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Qualitative Analysis of Bile Acids by Gas Chromatography

Bile Acids and Steroids 124

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A method for the gas chromatographic analysis of bile acid methyl esters before and after the treatment with trifluoroacetic anhydride using the fluoroalkyl silicone QF-1 as stationary phase is described.

In connection with work on fecal bile acids a method was required for the separation of complex bile acid mixtures. A few papers have been published on the gas chromatography of bile acids [1-4] but the stationary phases previously described do not give separation factors large enough to allow the analysis of fecal bile acid mixtures. Recently VandenHeuvel, Haahti and Horning described the use of a fluoroalkyl silicone as stationary phase for the separation of steroids [5]. Dr. Horning has kindly sent us a sample of this phase (QF-1 Dow Corning Corp.) which has now been tested and found to be extremely valuable for separation of bile acid methyl esters and their trifluoroacetates [6].

EXPERIMENTAL

A Pye Argon chromatograph was modified so that a 6 foot × 5 mm U-tube column could be used. Column temperature was kept constant or was linearly programmed with a Model 40 temperature programmer (F & M Scientific Corp.). The standard ionization detector was kept in a separately heated oven at about 240°C. An independently heated metal block was placed as a flash heater around the region where samples were injected. Connections between column and detector were made by stainless steel tubing (1.8 mm o.d.), teflon tubing and through-hole silicone stoppers.

Column packings were prepared essentially as described previously [2] and approximately 0.5 % QF-1 [5] or a mixture of 0.5 % QF-1 and 0.05 % ethylene glycol isophthalate was applied on acid washed, acetone washed and siliconized Gas Chrom P (Applied Science Laboratories). Methyl esters of bile acids were made and injected into the column as described previously. Trifluoroacetates were prepared by heating 0.3 – 1 mg of the methyl ester in 0.2 ml of trifluoroacetic anhydride in a small glass stoppered test tube at 30°C for 15 min. The reagent was then evaporated under a stream of nitrogen and the residue dissolved in 0.1 ml of acetone.

RESULTS AND DISCUSSION

Compounds without trifluoroacetoxy groups were run with the flash heater at 280—300°C; when trifluoroacetoxy compounds were present the flash heater was kept at 250°C. The compounds with hydroxyl and/or keto groups showed no signs of degradation. Slight tailing was observed with di- and trihydroxy compounds but did not disturb qualitative analysis. A separation of disubstituted bile acid methyl esters and methyl cholate is shown in Fig. 1. Trifluoroacetates always gave very sharp and symmetrical peaks. In some cases these compounds showed minor signs of degradation on the column as evidenced by a slight rise in the base line just before the appearance of the peak (one exception noted below). Although quantitative studies have not yet been carried out it was evident that trihydroxy compounds gave significantly lower responses with the detector (see Ref.[7]). The trifluoroacetate of methyl $3\beta,7\alpha,12\alpha$-trihydroxycholanate gave no response or a negative peak unless 1500 V was applied to the detector. Other trifluoroacetates tested always gave positive peaks with the potentials applied (1000—1500 V).

The relative retention times of the compounds tested are summarized in Table 1. As pointed out by VandenHeuvel, Haahti and Horning [5] the ketones are generally retained much longer than the corresponding hydroxy compounds on QF-1. From the results obtained with mono- and disubstituted compounds it is seen that substitution at C12, C7 and C3 increase the retention time in this order. Exceptions are the 3,7-diketo- and 3,12-diketocholanic acid methyl esters that have about the same retention time. Furthermore it is seen that

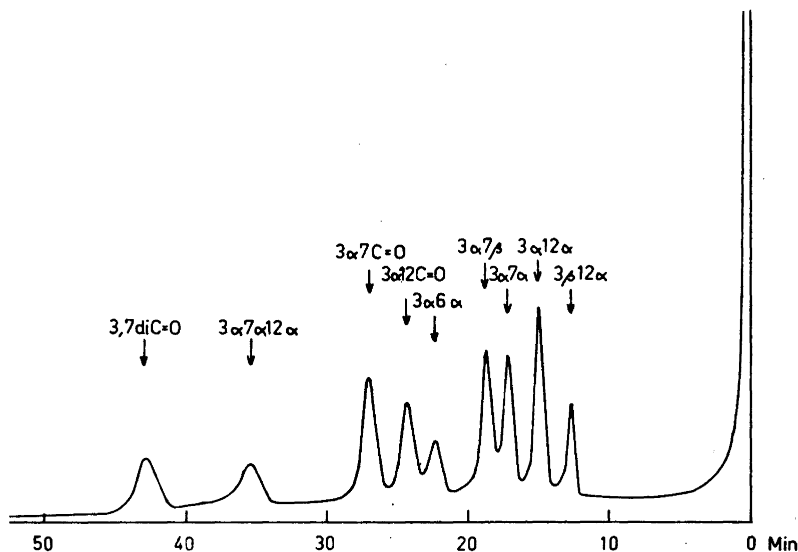

Fig. 1. Separation of some methyl esters of naturally occurring bile acids. Column: 0.5 % QF-1 on Gas-Chrom P. Temperature: 219°C. Argon pressure: 1.0 kg/cm².

Table 1. Retention times relative to methyl deoxycholate of various bile acid methyl esters before and after treatment with trifluoroacetic anhydride. Conditions were as described in Fig. 1.

Substituents	Free hydroxyl(s) (ROH)	Trifluoroacetate (TFA)	Ratio ROH/TFA
12α	0.31	0.22	1.41
12β	0.34	0.25	1.36
7α	0.35	0.26	1.35
7β	0.39	0.32	1.22
3β	0.44	0.41	1.07
3α	0.49	0.42	1.17
12-keto	0.49	—	—
7-keto	0.57	—	—
3-keto	0.95	—	—
3β,12α	0.85	0.58	1.47
3α,12α	1.00	0.67	1.50
3α,12β	1.08	0.78	1.39
3α,7α	1.15	0.85	1.35
3α,7β	1.25	1.00	1.25
3α,6α	1.47	0.98	1.50
3α,12-keto	1.62	1.54	1.05
3-keto,12α	1.83	1.42	1.29
3α,7-keto	1.79	1.68	1.07
3-keto,7α	2.17	1.76	1.23
3,12-diketo	2.86	—	—
3,7-diketo	2.83	—	—
3α,7α,12α	2.33	1.39	1.68
3α,7β,12α	2.44	1.33	1.83
3β,7α,12α	1.86	1.17	1.59
3α,6α,7α	2.61	1.24	2.10
3α,6β,7α	2.39	0.85	2.81
3α,6β,7β	2.27	1.50	1.51
3α,12α,7-keto	3.70	2.54	1.46
3α,7α,12-keto	4.00	2.80	1.43
3-keto,7α,12α	4.79	2.79	1.72
3α,7,12-diketo	4.48	4.30	1.04
3,7,12-triketo	6.33	—	—

an equatorial hydroxyl group gives a longer retention time than an axial one at the same carbon atom. Similar but not so regular effects can also be seen with the trisubstituted compounds studied.

Trifluoroacetylation generally decreases the retention times of compounds containing hydroxyl groups. Trifluoroacetylation of a compound having only one hydroxyl group at C3, changes the retention time but slightly. The retention times of C7 and C12 monohydroxy and di- and trihydroxy compounds decrease markedly after treatment with trifluoroacetic anhydride. There is a tendency for this decrease to be more pronounced with hydroxyl groups at C12 than at C7. The change of retention time on trifluoroacetylation of trihydroxy compounds is of particular value since these compounds show only small differences in retention time. An exceptionally short retention time of methyl 3α,6β,7α-trihydroxycholanate is observed after treatment with trifluoroacetic

anhydride. The same retention time is obtained with the trifluoroacetate of $3\alpha,7\alpha$-dihydroxycholanic acid methyl ester. This might possibly be explained by an elimination of the 6β-trifluoroacetoxy group in the flash heater.

When analyzing unknown samples of bile acid methyl esters we have found it very useful to analyze the sample before and after treatment with trifluoroacetic anhydride. When using QF-1 as stationary phase this gives much information on the nature of the bile acid. If the trifluoroacetylation is carried out at room temperature it is also possible to follow the course of the reaction and hydroxyl groups that are difficult to acylate can thus be detected. Certain hydroxyls (*e.g.*, 12α) react so slowly that a partial acylation can be effected.

For special purposes we have found a mixture of 0.5 % QF-1 and 0.05 % polyethylene glycol isophthalate useful. This stationary phase gives as good peaks as QF-1 alone. The differences in retention times between hydroxyl groups and keto groups are diminished and the decrease in retention time after treatment with trifluoroacetic anhydride becomes more pronounced. In some cases the separation factor between two compounds is increased and it is possible for example to separate partially the methyl esters of 12β-hydroxy- and 7α-hydroxycholanic acids which is not possible with QF-1 alone.

An increased resolution can also be obtained by temperature programming [8]. The flash heater and the detector are kept at the constant temperatures already described. The column temperature at which the analysis is started will depend to some extent on the type of bile acids to be separated. It was found suitable to increase the temperature by 1—2°C per minute.

The method described in this paper has been of great value for the analysis of fecal bile acids as will be described in following papers. It has made possible the isolation of a hitherto unknown naturally occurring bile acid to which the structure could be tentatively assigned on the basis of the chromatographic data [9].

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