Journal of Chromatography, 193 (1980) 444–450

CHROM. 12,697

Note

Gas–liquid chromatographic separation of bile acids and steroids on a nematic liquid crystal

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(Received January 17th, 1980)

Liquid crystals are becoming popular as stationary phases in gas–liquid chromatography (GLC) for the separation of rigid geometric isomers[1,2]. Recently, biomedical research on steroids, bile acids and their derivatives has been aided by the availability of new chromatographic techniques for separation and purity determinations[3–6]. Although thin-layer chromatography[7–11], and more recently high-performance liquid chromatography[12–16], have been used with varying degrees of success, GLC remains the most effective technique available for the analysis of complex multicomponent mixtures. Packed columns have been widely used in this work[4–6,10,11,17–20], but the resolution of bile acid and steroid epimers proved difficult, and almost invariably required the preparation of a variety of volatile derivatives. Interference by such compounds as cholesterol complicates the analysis of bile acids in biological material[21]. Recent advances in the development of open-tubular glass capillary columns and the coupling of GLC with mass spectrometry[17–25] have greatly enhanced the resolution of complex bile acid and steroid mixtures. Open-tubular capillary columns exhibit greater resolution power than can be obtained by conventional packed columns. However, since no single GLC column will satisfactorily resolve all the commonly encountered bile acid and steroid mixtures, a careful selection of two or more columns is often necessary. The need for derivatization further complicates GLC analysis due to potential loss of material and the production of artifacts.

The chromatographic behavior of steroids and bile acid methyl esters on N,N′-bis(*p*-phenylbenzylidene)-α,α′-bi-*p*-toluidine (BPhBT) together with the major advantages and disadvantages of BPhBT *versus* other stationary phases is discussed.

EXPERIMENTAL

GLC was carried out on a Hewlett-Packard 7610 instrument equipped with a flame-ionization detector. Chromatograms were generated on a 1-mV f.s. strip chart recorder using an electrometer setting of 4×10^2. Carrier gas flow was monitored by

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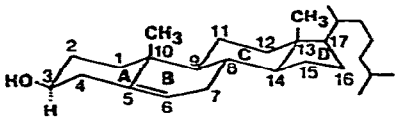
a calibrated Brooks 5840 dual GC mass flow controller. Columns were 2 mm I.D. borosilicate glass. The column packing (2.5% BPhBT on 100–120 mesh Chromosorb W HP) was prepared by the solvent slurry technique and fluidized drying with nitrogen. BPhBT was only slightly soluble in chloroform, but formed a fine suspension which could be used for slurry coating.

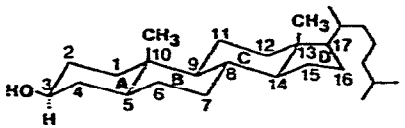
The nematic liquid crystal used as the GLC stationary phase, BPhBT, was synthesized and its structure verified according to procedures described previously[26]. All bile acid samples used in this study were kindly provided by Dr. M. I. Kelsey (NCI, Frederick Cancer Research Center, Frederick, MD, U.S.A.), except for 5β-cholanic acid-3,7,12-trione, 5β-cholanic acid-3α-ol-7,12-dione and 5β-cholanic acid-3, 7,-diol-12-one, which were obtained from Steroloids (Wilton, NH, U.S.A.). The steroids were also obtained from Steroloids. Diazomethane was used to convert the bile acids to their respective methyl esters.

RESULTS AND DISCUSSION

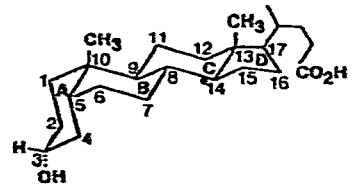
Steroids and bile acids are alike in possessing a reasonably rigid 1,2-cyclopentanophenanthrene ring system. The structures of four typical representatives of these naturally occuring compounds, together with the numbering system used throughout the text are shown in Fig. 1. Steroid epimers differing in A/B ring configuration and in axial/equatorial configuration have been successfully separated on a N,N′-bis(*p*-methoxybenzylidene)-α,α′-bi-*p*-toluidine (BMBT) liquid crystal column with separation factors exceeding those reported elsewhere[17]. BMBT, however,



Cholesterol (Δ5-Cholesten-3β-ol)



Cholestanol (5α-cholestan-3β-ol)



Isolithocholic acid (5β-Cholanic acid-3β-ol)

Fig. 1. Configurational structures and numbering for cholesterol, cholestanol and isolithocholic acid.

suffers from excessive column bleeding at the elevated temperatures (>250 °C) necessary for the elution of cholestane derivatives and bile acid methyl esters. We therefore examined the properties of BPhBT for use in liquid crystal columns and found that BPhBT exhibits retention characteristics similar to BMBT for steroids and bile acid methyl esters with significantly diminished bleed levels.

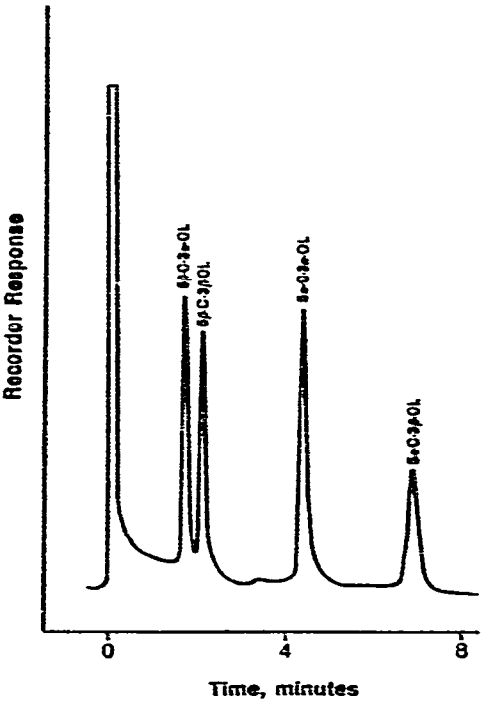


Fig. 2. Chromatogram of the 5α/β-cholestan-3α/β-ols. Column, 6 ft. × 2 mm I.D.; packing, 2.5% BPhBT. Conditions, oven 270°C; injector and detector 275°C; flow-rate 15 ml/min. C stands for cholestane.

As would be expected when liquid crystals are used as shape-selective stationary phases, solvents with larger length-to-breadth ratios are retained longer. (For example, Fig. 2 shows the separation of 5α/β-cholestan-3α/β-ols.) Since the 5α configuration (A/B *trans*) is more planar than the 5β configuration (A/B *cis*), then the 5α epimers are invariably retained longer. The same retention behavior is observed on conventional stationary phases[3], but the retention mechanism on liquid crystals is dominated by shape selectivity, and consequently the separation factors for 5α/β epimers are considerably larger on liquid crystal columns. For example, the separation factor for the isomeric pair 5α-cholestan-3β-ol/5β-cholestan-3β-ol is 1.11 on non-polar SE-30, 1.12 on polar NGS and QF-1[3], but it is 3.53 on BMBT[27] and 3.47 on BPhBT. For isomers differing in stereochemistry at the 3-position the separation on conventional phases is highly dependent on the nature of the phase. To illustrate: the retention ratio of the two epimers 5β-cholestan-3α-ol/5β-cholestan-3β-ol is close to 1.0 on SE-30, 1.06 on NGS and 1.14 on QF-1[3]. In contrast, the elution order of these two isomers (5β, 3α *vs.* 5β, 3β) on BPhBT is reversed with the 3β isomer retained

longer, because the axial 3β-hydroxy group lies more in the plane of the molecule than does the equatorial 3α analogue. Even larger separation factors are observed for the 3α/β epimers of the A/B *trans* (5α) configuration, because the 3β-hydroxy group is equatorial to the A ring in the 5α configuration and therefore it possesses a greater length-to-breath ratio than its 3α analogue. Since the separation factors for the 5α/β epimers on liquid crystal phases is much larger than for the 3α/β epimers, the order of elution of steroid stereoisomers is always 5α-3β>5α-3α>5β-3β>5β-3α. This is not the case on conventional phases, where the order of elution depends on the nature of the liquid phase and the types of derivatives used[3].

It should be emphasized that despite the improved separations obtained with BPhBT, its use is limited to near, or above, its solid–nematic transition temperature of 257 °C. Below this temperature, broad and unresolved peaks are observed[26]. Because of this temperature limitation, steroids of the androstane class elute inconveniently close to the solvent peak. Nonetheless, di- and trihydroxy and/or keto derivatives of androstane are sufficiently retained to be adequately separated. Fig. 3 shows the separation of these closely related steroids that differ in the number and position of double bonds in the steroid skeleton. Separation of steroid mixtures differing with respect to unsaturation is difficult, even with the use of open-tubular capillary columns[23].

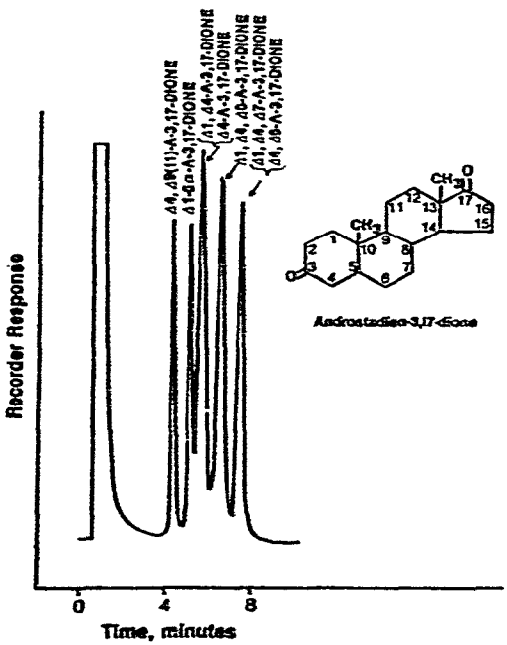


Fig. 3. Chromatogram of a composite mixture of isomeric androstadien-3,17-diones. Column, packing and conditions same as in Fig. 2. A stands for androstadien.

Analysis of bile acids by GLC is often complicated by the presence of interfering sterols such as cholesterol[21]. For example, Laatikainen and Hesso[22] were unable to resolve cholesterol and lithocholic acid (5β-cholanic acid-3α-ol) on OV-101,

even with the use of capillary columns. Miyazaki *et al.*[28] observed that cholesterol elutes simultaneously with deoxycholic acid (5β-cholanic acid-3,12-diol) on Hi-Eff 8B and on Poly I-110. These difficulties were resolved using BPhBT which separated cholesterol and other sterols from bile acids in a relatively rapid analysis.

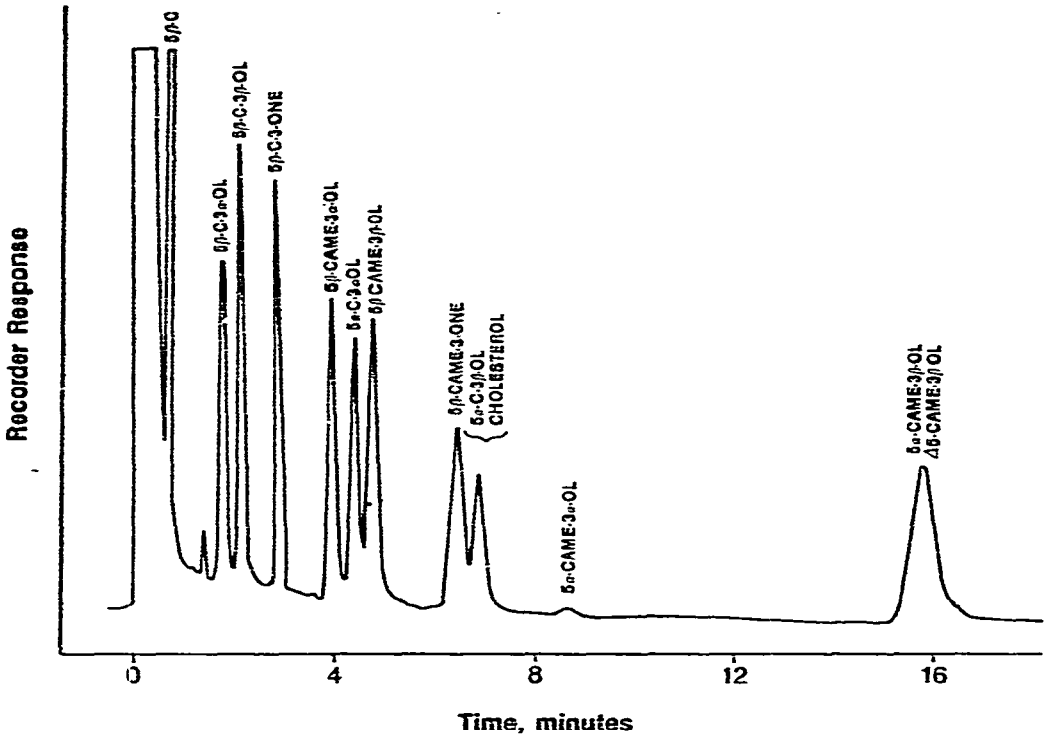


Fig. 4. Chromatogram of a synthetic mixture of monofunctional CAME, monofunctional cholestanes and cholesterol. Column, packing, and conditions as in Fig. 2. C stands for cholestane.

Fig. 4 shows the isothermal separation of monofunctional cholanic acid methyl esters (CAME) from underivatized monofunctional cholestanes of comparable molar mass. Fig. 5 gives the chromatographic profile of a composite mixture of 17 mono-, di-, and trihydroxy and/or keto derivatives of cholic acid methyl ester and cholesterol, under the same chromatographic conditions as in Fig. 4. The shapes of the peaks for steroids and monofunctional bile acids appear symmetrical; however, the peaks of the di- and trihydroxy and/or keto bile acids show diffuse tailing, which is more pronounced for the tri-substituted derivatives. Together with this non-symmetrical effect, a decrease in retention with increasing sample size is observed, indicating that a Langmuir-type adsorption contributes to the retention mechanism for these solutes on BPhBT. The retention ratios for 5β-CAME-3α-ol, 5β-CAME-3β-ol, 5β-CAME-3-one, 5α-CAME-3β-ol obtained from Fig. 5 were 1.00, 1.20, 1.65, 4.0, respectively. In contrast, the retention ratios of the same solutes in the same order on QF-1, which is considered to be the most selective of conventional phases, were 1.00, 0.89, 2.04, 1.12, respectively[6]. The separation factor for the 3α/β epimers of 5β-CAME on

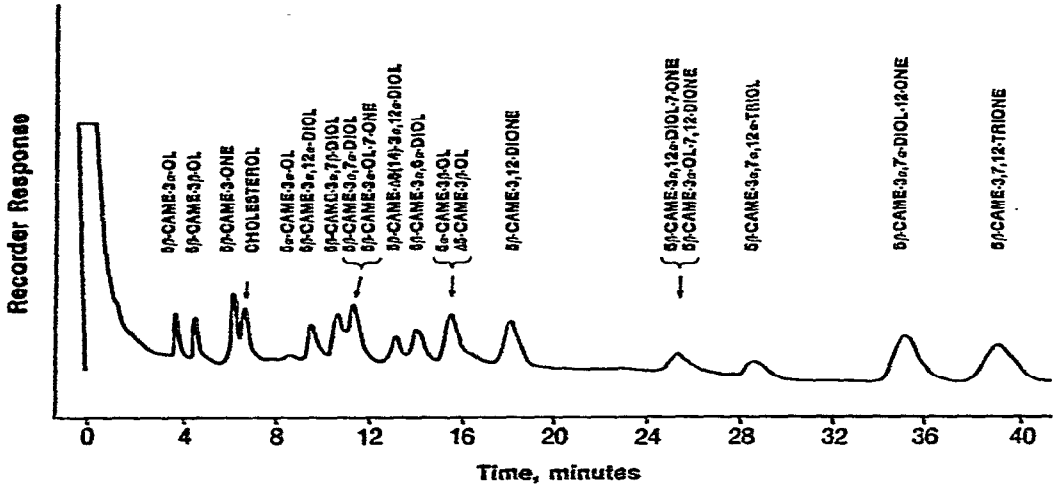


Fig. 5. Chromatogram of seventeen mono-, di- and trihydroxy and/or keto CAME and cholesterol. Column, packing and conditions as in Fig. 2.

BPhBT is different from that obtained by GLC on conventional phases, with or without derivatization. A larger separation factor is also observed for the $5\alpha/\beta$ epimers.

No liquid phase is entirely satisfactory for steroid and bile acid determinations[4–6]. BPhBT is no exception; however, it does offer unique separation properties without the need for complex derivatizations other than esterification of the carboxylic acid group. It is generally accepted that retention data correlation between an unknown and a selected reference compound on different columns of different "polarity" is sufficient for positive identification. Thus, the unique chromatographic elution order of bile acids and steroids on BPhBT greatly improved the reliability of identification of these compounds by GLC.

ACKNOWLEDGEMENT

This research was sponsored by the National Cancer Institute under Contract NO1-CO-75380 with Litton Bionetics.

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