liquid chromatography [1]. In a previous study we described their preparation and characterization, using the reagents sodium methylsulfinylmethanide and methyl iodide [1]. While this procedure has the advantage of quantitatively forming BAMME derivatives in a single step, it is relatively slow, taking several hours for reaction and work-up.

The quaternary base, trimethylanilinium hydroxide (TMAH), has been used in the preparation of alkyl esters of bile acids in a room temperature reaction with alkyl halides [2]. It has also been used for the derivatization of carboxylic acids and amino groups in the vapour phase by pyrolytic methylation. For instance, N,N-dimethyl derivatives of phenobarbitone can be formed in the hot injector port of a gas chromatograph [3]. Other quaternary bases such as tri-

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methylammonium hydroxide and (*m*-trifluoromethylphenyl)trimethylammonium hydroxide have been used to form methyl esters of fatty acids in a similar way [3,4].

In this study we report an on-column derivatization procedure for the quantitative analysis of unconjugated bile acids which uses TMAH for the pyrolytic methylation of both the hydroxyl and carboxylic acid moieties in the vapour phase in the hot injector. Advantage was taken of the splitless injection technique for capillary gas—liquid chromatography which conveniently causes the condensation of the derivatives into a sharply defined starting zone prior to the start of the temperature program sequence.

MATERIALS AND METHODS

Materials

The sources of the bile acids used in this investigation are given in Table I. Each gave a single spot when 30 μg was analyzed by thin-layer chromatography (TLC). 23-Norcholic acid (nor-24-3α-7α,12α-trihydroxy-5β-cholan-23-oic acid), however, gave three spots and was purified by preparative TLC on silica gel G (Rediplates, Fisher Chemical, Norcross, GA, U.S.A.) using hexane—ethyl acetate—acetic acid—propan-2-ol (2:1:1:0.1, v/v) as the mobile phase. 5α-Cholestane and *n*-alkane standards were purchased from Applied Science Labs. (College Park, PA, U.S.A.).

Chromatography grade methanol and dimethylformamide were obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Dimethylsulfoxide was distilled over calcium hydride. Methyl iodide and N,N-dimethylaniline were obtained from Fisher Chemical.

Commercial TMAH was purchased from Applied Science Labs. and Pierce (Rockford, IL, U.S.A.). TMAH was also prepared by first reacting methyl iodide and dimethylaniline to form trimethylanilinium iodide. The latter was recrystallised four times from methanol and then was converted to the hydroxide by passing a solution of the compound in dry methanol over a column of AG-1 ion-exchange resin (OH^- form), previously thoroughly equilibrated with methanol. The final concentration was adjusted to 2 *M* by the addition of anhydrous methanol.

Gas chromatography

A Hewlett-Packard 5880 gas chromatograph, equipped with a flame ionization detector and capillary column splitless injector was used. The glass capillary column, 10 m × 0.25 mm, was wall-coated with the liquid phase Poly S-179 (Applied Science Labs.). Preliminary studies were also carried out with the phase SP-2100 coated onto a 12 m× 0.2 mm fused silica capillary column (Hewlett-Packard). The injector port temperature was varied between 200—350°C and the samples were injected at an oven temperature of 60°C. The inlet purge flow of 60 ml/min was interrupted for 18 sec after the moment of injection. The oven temperature was programmed at 30°C/min to 220°C and then at 3.5°C/min to 260°C. The column was then heated to 300°C for 5 min. Hydrogen was used as the carrier gas at an inlet pressure of 15 p.s.i. which gave a flow-rate of 10 ml/min through the column at 60°C and 3 ml/min at 260°C.

TABLE I

KOVÁTS' RETENTION INDICES (KRI) OF BILE ACID METHYL ESTER METHYL ETHER DERIVATIVES

Bile acid*	Source**	KRI
Dinor-5αB-3β-ol	2	36.65
Dinor-B^{5}-3β-ol	2	36.63
Nor-5βB-3α,12α-diol	7	38.26
Nor-5βB-3α,7α,12α,triol	2	39.94
5αB-3β-ol	2	40.19
5βB	2	35.28
5βB-3α-ol	1	39.37
5βB$^{9-11}$-3α-ol	3	38.94
5βB^{11}-3α-ol	3	39.26
B^{5}-3β-ol	2	40.13
5βB$^{9-11}$-3β-ol	3	38.93
5βB^{11}-3β-ol	3	38.43
5βB-3β-ol	3	38.39
5βB-7α-ol	4	36.60
5βB-12α-ol	4	35.93
5βB^{3}-12α-ol	3	36.44
5βB-3α,6α-diol	1	41.94
5βB-3α,7α-diol	1	40.73
5βB-3α,7β-diol	6	41.36
5βB-3β,7α-diol	3	39.49
5βB-3β,7α-diol	3	40.59
5βB-3α,12α-diol	1	39.76
5βB$^{8-14}$-3α,12α-diol	3	40.04
5βB-3α,12β-diol	3	39.71
5βB-3β,12α-diol	3	38.97
5βB-3β,12β-diol	3	38.85
5βB-3α,6α,7α-triol	2	41.75
5βB-3α,6β,7β-triol	2	40.89
5βB-3α,7α,12α-triol	1	41.44
5βB-3α,7α,12β-triol	3	40.91
5βB-3β,7α,12α-triol	5	40.40
5βB-3β,7α,12β-triol	3	39.75

*Abbreviated nomenclature: B = unsubstituted cholan-24-oic acid; the 5(αβ) prefix indicates proton orientation at the C_5 position; the superscript gives the position of a double bond.

**1 = Sigma, St. Louis, MO, U.S.A.
2 = Research Plus Steroids Laboratory, Denville, NJ, U.S.A.
3 = F. Chang, Department of Biochemistry, University of South Alabama, Mobile, AL, U.S.A.
4 = W.H. Elliott, Department of Biochemistry, St. Louis University Medical School, St. Louis, MO, U.S.A.
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The flame ionization detector temperature was 300°C. Nitrogen at 30 ml/min was the make-up gas. Calculation of peak areas was performed by the data system which was part of the HP-5880 gas chromatograph.

Retention times of BAMME derivatives were expressed as Kováts' retention indices using *n*-alkanes from C_{32} to C_{44}.

Choice of solvent and injector port temperature

The following solvents for the bile acids were used: methanol, ethyl acetate, dimethylformamide and dimethylsulfoxide. Since the trihydroxy bile acids (with four groups to be methylated) were thought to be the most difficult to derivatize, optimization studies were done with cholic acid. The area of the cholic acid BAMME peak was expressed as a ratio to 5α-cholestane (20 ng injected as internal standard). The injector port temperature was varied from 200—375°C.

Injection technique

Initially, 1 μl of TMAH was withdrawn into a 10-μl glass syringe. A 0.2—1.0 μl aliquot of the sample to be analyzed was then taken up followed by a further 1.0 μl of TMAH. This was performed immediately prior to injection.

RESULTS AND DISCUSSION

The use of different solvents for the bile acids had only a minor effect on the chromatograms. Methanol was adopted for the studies since it gave the smallest solvent front when the Poly S-179 coated capillary column was used, and was a good solvent for TMAH. It is probable that any effects the other solvents might have had were overcome by the preponderance of methanol in the TMAH reagent.

The injector port temperature was of far greater importance. Below 200°C no derivatives, partial or permethylated, were detected. Whilst the mono- and dihydroxy bile acids were converted to the permethylated forms at 250°C, maximum conversion of cholic acid to its permethylated form did not occur until 350°C, similar to results obtained for the methylation of fatty acids [5]. The small amount of partially methylated material was a problem when using the SP-2100 fused silica capillary column, since the very low interaction between the column and the free hydroxyl groups did not cause much change in retention volume. However, on Poly S-179 the difference between the partially methylated and permethylated forms was substantial and thus any incompletely methylated bile acid in no way interfered with the analysis. Although a sandwich injection technique was adopted, equally good data were obtained when injecting with the TMAH in front of or behind the sample. Premixing of TMAH and the sample resulted in no derivative peaks at all.

Application of this method to 32 different bile acid standards produced a single peak for each bile acid in the region of the chromatogram assigned to BAMME derivatives prepared as described previously [1]. Most could be resolved by the temperature program, 60 to 220°C at 30°C/min, 220 to 260°C at 3.5°C/min (Table I). The use of hydrogen as carrier gas enabled rapid analysis, the cycle time for each analysis being 18—20 min.

The method was reproducible and quantitative. The coefficient of variation for a 100-pmol sample injection ranged from 2.5% for chenodeoxycholate to 8.7% for cholate. Compared to authentic BAMME derivatives, derivatization was complete for the monohydroxy bile acids, 70% for dihydroxy bile acids and 50% for trihydroxy bile acids.

It has been claimed that TMAH is unstable. This has not been our experience. No attempt was made to keep the reagent cool, or to rigorously maintain anhydrous conditions. The original batch of reagent has been used for more than 15 months. Unlike commercial TMAH, no smell of dimethylaniline was detected in our reagent.

The use of commercial TMAH resulted in the appearance of contamination peaks in the region where BAMME derivatives were eluted. By contrast, TMAH synthesized in this laboratory was free of this contamination. It should be stressed that the contaminating peaks represent an extremely small percentage of the TMAH reagent and would not interfere necessarily with assays of substances of differing elution volumes to BAMME derivatives.

From comparison of the Kováts' retention indices for combination of bile acid derivatives, the contributions of the functional groups were determined (Table II). It can be seen that the greatest effects were caused by introduction of a hydroxyl group at the 3-position, being greater at 3α (4.02) than at 3β (3.01). Hydroxylation at the 12-position caused quite small changes, again being greater at 12α (0.67) than at 12β (0.31). At the 7-position, the β-orientated group (2.10) had a greater effect than the α-group (1.28). Hydroxylation at the 6-position had a more variable effect; in hyodeoxycholate (3α,6α-dihydroxy-5β-cholan-24-oate) the 6α-group caused a large change (2.56) whereas in hyocholate (3α,6α,7α-trihydroxy-5β-cholan-24-oate) the change was small (1.02). The latter could be due to proximity of the 7α-hydroxy group, thus reducing the effect. This was more so in the case of β-muricholate (3α,6β,7β-trihydroxy-5β-cholan-24-oate) which eluted more rapidly than hyodeoxycholate

TABLE II

EFFECTS OF SUBSTITUENTS ON KOVÁTS' RETENTION INDICES FOR BILE ACID DERIVATIVES

Substituent	Change (mean ± S.E.M., No. of comparisons)
3α-ol	4.02 ± 0.10 (3)
3β-ol	3.01 ± 0.07 (3)
6α-ol	1.02, 2.56
6β-ol	−0.47
7α-ol	1.28 ± 0.09 (7)
7β-ol	1.99, 2.20
12α-ol	0.67 ± 0.11 (4)
12β-ol	0.31 ± 0.06 (4)
Nor	−1.50, −1.50
Dinor	−3.50, −3.54
5α/5β	1.80
B^{3*}	0.51
B^{5}	1.74
B^{9-11}	−0.43, 0.54
B^{11}	−0.12, 0.05

*B^{n} = double bond between carbon atoms n and $n+1$, or as indicated.

or ursodeoxycholate, the 6β-group contributing a small negative effect (0.47). Removal of one or two methylene groups from the side chain caused consistent reduction in the retention index. Removal of two hydrogen atoms to form double bonds had a small and variable effect, the largest being due to the 5,6 double bond at the A/B ring junction. This double bond drastically alters the molecular shape by swinging the A-ring into the plane of the B-, C- and D-rings.

The application of TMAH for the pyrolytic formation of BAMME derivatives provides a simple, rapid and quantitative procedure for the analysis of unconjugated bile acids.

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