Journal of Chromatography, 351 (1986) 604–607
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 305

Note

Facile high-performance liquid chromatographic resolution of (25*R*)- and (25*S*)-26-hydroxycholesterol-3,26-di-*p*-bromobenzoates

T. ARUNACHALAM
Worcester Foundation for Experimental Biology, 222 Maple Avenue, Shrewsbury, MA 01545 (U.S.A.)
J. C. HODGIN
Micromeritics Instrument Company, 5680 Goshen Spring Road, Norcross, GA 30093 (U.S.A.)
and
E. CASPI*
Worcester Foundation for Experimental Biology, 222 Maple Avenue, Shrewsbury, MA 01545 (U.S.A.)
(First received September 10th, 1985; revised manuscript received October 31st, 1985)

For ongoing biosynthetic and metabolic studies, a method was required for facile resolution and differentiation of (25*R*)- and (25*S*)-26-hydroxycholesterol esters. Redel[1,2] achieved resolution of the two epimeric diacetates by recycling them multiple times in a Waters Assoc. high-performance liquid chromatography instrument equipped with two serially-connected μPorasil (30 cm × 4.0 mm I.D.) columns. In our hands, in using a Micromeritics instrument in the recycling mode, no resolution of the epimers was achieved. Usually, on recycling, the peak of the eluted material became diffused and the components were not resolved.

We report a simple and efficient method for single path resolution and identification of the (25*R*)- and (25*S*)--26-hydroxycholesterols as 26-*p*-bromobenzoates and/or 3,26-di-*p*-bromobenzoates. The di-*p*-bromobenzoates were resolved nearly to the base line. The 26-mono-*p*-bromobenzoates are eluted considerably slower. In all instances, the (25*S*)-3,26-di-*p*-bromobenzoate esters were eluted ahead of the corresponding (25*R*)--di-esters.

EXPERIMENTAL

Reagent grade solvents were used in synthetic preparations. Silica gel [(HF60; 254+366) Merck, Darmstadt, F.R.G.] was used for thin-layer chromatography (TLC). NMR spectra were recorded on a Varian 390 instrument in C^2HCl_3 using tetramethylsilane (TMS) as internal standard. For HPLC, reagent grade solvents were redistilled and degassed prior to use.

(25RS)-26-Hydroxycholesterol-3,26-bis-p-bromobenzoate (I)

A mixture of (25*RS*)-26-hydroxycholesterol[3,4] (8.5 mg), pyridine (100 μl) and *p*-bromobenzoylchloride (40 mg) in dry benzene (3 ml) was stirred (12 h) at room temperature. Diethyl ether (10 ml) and water (10 ml) were then added and stirring was continued for 1 h. The product was extracted with diethyl ether, the ether extract

0021-9673/86/$03.50

was washed with aqueous sodium bicarbonate, water, dried and the solvent removed. The residue was fractionated by preparative TLC [silica gel; hexane–ethyl acetate (3:1)] to furnish the bis-*p*-bromobenzoate.

(25R)-26-Hydroxycholesterol-3,26-bis-p-bromobenzoate (derived from diosgenin) (II)

A mixture of (25*R*)--26-hydroxycholesterol[5] (derived from diosgenin) (100 mg), pyridine (1 ml), *p*-bromobenzoylchloride (500 mg) and benzene (10 ml) was stirred for 48 h at room temperature. The mixture was diluted with diethyl ether (10 ml) then 5% aqueous sodium bicarbonate (20 ml) was added and the mixture was stirred for 12 h. The organic layer was separated and the aqueous layer was re-extracted with diethyl ether. The combined ether extract was washed with water, dried and concentrated to give a crystalline residue. Purification by preparative TLC [silica gel; hexane–ethyl acetate (2:1)] furnished the title product (54 mg); m.p. 94–95°C (from methanol); NMR δ = 0.65 (s, 3H, C_{18}-H), 0.9 (3H, d, J = 6Hz, C_{21}-H), 0.99 (3H, s, C_{19}-H), 0.98 (3H, d, J = 8Hz, C_{27}-H), 3.5 (bm, 3α-H), 4.12 (2H, m, C_{26}-H), 5.34 (m, C_5-H) and also an A_2B_2 pattern for the aromatic protons.

(25R)-26-Hydroxycholesterol-26-p-bromobenzoate (III) and (25S)-26-hydroxycholesterol-26-p-bromobenzoate (IV) (from synthetic samples)

Synthetic samples of (25*R*)- and (25*S*)-26-hydroxycholesterol-3-tetrahydropyranyl (THP) ether were obtained from the late Professor Henry W. Kircher of the University of Arizona (Tucson, AZ, U.S.A.).

To a solution of (25*R*)-26-hydroxycholesterol-3-THP ether (25 mg, m.p. 131–132°C) in pyridine (2 ml) and benzene (5 ml), *p*-bromobenzoylchloride (100 mg) was added. The mixture was stirred for 24 h at room temperature and processed in the usual manner. For the removal of the THP-ether the crude product was dissolved in methanol (10 ml), containing concentrated hydrochloric acid (two drops) and the mixture was stirred at 20°C (4 h). The solvent was distilled under reduced pressure, and the residue was dissolved in diethyl ether. The ether extract was washed with aqueous sodium bicarbonate, water, dried and concentrated. The obtained product was purified by preparative TLC [silica gel; hexane–ethyl acetate (3:1)] and then crystallized from methanol (18.5 mg).

The (25*S*)-26-hydroxycholesterol-3-THP ether (20 mg) was processed in a similar manner to yield (25*S*)-26-hydroxycholesterol-26-*p*-bromobenzoate.

(25R)- (V) and (25S)-26-Hydroxycholesterol-3,26-bis-p-bromobenzoate (VI) (from synthetic samples)

The above "synthetic" (25*R*)-26-hydroxycholesterol-26-*p*-bromobenzoate (5 mg) was stirred with *p*-bromobenzoylchloride (20 mg) in pyridine (0.5 ml), and benzene (1 ml) at room temperature for 15 h. After the usual work-up and purification by preparative TLC [silica gel; hexane–ethyl acetate (3:1)] (25*R*)-26-hydroxycholesterol di-*p*-bromobenzoate was obtained. HPLC analysis indicated that the product was at least 98% pure.

In a similar manner, the (25*S*)-26-hydroxycholesterol-26-*p*-bromobenzoate was converted to the di-*p*-bromobenzoate. HPLC analysis indicated that the product was mostly (25*S*)-isomer contaminated with a small amount of (25*R*)-isomer.

TABLE I

HPLC OF "SYNTHETIC" SAMPLES OF (25*R*)- AND (25*S*)-26-HYDROXYCHOLESTEROL-DI-*p*-BROMOBENZOATES

Solvent	*Flow-rate (ml/min)*	*Retention time (min)*	
		(25S)	*(25R)*
Dichloromethane–hexane (1:1)	1.5	11.25	11.63
Dichloromethane–hexane (1:1)	1.0	17.30	18.30
Dichloromethane–hexane (2:3)	1.25	21.0	22.4
Dichloromethane–hexane (3:7)	1.3	45.0	47.0

HPLC

A Micromeritics instrument, equipped with a Micromeritics Model 750 solvent delivery system, and a Micromeritics dual Model 788 variable-wavelength UV detector was used. The detector was set at 254 nm. The flow-rate was adjusted as indicated in Table I. An Altech 5-μm silica gel column (20 cm × 4.5 mm I.D.) was used throughout. The column was eluted with the indicated mixtures of dichloromethane–hexane.

DISCUSSION

Our initial attempts at resolving the (25*RS*)-26-hydroxycholesterol-di-*p*-bromobenzoate on a 10-μm Microsil-NH_2 bonded[6] column (30 cm × 5 mm I.D.) and a Whatman 10-μm Partisil PXS 1025 column (25 cm × 4.5 mm I.D.), using mixtures of dichloromethane–hexane as the mobile phase, were not successful. In both cases low resolution was obtained and the analysis time on the Partisil PXS 1025 column was almost double the NH_2-bonded column. Even coupling of four Partisil PXS 1025 columns in series did not improve the separation.

However, when the sample of the (25*RS*)-di-*p*-bromobenzoate was analyzed on the Altech 5-μm silica gel column, using conditions specified in Fig. 1a, a reasonably good resolution of the diastereoisomers was obtained. To identify the isomers, (25*R*)-26-hydroxy-di-*p*-benzoate derived from diosgenin was analyzed. The product (Fig. 2) contained mainly the (25*R*)-isomer and a small amount of the (25*S*)-isomer and a minor unknown impurity. Spiking of the (25*RS*)-diester with (25*R*)-diester (originating from diosgenin) revealed that the (25*S*)-isomer is eluted first (Fig. 1b). Addition of (25*S*)-di-*p*-bromobenzoate (see below) to the (25*RS*) (Fig. 1a) enhanced the faster moving peak (Fig. 1c).

The late Professor H. W. Kircher had sent us two samples of synthetic 26-hydroxycholesterol-3-THP ethers for analysis and confirmation of their C-25 configurations. The samples were converted to 26-mono- and 3,26-di-*p*-bromobenzoates, as described in the Experimental section. The sample, which was thought to be the (25*R*)-diester, proved to be essentially homogeneous (Fig. 3b), while the (25*S*)-di-*p*-bromobenzoate contained a significant amount (10–15%) of the (25*R*)-isomer (Fig. 3a). A mixture of the two isomers was resolved as seen in Fig. 3c. The retention times of the "synthetic" diesters, with different solvent mixtures, are given in Table I. The

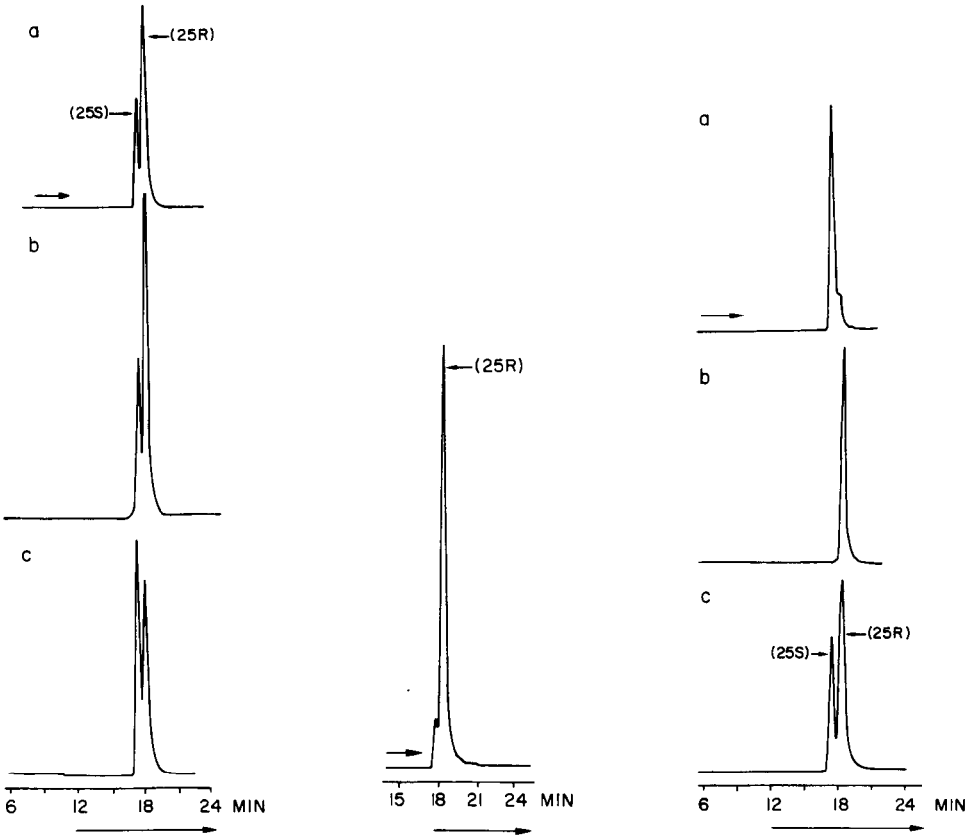

Fig. 1. An Altech 5-μm silica gel (20 cm × 4.5 mm I.D.) column was used. The column was eluted with dichloromethane–hexane (1:1) at a flow rate of 1 ml/min. (a) (25*RS*)-Cholesterol-di-*p*-bromobenzoate (I); (b) (25*RS*)-3,26-diester (I) spiked with (25*R*)-26-hydroxycholesterol diester (II); (c) (25*RS*)-diester (I) spiked with (25*S*)-3,26-diester (VI) (derived from the synthetic sample).

Fig. 2. Chromatography of (25*R*)-3,26-diester (II) (derived from diosgenin).

Fig. 3. Chromatography of 3,26-diester (V) and (VI) derived from synthetic samples: (a) (25*S*)-3,26-di-*p*-bromobenzoate (VI); (b) (25*R*)-3,26-di-*p*-bromobenzoate (V); (c) mixture of (25*R*) (V) and (25*S*) (VI) di-*p*-bromobenzoates.

26-mono-*p*-bromobenzoates were purified in this system, but their elution was considerably slower (*ca.* 60 min).

The described procedures are very likely applicable to other steroidal 26-hydroxy analogues.

REFERENCES

1 J. Redel and J. Capillon, *J. Chromatogr.*, 151 (1978) 418.
2 J. Redel, *J. Chromatogr.*, 168 (1979) 273.
3 R. K. Varma, J. A. F. Wikramasinghe and E. Caspi, *J. Biol. Chem.*, 244 (1969) 3951.
4 R. K. Varma, M. Koreeda, B. Yagen, K. Nakanishi and E. Caspi, *J. Org. Chem.*, 40 (1975) 3680.
5 T. Arunachalam, P. J. MacKoul, N. M. Green and E. Caspi, *J. Org. Chem.* 46 (1981) 2966.
6 A. Wikby, A. Thalén and G. Oresten, *J. Chromatogr.*, 157 (1978) 65.