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EVALUATION OF POLYETHYLENE GLYCOL-HT AS A STATIONARY PHASE FOR CAPILLARY COLUMN GAS–LIQUID CHROMATOGRAPHY OF TRIMETHYLSILYL ETHERS OF BILE ACID METHYL ESTERS

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SUMMARY

Polyethylene glycol-HT was introduced as a polar stationary phase for bile acid analysis in capillary column gas–liquid chromatography. It has an excellent thermal stability compared with polyethylene glycol 20,000. Helium can be used as carrier gas. These characteristics show the superiority of this liquid phase to polyethylene glycol 20,000 which is of similar polarity. The relative retention times of some bile acid derivatives on a polyethylene glycol-HT capillary column are reported.

INTRODUCTION

Capillary column gas–liquid chromatography is a well established method for bile acid analysis[1,2]. Several non-polar or weakly polar stationary phases such as SE-30, OV-1, OV-101 and SP-2250 have been used for this purpose[2–5]. Although an attempt was made to produce polar capillary columns[6], polyethylene glycol 20,000 (PEG 20,000) is the only polar stationary phase available commercially for bile acid analysis[1]. Hydrogen is used as a carrier gas for the PEG 20,000 capillary column, which is potentially hazardous. Furthermore, in our experience, satisfactory results are not obtained using a PEG 20,000 capillary column to analyze trimethylsilyl (TMS) ethers of bile acid methyl esters, which indicates the possible instability of this stationary phase for bile acid analysis by gas chromatography.

Polyethylene glycol-HT (PEG-HT) is a derivative of polyethylene glycol having a molecular weight higher than 20,000. It has the similar polarity as PEG 20,000 and has been used as a stationary phase for fatty acid and sugar analyses[7]. We have evaluated PEG-HT for bile acid analysis in capillary column gas–liquid chromatography. The relative retention times of such derivatives as TMS ethers of bile acid methyl esters and ketonic bile acid methyl esters on both PEG-HT and OV-1 capillary columns are described.

MATERIALS AND METHODS

PEG-HT and OV-1 support-coated open tubular glass capillary columns were obtained from Gasukuro Kogyo Co. Ltd. (Tokyo, Japan). The sources of standard

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bile acids used in this study were as reported before[8]. In addition, 3α,6β,7β-trihydroxy-5β-cholanoic acid was purchased from Steraloids (Wilton, NH, U.S.A.). 3-Keto-5β-cholanoic acid and 7-keto-5β-cholanoic acid were synthesized by chromium oxidation from their parent hydroxycholanoic acids[9]. All compounds were confirmed by combined gas chromatography–mass spectrometry (GC–MS). Pyridine, hexamethyldisilazane and trimethylchlorosilane were obtained from Pierce (Rockford, IL, U.S.A.), and the remaining reagents and solvents were of analytical grade from Wako (Osaka, Japan).

The standard bile acids were dried at room temperature in vacuum overnight. They were methylated with freshly distilled diazomethane, and trimethylsilylation of hydroxycholanoic acid methyl esters was performed with pyridine–hexamethyldisilazane–trimethylchlorosilane (3:2:1) at 45°C for 30 min[10]. Immediately before the application of bile acid derivatives on to the capillary column, the TMS reagent was removed with a stream of nitrogen and the samples were redissolved in *n*-hexane. The purity of such standard bile acids as lithocholic acid, deoxycholic acid, chenodeoxycholic acid, ursodeoxycholic acid and cholic acid was checked by thin-layer chromatography and gas–liquid chromatography using a QF-1 packed column. All except lithocholic acid had no appreciable amount of impurity. Lithocholic acid had about 4% of impurity, but was used without further purification.

A gas chromatograph (Shimadzu GC-7A) was equipped with a flame ionization detector and an all-glass solid injector (Shimadzu, Kyoto, Japan). Helium was used as a carrier gas at a flow-rate of about 1.5 ml/min. The temperature of the oven was kept at 230°C during the isothermal analysis. The injector and detector temperatures were 280°C. The signals from the detector were processed by an integrator (Model 7000A; System Instruments Co., Ltd., Tokyo, Japan). Combined GC–MS was performed using Hitachi 063 and Hitachi M-60 instruments (Hitachi Ltd., Tokyo, Japan). The temperatures of the molecular separator and ion source were 250 and 200°C, respectively.

The relative retention times were determined in triplicate using the standard bile acids. The detector response and reproducibility were studied using mixtures of lithocholic acid, deoxycholic acid, chenodeoxycholic acid, ursodeoxycholic acid and cholic acid. 5α-Cholestane was used as an internal standard. 0.2-nmol amounts of all compounds were injected. The results were calculated from the areas of the peaks obtained from the successive injections of five mixtures, the area of 5α-cholestane being taken as 1.00 in each case.

RESULTS AND DISCUSSION

A PEG-HT capillary column is made by coating with a specially pretreated polyethylene glycol which has a molecular weight higher than 20,000. Both support-coated open tubular glass and silica capillary columns are available having maximum operating temperatures of 240 and 250°C, respectively. Thus, their thermal stability is superior to that of the PEG 20,000 capillary column. Generally, bile acid analysis by gas–liquid chromatography requires a high column temperature, from which point of view the thermal stability of PEG-HT is of particular use. The major fragment ions of PEG-HT were *m/z* 207, 281, 355, 429, 503 and 577 by mass spectrometry. The most intense one was at *m/z* 207. Thus, the background ions of PEG-HT would not inter-

fere with the MS analysis of bile acid TMS ether derivatives. The use of hydrogen as a carrier gas can be hazardous. In this study helium was used for the PEG-HT capillary column, which would be safe and available in most laboratories without any additional change of gas chromatographic systems.

With regard to the column performance of PEG-HT, as shown in Fig. 1 the analysis time was short and a baseline separation was nearly obtained for the main TMS ethers of bile acid methyl esters under the isothermal conditions. The separation of these compounds will be improved by the temperature programming technique. The absolute retention times of these compounds did not change over a period of at least 5 months on the same capillary column. Therefore, it was not necessary to alter

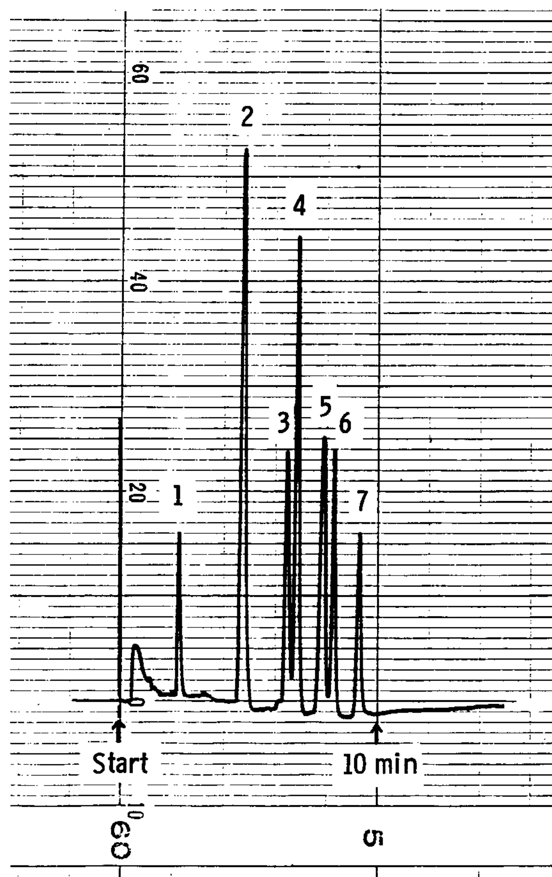

Fig. 1. Gas chromatogram of trimethylsilyl ethers of standard bile acid methyl esters and 5α-cholestane as internal reference on a 20-m PEG-HT support-coated open tubular glass capillary column. Peaks: 1 = 5α-cholestane; 2 = cholic acid and 7α-monohydroxy-5β-cholanoic acid; 3 = deoxycholic acid; 4 = chenodeoxycholic acid; 5 = hyodeoxycholic acid; 6 = lithocholic acid; 7 = ursodeoxycholic acid. Note the overlapping of the trimethylsilyl ethers of cholic acid methyl ester and of 7α-monohydroxy-5β-cholanoic acid methyl ester. The internal diameter of the column was 0.28 mm. Temperatures: oven, 230°C; injector and detector, 280°C. Carrier gas (helium) flow-rate, about 1.5 ml/min. A Van den Berg solventless injector was used.

the parameters of retention times for the integrator during those 5 months in order to analyze biological samples.

The relative retention times of several TMS ethers of bile acid methyl esters and ketonic bile acid methyl esters on PEG-HT are shown in Table I together with those on OV-1. The polarity of PEG-HT is almost the same as that of PEG 20,000. Accordingly, they afford similar relative retention times for some of the bile acid derivatives[1]. A major characteristic of PEG-HT was the retardation of ketonic bile acid methyl esters. For instance, 3,7,12-triketo-5β-cholanoic acid methyl ester was not eluted from a 10-m PEG-HT capillary column within 3 h. This was also the case for ketonic TMS ethers of cholanoic acid methyl esters, whose retention times were increased remarkably by the presence of ketonic group(s). Since the advantage of the PEG-HT capillary column was a quick elution of the compounds, the analysis of di- or triketocholanoic acid methyl esters did not seem practical. Another disadvantage was the overlapping of the TMS ethers of cholic acid methyl ester and of 7α-monohydroxy-5β-cholanoic acid methyl ester. When GC–MS was used, however, this overlapping could easily be resolved by the selective ion monitoring technique.

The detector response and reproducibility are shown in Table II. The detector response for the PEG-HT capillary column did not correspond with the weight of the

TABLE I

RELATIVE RETENTION TIMES OF BILE ACID METHYL ESTER DERIVATIVES ON A 20-m PEG-HT AND A 10-m OV-1 SUPPORT-COATED OPEN TUBULAR GLASS CAPILLARY COLUMN

The oven temperature for OV-1 was 240°C; other operating conditions as in Fig. 1.

*Functional groups of bile acid methyl ester**	*RRT***	
	PEG-HT	*OV-1*
None	0.35	0.41
3α-TMS	1.27	0.85
3β-TMS, 5-cholenoic acid	1.66	0.98
3-Keto	4.08	0.86
7α-TMS	0.72	0.61
3α,6α-Di-TMS	1.21	1.14
3α,7α-Di-TMS	1.06	1.07
3α,7β-Di-TMS	1.46	1.21
3α,12α-Di-TMS	1.00	1.00
3α-TMS, 7-keto	4.06	1.36
3,7-Diketo	13.10	1.30
3,12-Diketo	12.92	1.30
3α,6α,7α-Tri-TMS	0.95	1.40
3α,6β,7β-Tri-TMS	1.14	1.49
3α,7α,12α-Tri-TMS	0.72	1.11
3α,7α-Di-TMS, 12-keto	2.97	1.70
3α,12α-Di-TMS, 7-keto	3.21	1.64
3α-TMS, 7,12-Diketo	13.56	1.93
3,7,12-Triketo	–	1.81

* In 5β-cholanoic acid methyl ester unless otherwise indicated. TMS = Trimethylsilyl ether.

** Retention times relative to trimethylsilyl ether of deoxycholic acid methyl ester at 393 and 1308 sec on PEG-HT and OV-1, respectively.

TABLE II

DETECTOR RESPONSE OF TRIMETHYLSILYL ETHERS OF BILE ACID METHYL ESTERS ON A 20-m PEG-HT SUPPORT-COATED OPEN TUBULAR GLASS CAPILLARY COLUMN

The operating conditions were as in Fig. 1. 5α-Cholestane was used as an internal standard. 0.2-nmol amounts of compounds were injected. The results were calculated as mean ± S.D. of the ratios of the area of each bile acid peak to the area of 5α-cholestane, from five standard mixtures. The actual areas of 5α-cholestane in those samples as determined by the integrator were 21,232 ± 1276 (mean ± S.D.). TMS = Trimethylsilyl ether; ME = methyl ester.

Compound	*Detector response (mean ± S.D.)*
5α-Cholestane	1.00 –
Lithocholic acid TMS ME	1.23 ± 0.10
Deoxycholic acid TMS ME	1.43 ± 0.07
Chenodeoxycholic acid TMS ME	1.63 ± 0.08
Ursodeoxycholic acid TMS ME	1.59 ± 0.10
Cholic acid TMS ME	2.00 ± 0.04

unsubstituted parent compounds, but did roughly with the actual injected weight of the substituted compounds. The reproducibility was satisfactory. Linearity of the detector response was demonstrated in the examined range of 0.05–0.8 nmol of the bile acids listed in Table II.

In conclusion, PEG-HT is one of the very few polar liquid phases suitable for bile acid analysis by capillary column gas–liquid chromatography. It offers a short analysis time by its excellent thermal stability, and is particularly useful for most of the hydroxy bile acids in biological samples.

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