Gas–liquid chromatography of hyocholic acid

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SUMMARY Partial derivatives of hyocholic acid were formed under the usual conditions for trifluoroacetylation of bile acids (trifluoroacetic anhydride, 35°C for 20 min). Complete trifluoroacetylation of hyocholic acid was achieved at 80°C for 30 min, or at 60°C for 30 min when a trace of pyridine was added to the reaction mixture.

SUPPLEMENTARY KEY WORDS bile acids · methyl esters · trifluoroacetylation · partial derivatives · steric hindrance · pyridine

Several derivatives have been suggested for use in gas–liquid chromatographic analysis of bile acids. Methyl esters of bile acids may be separated by GLC on a 3% polysulfone liquid phase (1). However, in our hands considerable loss of the sterol esters was found at the necessarily high column temperatures (315°C). Hyocholic acid methyl ester (methyl 3α,6α,7α,trihydroxy 5β-cholanic acid) was almost completely degraded on the column and considerable losses were also found for methyl cholate, with smaller losses for mono- and dihydroxy acids. A marked improvement in quantitation of bile acids has been obtained with trifluoroacetyl and silyl derivatives (2, 3). It has been reported that trifluoroacetyl (TFA) derivatives of most bile acids can be obtained at 35°C for 20 min with trifluoroacetic anhydride (TFAA) (4). When these conditions were employed in our laboratory for analysis of hog bile, which is unusual in its high level of hyocholic acid, an unidentified peak appeared with a retention time longer than that reported for hyocholic acid. A hyocholic acid standard which was trifluoroacetylated under the same conditions yielded two distinct peaks, one corresponding to hyocholic acid and the other to the unidentified peak from hog bile. Thus, it appeared that a partial derivative of hyocholic acid was being formed and stronger reaction conditions would be required for complete trifluoroacetylation. This report describes the optimum reaction conditions for trifluoroacetylation of hyocholic acid.

Methyl esters were made from about 100 μg of hyocholic acid (purity 95%, Mann Research Laboratories Inc., New York) in 5-ml screw-capped tubes with Teflon liners by reaction with diazomethane in alcoholic ether at room temperature. Diazomethane was generated from "Diazald" (Aldrich Chemical Co., Inc., Milwaukee, Wis.) (5). After 15 min the reaction mixture was evaporated under N_2, and 0.5 ml of trifluoroacetic anhydride was added. The tightly sealed tubes were heated for 30 min at the specified temperatures and the excess TFAA was removed with a stream of dry N_2.[1] The residue was dissolved in CS_2 or chloroform and analyzed on an F & M 400 gas chromatograph equipped with a hydrogen flame detector. Peak areas were calculated by disc integration.

The reaction mixture was also applied in chloroform on to a 0.5-mm thickness of Adsorbosil-5 (Applied Science Laboratories Inc., State College, Pa.) on a 20 × 20 cm glass plate. The plate was developed in benzene–acetone 95:5, air dried, and sprayed with a 0.05% solution of pyrene in hexane (6). Two distinct spots with R_F values of 0.5 and 0.65 were detected under long wave UV light which corresponded to the two peaks found by GLC. This eliminated the possibility that the second peak was produced by thermal decomposition of the derivative on the GLC column.

Fig. 1 shows chromatograms of methyl hyocholate treated with TFAA at 35°C for 20 min and 80°C for 30 min. The retention of the two major products of trifluoroacetylation at 35°C relative to a methyl deoxycholate TFA derivative were 1.67 (peak *A*) and 2.43 (peak *B*). Smaller peaks, which appeared between peaks *A* and *B* and following *B*, also were probably various partial derivatives of hyocholic acid, since they were not present at the 80°C reaction temperature. The relative amounts of peaks *A* and *B* formed under various temperatures are given in Fig. 2. Other bile acids tested, such as cholic, deoxycholic, lithocholic, and hyodeoxycholic methyl esters, were completely trifluoroacetylated at 35°C for 20 min and showed no further changes at the higher reaction temperatures.

It has been reported that α-muricholic acid (3α,6β,7α-trihydroxy 5β-cholanic acid), which is found in rat bile, when methylated and trifluoroacetylated, undergoes thermal decomposition on the GLC column and forms a second compound with a very short retention time (7). We also found two peaks when methyl α-muricholic acid was trifluoroacetylated at 35°C for 20 min and analyzed by GLC. (α-Muricholic acid was kindly supplied by Dr. S. L. Hsia, Department of Biochemistry, University of Miami.) At the 80°C-trifluoroacetylation reaction temperature only the peak with the short relative retention time (1.16) was detected. The very short relative retention time could be explained by

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Abbreviations: GLC, gas–liquid chromatography; TFA, trifluoroacetyl; TFAA, trifluoroacetic anhydride.

[1] CAUTION: The tubes are under pressure since the boiling range of TFAA is 40–42°C. All manipulations should be done with rubber gloves in a ventilating hood because TFAA liquid or vapor can cause severe burns.

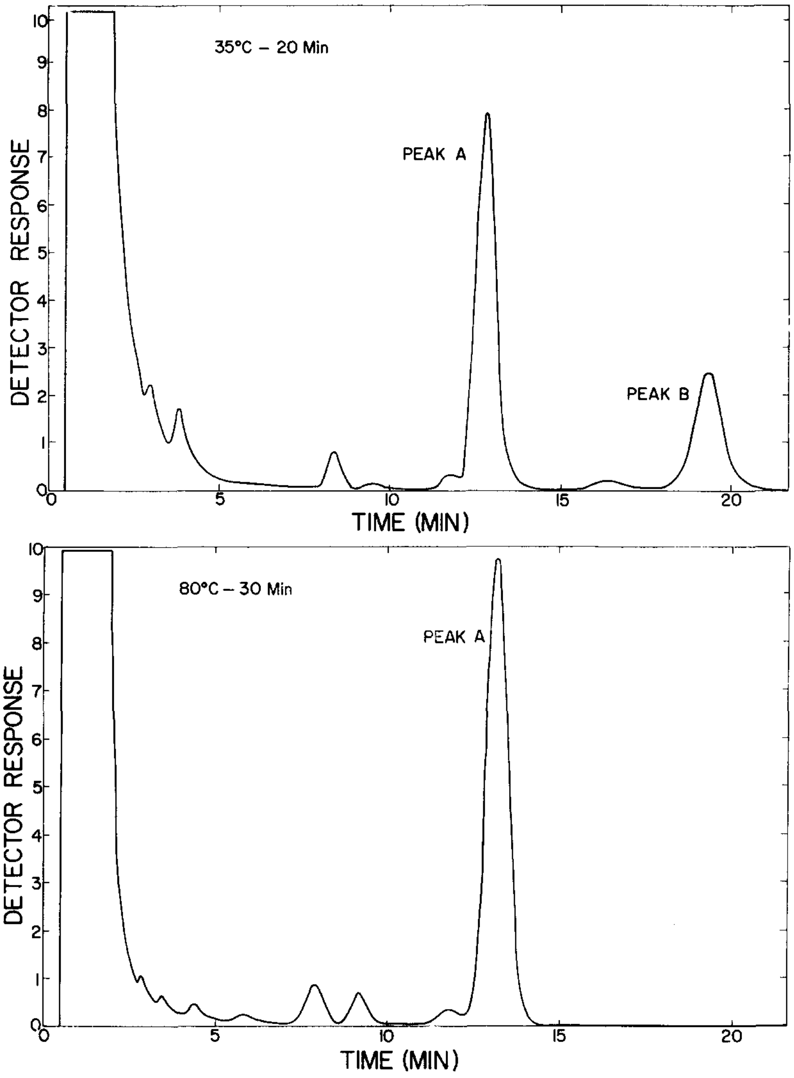

FIG. 1. GLC separation of methyl hyocholate (100 μg), trifluoroacetylated at 35°C for 20 min and at 80°C for 30 min. Conditions: 3% QF-1 on 100–120 mesh Gas-Chrom Q; 6 ft × $^1/_4$ inch O.D. glass column: 230°C isothermal with on-column injection. Helium carrier gas was set at a flow rate of 75 ml/min.

the general characteristic that bile acids with axial groups are usually eluted from the GLC column more readily than bile acids with equatorial groups at the same positions (8). The 6β and 7α hydroxyls of α-muricholic acid are diaxial and would be expected to decrease the relative retention time compared to the other stereoisomers. Dehydration at the 6- or 7-position during trifluoroacetylation and formation of a TFA derivative of the resulting enol is also a possible explanation.

The difficulty in trifluoroacetylation of hyocholic acid relative to other bile acids may be explained by the vicinal interaction of hydroxyl groups at carbons 6 and 7. In hyocholic acid the 3 and 6 hydroxyls are equatorial (relative to rings A and B, respectively) and the 7 hydroxyl is axial (9). Since the less sterically hindered equatorial hydroxyl groups are probably trifluoroacetylated more readily than the axial hydroxyl, it is reasonable to assume that the diequatorial 3α,6α,di-trifluoroacetyl derivative would be formed more readily than either of the other diesters. The partial acetylation of hyocholic acid with acetic anhydride has been explained by the same rationale (10). Certainly, esterification at either the 6 or 7 hydroxyl of hyocholic acid with a bulky substituent such as the TFA group would decrease the reactivity of the adjacent hydroxyl. Examination of molecular models supports this concept.

We also found that pyridine catalyzes trifluoroacetylation as suggested by Kuksis (4). Less than 10 μl of dry pyridine effected nearly complete trifluoroacetylation of methyl hyocholate at 60°C for 30 min. Pyridine also reacted with the anhydride, probably forming a pyridinium salt, so only small amounts of the base were added to prevent injection of the precipitate on the GLC column.

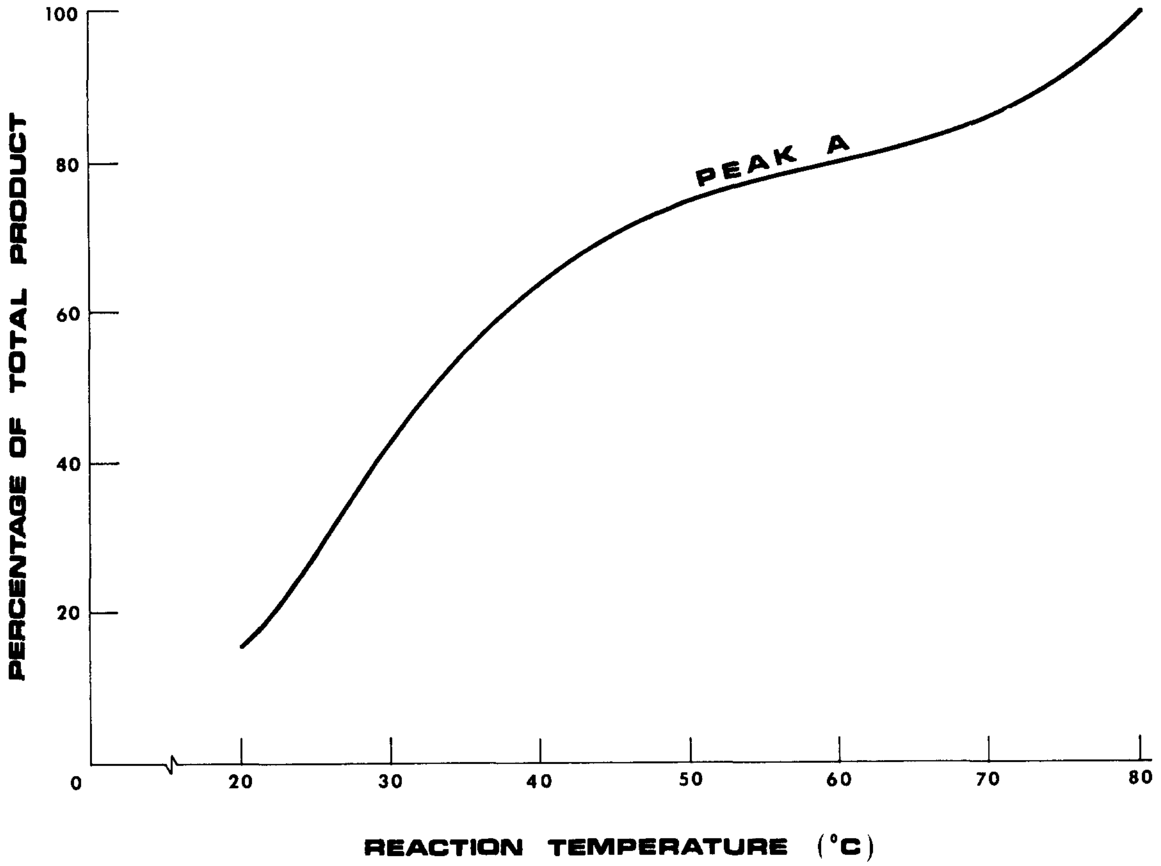

FIG. 2. GLC analysis of TFA derivatives of methyl hyocholate at various reaction temperatures from 20–80°C in 10-degree increments. Peak *A* expressed as percentage of the sum of the areas of peak *A* plus peak *B*. Conditions as in Fig. 1.

No great advantage could be found in using pyridine because complete trifluoroacetylation of methyl hyocholate could be achieved at a slightly higher temperature (80°C for 30 min) while avoiding the troublesome precipitate.

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