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Note

Enantiomer separation of diacid-diacylglycerol mixtures by high-performance liquid chromatography on a chiral column

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Recently, we reported direct enantiomer separations of diacylglycerols as their 3,5-dinitrophenylurethane (3,5-DNPU) derivatives by high-performance liquid chromatography (HPLC) on the chiral stationary phases Sumichiral (previous name Sumipax) OA-2100 and 4100 [1,2]. The structures of diacylglycerol enantiomers are shown in Fig. 1.

The previous papers [1,2] mainly described enantiomer separations of individual monoacid-diacylglycerol ($R_1 = R_2$) and saturated monoacid-diacylglycerol homologue mixtures. In this paper, HPLC procedures for enantiomer separations of diacid-diacylglycerol ($R_1 \neq R_2$) mixtures are presented, as the separations of diacid-diacylglycerols are usually more important in analyses of natural lipid components.

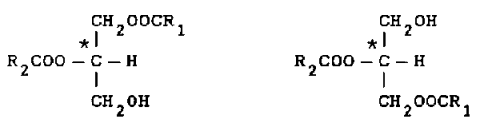
EXPERIMENTAL

Samples

Enantiomers of 1,2- and 2,3-diacyl-*sn*-glycerols were synthesized by the method of Howe and Malkin [3]. Diacylglycerol racemates were obtained by inter-esterification of fatty acid methyl esters with glycerol in dimethylformamide medium. Diacylglycerols were separated by thin-layer chromatography (TLC) and converted to the 3,5-DNPUs as described previously [1,2].

HPLC

HPLC separations were carried out with a Hitachi (Tokyo, Japan) L-6200 instrument equipped with a chiral column (stainless-steel, 50 cm × 4 mm I.D.) packed



1,2-diacyl-sn-glycerol 2,3-diacyl-sn-glycerol

Fig. 1. Structures of diacylglycerol enantiomers. RCO = acyl group.

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with 5-μm particles of N-(*R*)-1-(α-naphthyl)ethylaminocarbonyl-(*S*)-valine chemically bonded to Sumichiral OA-4100 γ-aminopropylsilanized silica (Sumitomo Chemical, Osaka, Japan). The analyses were done isocratically using a mixture of HPLC-grade hexane, dichloroethane and ethanol as the mobile phase at ambient temperature. A Hitachi 638-0805 recycle valve was used for recycling.

Analysis of methyl esters of diacylglycerols

The diacylglycerol 3,5-DNPU peak fraction separated by HPLC was dissolved into 1 ml of diethyl ether and 25 μl of a 1 M solution of sodium methoxide in methanol were added. The mixture was kept at ambient temperature for 5 min, then the product was extracted with diethyl ether. The extract was refined by column chromatography with a silicic acid column using diethyl ether for development and analysed by open-tubular gas chromatography (GC) using a Shimadzu (Kyoto, Japan) GC-6AM instrument equipped with a flame ionization detector and a glass capillary WCOT column (50 m × 0.28 mm I.D.) coated with SP 2300 (Supelco, Bellefonte, PA, U.S.A.). The column temperature was 190°C.

RESULTS AND DISCUSSION

Separation of saturated diacid-diacylglycerols

Fig. 2 shows enantiomer separations of a saturated 1,2-diacylglycerol homologue mixture with a difference of two carbons as their 3,5-DNPUs on Sumichiral OA-4100. Better separations were obtained at lower concentrations of dichloroethane (Fig. 2A–C), and it permitted the separation of ten enantiomers of the five diacylglycerol homologues (Fig. 2C). In the previous study [1,2], the separations between homologues differing by four acyl carbons were 1.1 in the peak resolution and 1.1 in the separation factor (α, the ratio of the capacity factors). In this study, the peak resolution of the homologues differing by two acyl carbons was higher than 1.1 for each *sn*-1,2- and *sn*-2,3-enantiomer under the conditions in Fig. 2C.

Fig. 3 shows a linear relationship between the logarithm of retention volumes and acyl carbon number for homolgous series of each enantiomer. This linear relationship in the chromatography of homologues was reported by James and Martin [4] for the first time in the GC of the lower fatty acids on a silicone–stearic acid column. In the HPLC of acylglycerols, this linear relationship has been reported in the analysis of triacylglycerols using a non-aqueous reversed-phase system [5]. The two straight lines for *sn*-1,2- and *sn*-2,3-enantiomers in Fig. 3 are approximately parallel, and the relationship between retention volumes and acyl carbon numbers can be expressed by the following equations:

$$\log V_r(sn\text{-}1,2) = HN + A \quad (1)$$

$$\log V_r(sn\text{-}2,3) = HN + B \quad (2)$$

where $V_r(sn\text{-}1,2)$ and $V_r(sn\text{-}2,3)$ are the retention volumes of 1,2- and 2,3-diacyl-*sn*-glycerol enantiomers, respectively, with the acyl carbon number N and H (carbon number separation factor) and $B - A$ (E = enantiomer separation factor) are the slopes of the lines and the distance between the two lines along the vertical axis, respectively.

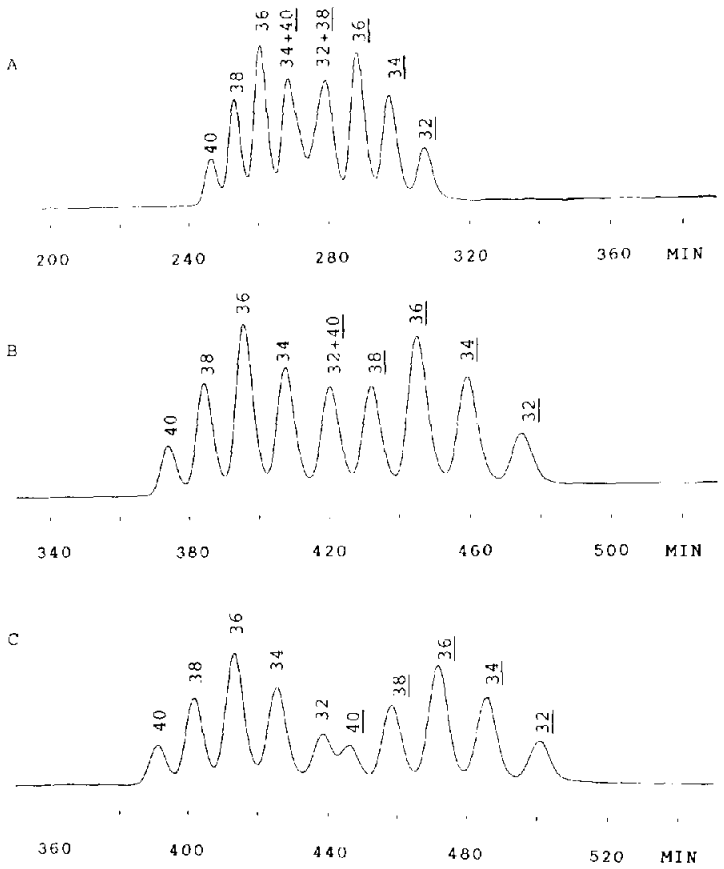

Fig. 2. HPLC separation of saturated diacylglycerols synthesized from palmitic, stearic and arachidic acids as their 3,5-DNPUs on an OA-4100 chiral column. Flow-rate: 0.25 ml/min. Mobile phase: hexane–dichloroethane–ethanol. (A) 170:40:1, (B) 170:20:1 and (C) 170:10:1. Recycle: after first recycle. Above each peak is given the acyl carbon number of the diacylglycerol. Underlined numbers are the acyl carbon numbers of *sn*-2,3-enantiomers.

Fig. 3 also shows the dependence of retention volumes on the composition of the mobile phase. Better separations were obtained with a lower concentration of dichloroethane. An increase or decrease in the hexane content in the mobile phase did not improve the peak resolution. An increase in the ethanol content in the mobile phase hardly improved the peak resolution, and a decrease in the ethanol content lowered the E value considerably. Dichloroethane contains negatively charged chlorine atoms, which bond reversibly to the positively charged hydrogen of the NH groups in the stationary phase and the urethane part of the diacylglycerol derivatives by hydrogen bonding. Dichloroethane will play an important role in the resolution of the chiral components by influencing the reversibly competitive bonding with the urethanes and the stationary phase [6].

Reducing the flow-rate did not improve the resolution appreciably in the region below 0.25 ml/min. Recycling procedures improved the resolution of the diacylglycerol

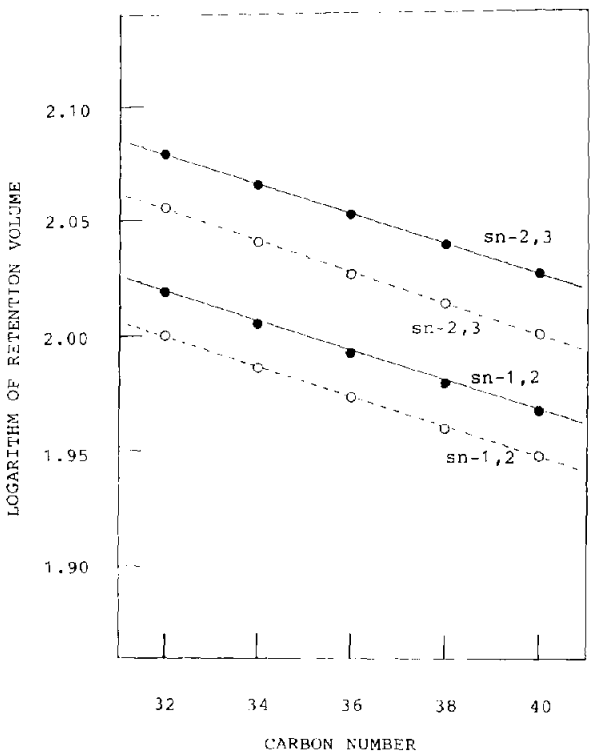

Fig. 3. Plot of logarithm of retention volume *vs.* acyl carbon number for saturated diacylglycerol enantiomers synthesized from palmitic, stearic and arachidic acids as their 3,5-DNPUs separated by HPLC. Flow-rate: 0.25 ml/min. Mobile phase: hexane–dichloroethane–ethanol, full lines 170:10:1, dashed lines 170:20:1. Recycle: after first recycle.

enantiomers. The peak resolution between 1,2-di-16:0- and 2,3-di-20:0-*sn*-glycerols was much improved after the second recycle (Fig. 4).

HPLC of diacylglycerols prepared from saturated and unsaturated acids

Fig. 5 shows the enantiomer separation of the diacid-diacylglycerols synthesized from stearic acid and one of the 18:1, 18:2, 18:3 and 20:5 unsaturated fatty acids with glycerol by HPLC on the chiral stationary phase. Each chromatogram shows six peaks for the 1,2- and 2,3-diacyl-*sn*-glycerol enantiomers containing two saturated (SS-DG), one saturated, one unsaturated (SU-DG) and two unsaturated acyl groups (UU-DG), where S and U represent saturated and unsaturated acyl groups, respectively.

The diacylglycerols can be effectively separated by using the mobile phase hexane–dichloroethane–ethanol (250:20:1) (Fig. 5A, C and D), but 1,2-di-18:2- and 2,3-18:0-18:2-*sn*-glycerols overlapped completely under these conditions. The same mobile phase in the proportions 170:10:1 resulted in their preferential separation (Fig. 5B).

The critical pair formed by 16:0 and 18:1 acyl groups has been found in the HPLC of monoacylglycerols as their 3,5-DNPU derivatives on OA-2100 chiral stationary phase, and they have been separated as free monoacylglycerols by TLC on

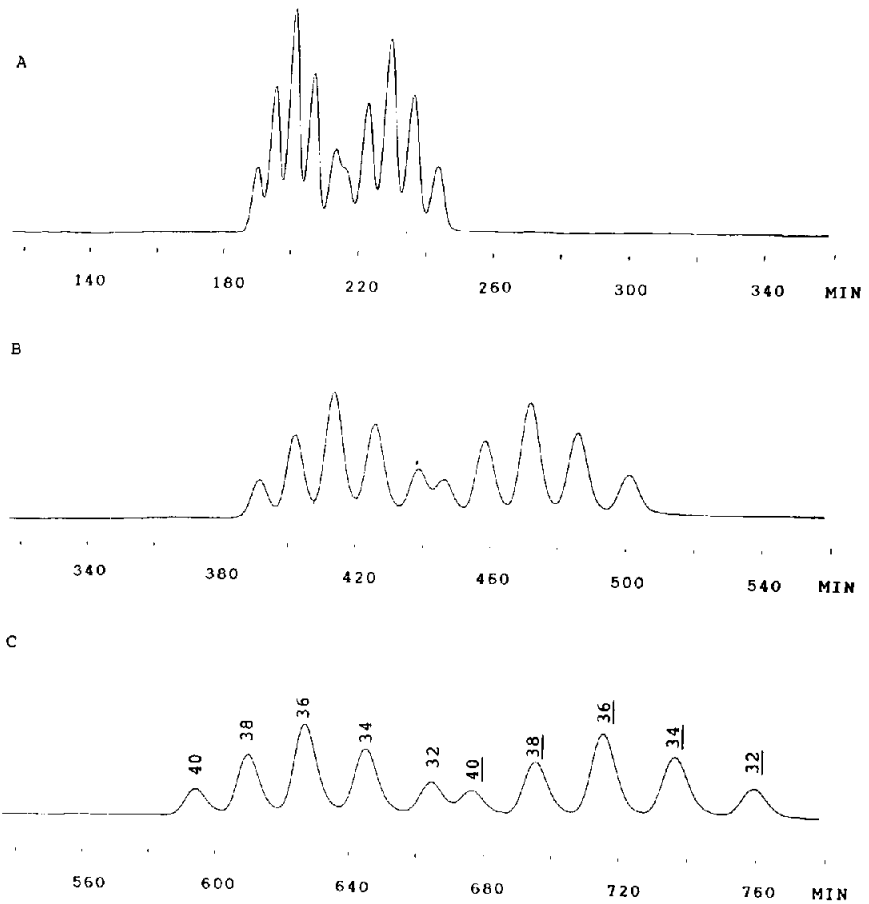

Fig. 4. HPLC separation of saturated diacylglycerols synthesized from palmitic, stearic and arachidic acids as their 3,5-DNPUs recycling through an OA-4100 chiral column. Flow-rate: 0.25 ml/min. Mobile phase: hexane–dichloroethane–ethanol, 170:10:1. Recycle: (A) original chromatogram; (B) after first recycle; (C) after second recycle. Above each peak in (C) is given the acyl carbon number of the diacylglycerol. Underlined numbers are acyl carbon numbers of *sn*-2,3-enantiomers.

silver nitrate- and boric acid-impregnated silicic acid plates in a preliminary separation [7]. Similar critical pairs have been found with the diacylglycerols such as the pair of 1,2-18:1-18:0- and 1,2-16:0-18:0-*sn*-glycerols. The pair can be separated by the silver complex method using HPLC or TLC.

Fig. 6 shows linear relationships between the logarithm of the retention volume (V_r) and the number of unsaturated acyl groups for each homologous series of enantiomers. The straight lines are approximately parallel for each homologous series. Therefore, the relationship between retention volumes of *sn*-1,2- and *sn*-2,3-enantiomers can be expressed by the following equations:

$$2 \log V_r(\text{SU-}sn\text{-}1,2) = \log V_r(\text{SS-}sn\text{-}1,2) + \log V_r(\text{UU-}sn\text{-}1,2) \quad (3)$$

$$2 \log V_r(\text{SU-}sn\text{-}2,3) = \log V_r(\text{SS-}sn\text{-}2,3) + \log V_r(\text{UU-}sn\text{-}2,3) \quad (4)$$

$$E = \log V_r(sn\text{-}2,3) - \log V_r(sn\text{-}1,2) \quad (5)$$

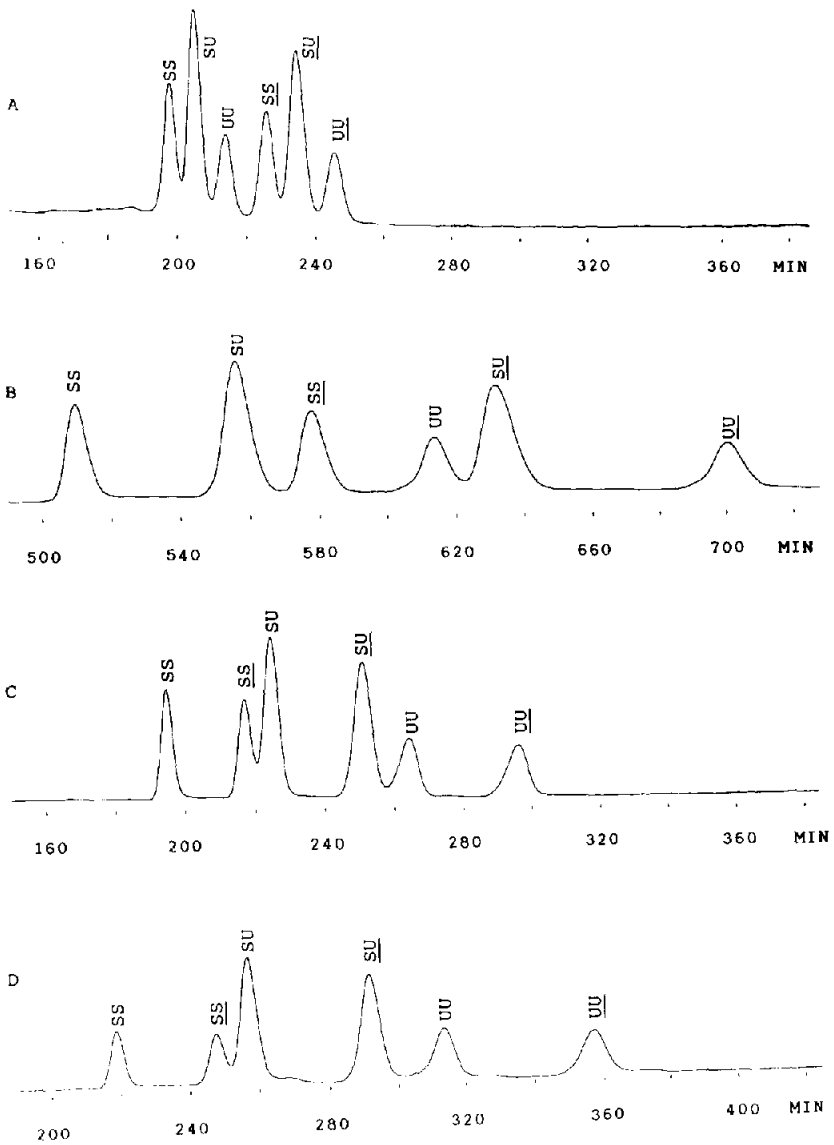

Fig. 5. HPLC separation of diacylglycerols synthesized from saturated and unsaturated acids as their 3,5-DNPUs on an OA-4100 chiral column. (A) Synthesized from stearic and oleic acids; (B) synthesized from stearic and linoleic acids; (C) synthesized from stearic and linolenic acids; (D) synthesized from stearic and eicosapentaenoic acids. Flow-rate: (A), (C) and (D) 0.5 and (B) 0.25 ml/min. Mobile phase: hexane–dichloroethane–ethanol, (A), (C) and (D) 250:20:1 and (B) 170:10:1. Recycle: (A), (C) and (D) original chromatogram; (B) after first recycle. SS, SU snd UU: see text. Underlined letters are those for *sn*-2,3-enantiomers.

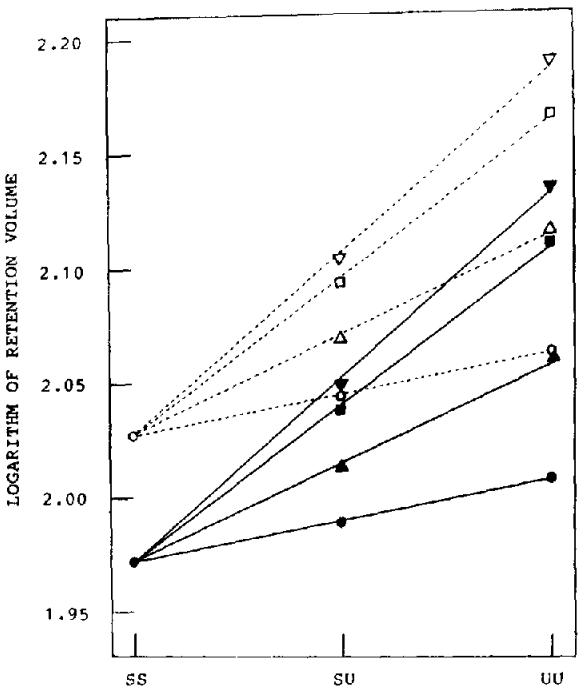

Fig. 6. Plot of logarithm of retention volume *vs.* diacylglycerols synthesized from saturated and unsaturated acids as their 3,5-DNPUs separated by HPLC. SS, SU and UU: see text. Full lines are for *sn*-1,2-enantiomers and dashed lines for *sn*-2,3-enantiomer lines. ●, ○ = Synthesized from stearic and oleic acids; ▲, △ = synthesized from stearic and linoleic acids; ■, □ = synthesized from stearic and linolenic acids; ▼, ▽ = synthesized from stearic and eicosapentaenoic acids. Flow-rate: 0.5 ml/min. Mobile phase: hexane–dichloroethane–ethanol, 250:20:1.

where V_r(SU-*sn*-1,2) and V_r(SU-*sn*-2,3) are the retention volumes of 1,2- and 2,3-diacyl-*sn*-glycerol enantiomers each having one saturated and one unsaturated acyl group and E is the enantiomer separation factor. The slope increases with increasing number of double bonds in the unsaturated acyl group (Fig. 6). Eqns. 3 and 4 are useful for calculating the retention times of the diacid-diacylglycerols with unsaturated and saturated acyl groups from those of the corresponding monoacid-diacylglycerols.

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