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Short communication

Highly sensitive high-performance liquid chromatographic method to discriminate enantiomeric monoacylglycerols based on fluorescent chiral derivatization with (*S*)-(+)-2-*tert*.-butyl-2-methyl-1,3-benzodioxole-4-carboxylic acid

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Abstract

As an extension of previous methods for enantiomer analyses of diacylglycerols, a highly sensitive HPLC method was developed for the determination of the absolute configuration and optical purity of monoacylglycerols. Chiral derivatization by a fluorescent (*S*)-TBMB carboxylic acid followed by a normal-phase HPLC separation of the derived diastereomeric di-(*S*)-TBMB-carbonyl-*sn*-1- and -*sn*-3-monoacylglycerols provided useful tools to determine the chirality of a series of saturated and unsaturated monoacylglycerols ($C_{12:0}$–$C_{18:0}$, $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$) [(*S*)-TBMB = (*S*)-(+)-2-*tert*.-butyl-2-methyl-1,3-benzodioxole]. In addition, the HPLC elution times of each diastereomeric isomer were correlated with the chain length (carbon number) and the double bond numbers of acyl groups.

1. Introduction

The development of a simple method to determine the enantiomeric distribution of monoacylglycerols (*sn*-1 and *sn*-3) will contribute to stereochemical studies of fatty acids, e.g., to study the stereoselectivity of lipase reactions or the biological functions of naturally occurring monoacylglycerols. Thomas et al. [1] developed a simple method to separate *sn*-1- and *sn*-3-monoacylglycerols from the *sn*-2-isomer by TLC on silica gel impregnated with boric acid. Other methods have been proposed for separating the homologous monoacylglycerols by reversed-phase HPLC without derivatization [2–4], but the enantiomeric separation of monoacylglycerols could not be achieved. Recently, Takagi and co-workers [5,6] reported the first successful separation of the enantiomeric monoacylglycerols using a chiral HPLC column coupled with di-3,5-dinitrophenylurethane derivatization.

Previously, we have reported highly sensitive methods for determining the optical purity and absolute configuration of diacylglycerols base on the fluorescent derivatization with (*S*)-(+)-2-*tert*.-butyl-2-methyl-1,3-benzodioxole [(*S*)-TBMB] carboxylic acid followed by HPLC separation of the derived diastereoisomers [7,8]. In this work, we extended this approach to mono-

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acylglycerols and propose a simple and highly sensitive HPLC method for separating the enantiomers. Various normal-phase silica gel HPLC modes were tested for the diastereomeric separation of a series of monoacylglycerols ($C_{12:0}$–$C_{18:0}$, $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$), and this paper describes the HPLC results, which allowed complete separation between the *sn*-1- and *sn*-3-isomers and also the *sn*-2-isomer within 80 min.

2. Experimental

2.1. Chemicals

Racemic monoacylglycerols with $C_{12:0}$–$C_{18:0}$, $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$ acyl groups were purchased from Sigma (St. Louis, MO, USA). Optically active 3-monopalmitoyl-*sn*-glycerol and 2-monopalmitoylglycerol were also obtained from Sigma. (*S*)-(+)-2,2-Dimethyl-1,3-dioxolane-4-methanol was obtained from Tokyo Kasei (Tokyo, Japan) for the preparation of homologous diastereomeric 1,2-di-O-(*S*)-TBMB-carbonyl-3-O-acyl-*sn*-glycerol derivatives ($C_{12:0}$, $C_{14:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$). (*S*)-TBMB-COOH (100% e.e.) was synthesized according to the previously described method [9].

2.2. Di-(S)-TBMB derivatization of monoacylglycerols

Monoacylglycerols were derivatized with (*S*)-TBMB carbonyl chloride [8] as follows. A dry pyridine solution [0.2 ml containing 10% of 4-dimethylaminopyridine (DMAP)] of (*S*)-TBMB-COCl (20 mg, 0.08 m*M*) was added to a solution of 1-monopalmitoyl-*rac*-glycerol (7.0 mg, 0.02 m*M*) in dry CH_2Cl_2 (2 ml) with stirring at room temperature. After 2 h, the reaction mixture was diluted with CH_2Cl_2 (10 ml) and washed with saturated $NaHCO_3$ solution (3 × 10 ml) and water (20 ml). The methylene chloride solution was dried over $MgSO_4$, the latter was removed by filteration and the solvent was evaporated in vacuo at 40°C to afford 1,2-di-O-(*S*)-TBMB-carbonyl-3-O-palmitoyl-*rac*-glycerol, which was purified by preparative TLC [*n*-hexane–ethyl acetate (10:1, v/v)] (13 mg, yield 81%).

Di-(*S*)-TBMB-carbonyl derivatizations of 3- and 2-monopalmitoyl-*sn*-glycerols and other commercially available racemic monoacylglycerols ($C_{12:0}$, $C_{14:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$) were conducted in the same manners.

Optically active other homologous diastereomeric 1,2-di-O-(*S*)-TBMB-carbonyl-3-O-acyl-*sn*-glycerols ($C_{12:0}$, $C_{14:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$) were prepared via five steps (benzylation, deisopropylidenation, di-(*S*)-TBMB carbonylation, catalytic debenzylation and acylation using the corresponding acyl chloride) from an optically active (*S*)-(+)-2,2-dimethyl-1,3-dioxolane-4-methanol in a similar manner to that described in a previous paper [8] for the preparation of the 3-O-(*S*)-TBMB-carbonyl-1,2-di-O-acyl-*sn*-glycerol derivatives.

2.3. HPLC separations

Prior to the HPLC injection, the reaction mixture of di-(*S*)-TBMB-carbonyl-monoacylglycerol derivatives was preliminarily purified on silica gel TLC sheet [*n*-hexane–ethyl acetate (10:1, v/v)]. The TLC band of the derivatives was cut off from the TLC sheet and extracted with the HPLC solvents.

HPLC separations were conducted with a Jasco (Tokyo, Japan) Model 880-PU instrument connected to a Tosoh Model FS-8010 fluorescent detector with excitation at 310 nm and emission at 370 nm. Separations were performed on a Develosil 60-3 (Nomura Chemical) silica gel column (stainless steel, 50 cm × 4.6 mm I.D.). The analyses were carried out isocratically using HPLC-grade *n*-hexane–*tert.*-butyl alcohol (250:1, w/w; flow-rate 0.6 ml/min) as the mobile phase at ambient temperature. For quantitative determination, peak areas were calculated with a Model 807-IT integrator (Jasco).

3. Results and discussion

(*S*)-TBMB-COOH used in this study is optically pure to determine directly the optical

purities of monoacylglycerols as their diasteromeric di-(*S*)-TBMB-carbonyl derivatives. Di-(*S*)-TBMB-carbonyl derivatization of monoacylglycerols was performed in more than 80% yield using more than a four-fold excess of (*S*)-TBMB-COCl according to the optimized reaction conditions detailed under Experimental (Fig. 1). The same derivatization procedure as described under Experimental could be applied for analytical purposes to a sub-μg level of monoacylglycerols. In this case, the reaction mixture was directly spotted on the TLC sheet and developed with *n*-hexane–ethyl acetate (10:1, v/v). The fluorescent spots ($R_F = 0.30$–0.35) corresponding to di-(*S*)-TBMB-carbonylated monoacylglycerols were cut off from the TLC sheet and extracted with the HPLC solvents (*n*-hexane–*tert.*-butyl alcohol) for direct HPLC injection. This simple and convenient work-up procedure prior to the HPLC analysis was also employed in our previous study for the determination of diacylglycerols [8]. This procedure, taking a few minutes using a ready-made aluminium TLC sheet (5 cm × 5 cm), is recommended for eliminating pyridine and DMAP and their salts, which are unfavourable towards the silica column.

In contrast to the case of diacylglycerols in our previous study [8], the separation of enantiomeric monoacylglycerols as the (*S*)-TBMB-carbonyl derivatives could not be achieved straightforwardly. Various investigations of the HPLC conditions led us finally to use a longer silica gel column (Develosil 60-3, 50 cm) and *n*-hexane-*tert.*-butyl alcohol (250:1, w/w) as the mobile phase. Under these conditions, the separation of enantiomeric monoacylglycerols was accomplished in 80 min with a resolution factor (R_s) above 1.50.

Fig. 2 shows typical HPLC profiles of monoacylglycerols as the fluorescent diastereomeric di-(*S*)-TBMB-carbonyl derivatives. The *sn*-2-monoacylglycerol isomer could also be completely separated both from the monoacyl *sn*-1- and *sn*-3-isomers. The HPLC studies using chiral monoacylglycerols with known configurations indicated that the *sn*-1-isomers were eluted faster than the *sn*-3-isomers for any saturated and

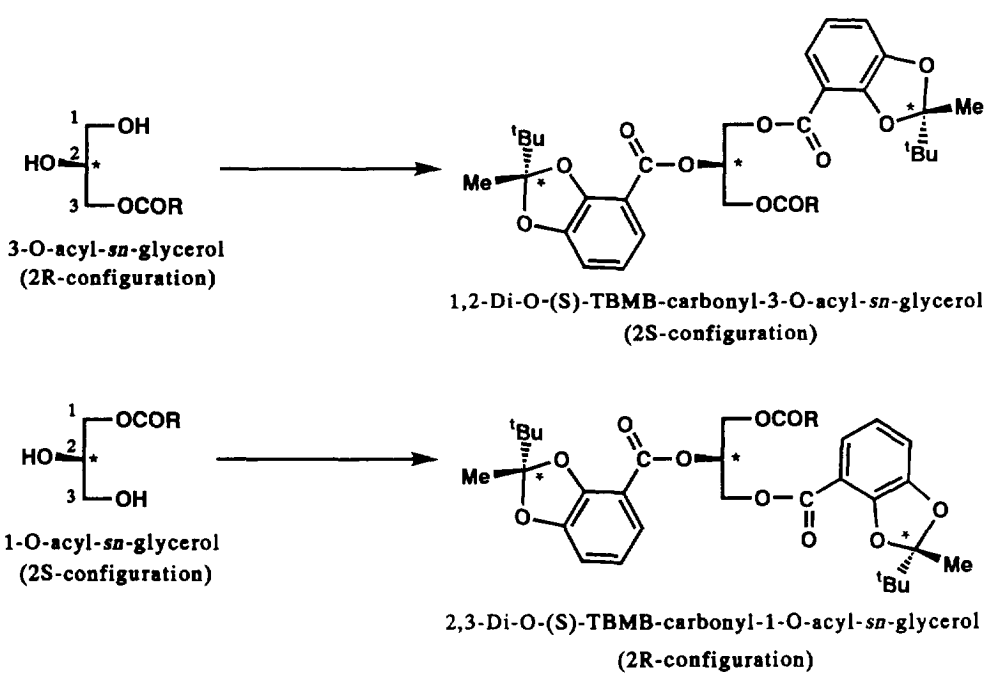

Fig. 1. Scheme for the direct derivatization of monoacylglycerols with (*S*)-TBMB-COCl forming diastereomeric derivatives: (*S*)-TBMB-COCl, pyridine, 4-dimethylaminopyridine (DMAP), room temperature.

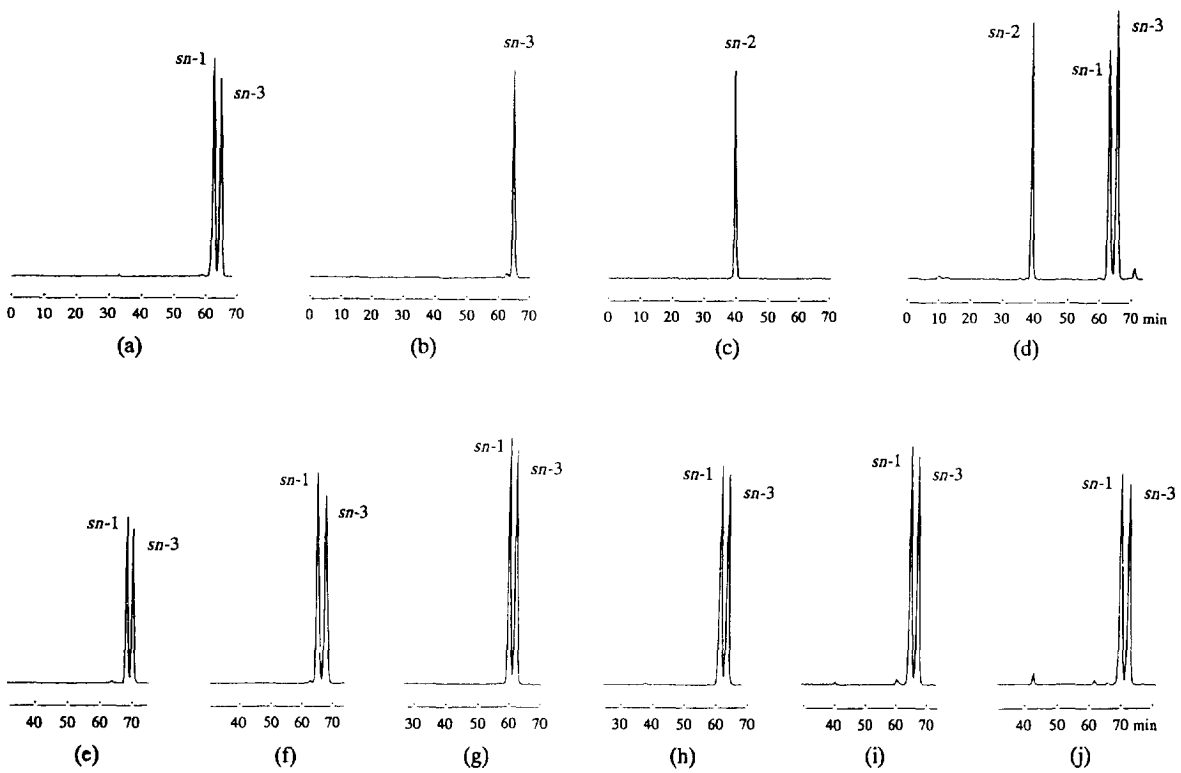

Fig. 2. Typical HPLC separations of each di-(*S*)-TBMB carbonylated homologous saturated and unsaturated monoacylglycerol. (a) *rac*-Monopalmitoyl-; (b) *sn*-3-monopalmitoyl-; (c) *sn*-2-monopalmitoyl-; (d) monopalmitoyl mixture [(a) + (b) + (c)]; (e) *rac*-monolauroyl-; (f) *rac*-monomyristoyl-; (g) *rac*-monostearoyl-; (h) *rac*-monooleoyl-; (i) *rac*-monolinoleoyl-; and (j) *rac*-monolinolenoyl-; *sn*-1, *sn*-2 and *sn*-3 in each chromatogram represent each position of the monoacyl group. HPLC conditions: silica gel column (Develosil 60-3, 50 cm × 4.6 mm I.D.); λ_{ex} = 310 nm, λ_{em} = 370 nm; eluent, *n*-hexane–*tert.*-butyl alcohol (250:1, w/w); flow-rate, 0.6 ml/min; temperature, 22–24°C.

unsaturated monoacylglycerols ($C_{12:0}$–$C_{18:0}$, $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$) examined here. Although the separation of enantiomeric monoacylglycerols took longer with a longer silica column compared with the analysis of diacylglycerols (Develosil 60-3, 25 cm, $R_s > 2.0$), all enantiomers of saturated and unsaturated monoacylglycerols studied here could be separated with nearly identical separation coefficients (α = 1.05) and peak resolutions (R_s = 1.60), as shown in Table 1.

In Fig. 3, we have plotted the logarithm of the retention volume (log V_r) versus the acyl carbon numbers (*CN*) and double (olefinic) bond numbers (*DN*) for each homologous series of isomeric *sn*-1- and *sn*-3-monoacylglycerols. Although some plots for the logarithmic retention volumes (log V_r) and various *DN* showed slightly positive deviations from the linear equation (Fig. 3B), their relationship could be approximated by the following equations: for the log V_r versus *CN* of saturated monoacylglycerols, log V_r (*sn*-1) = −0.011 *CN* + 1.67, log V_r (*sn*-3) = −0.012 *CN* + 1.70 and $E(CN)$ = log V_r (*sn*-3) − log V_r (*sn*-1) ≈ 0.03; and for log V_r versus *DN* of unsaturated monoacylglycerols, log V_r (*sn*-1) = 0.027 *DN* + 1.47, log V_r (*sn*-3) = 0.028 *DN* + 1.49 and $E(DN)$ = log V_r (*sn*-3) − log V_r (*sn*-1) ≈ 0.02, where E is the diastereomer separation factor.

In order to confirm the reproducibility and the quantitative aspects of the present method, monopalmitoylglycerols with known optical purities were derivatized with (*S*)-TBMB-COCl and subjected to the HPLC analysis (Table 2).

Table 1
Chromatographic data for homologous monoacylglycerols as their di-(*S*)-TBMB-carbonyl derivatives

Acyl group	Position	V_r (ml)	k'	α	R_s
Monolauroyl	*sn*-1	34.64	5.66	1.05	1.57
	sn-3	36.33	5.94		
Monomyristoyl	*sn*-1	32.88	5.37	1.05	1.60
	sn-3	34.54	5.64		
Monopalmitoyl	*sn*-2	18.38	3.00	1.69	19.95
	sn-1	31.11	5.08	1.05	1.58
	sn-3	32.56	5.32		
Monostearoyl	*sn*-1	29.75	4.86	1.04	1.55
	sn-3	31.01	5.07		
Monooleoyl	*sn*-1	31.03	5.07	1.04	1.54
	sn-3	32.40	5.29		
Monolinoleoyl	*sn*-1	33.16	5.42	1.05	1.60
	sn-3	34.90	5.70		
Monolinolenoyl	*sn*-1	35.77	5.85	1.05	1.67
	sn-3	37.57	6.14		

V_r = retention volume corrected by column void volume (6.12 ml); k' = capacity factor; α = separation coefficient; R_s = peak resolution.

Good agreement could be obtained for the optical purities assessed by the present HPLC method before and after the (*S*)-TBMB-carbonyl derivatization within the usual limit of variation (S.D. = 2.07, $n = 7$). The very small but significant deviation (ca. 2%) might be due mainly to the partial racemization of commercially available 3-monopalmitoyl-*sn*-glycerol during storage. In any event, this result showed that the peak areas of the two diastereomeric di-(*S*)-TBMB carbonylated monoacylglycerols can be used directly to determine the optical purities of original monoacylglycerols within a ca. 2% error without a calibration process. The detection limit of 3-monopalmitoyl-*sn*-glycerol (2*R*-configuration) as its di-(*S*)-TBMB carbonylate (2*S*-configuration) was 0.3 pmol on-column (signal-to-noise ratio = 3) owing to the fluorescence of the (*S*)-TBMB carbonyl chromophore.

We applied the method to check the racemization of chiral 3-monopalmitoyl-*sn*-glycerol under the acidic to basic conditions of pH 4.0 (phthalate buffer), 6.9 (phosphate buffer) and 9.2 (Na_2HCO_3 buffer). The results revealed that no racemization occurred in the pH range 4.0–9.2 at least for 1 week at room temperature; the optical purity of 3-monopalitoyl-*sn*-glycerol, initially ca. 96% e.e. as can be seen in Table 2, was kept constant at 95–96% e.e in all the pH solutions examined for 1 week. These results will be useful for studying the stereoselectivities of lipase-catalysed reactions at various pH values or the other enzymic and chemical reactions of glycerolipids.

In this work, we have extended our previous analytical strategy for diacylglycerols using (*S*)-TBMB-carbonyl labelling and HPLC analysis to monoacylglycerols to determine the optical purity and the absolute configuration. Under the present HPLC conditions using a normal-phase silica column (Develosil 60-3), the *sn*-1- and

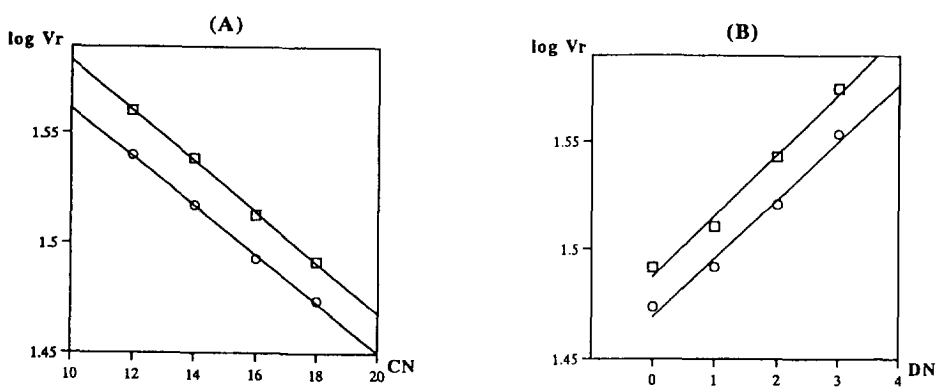

Fig. 3. Relationships between log V_r (retention volume) and (A) *CN* (number of acyl carbon atoms, $C_{12:0}$–$C_{18:0}$) and (B) *DN* (number of double bonds, $C_{18:0}$–$C_{18:3}$) for homologous and isomeric monoacylglycerols as their (*S*)-TBMB derivatives separated by HPLC on a silica gel column (Develosil 60-3). ○ = Log V_r (*sn*-1); □ = log V_r (*sn*-3).

Table 2
Comparison of optical purities before and after the derivatization of standard monoacylglycerols

Standard monoacylglycerol mixtures before derivatization[a]			After derivatization with (*S*)-TBMB-COCl[b]		
1-Monopalmitoyl-*rac*-glycerol (racemate) (mg)	3-Monopalmitoyl-*sn*-glycerol (*sn*-3) (mg)	Calculated optical purity (% e.e.)	Average observed optical purity (% e.e.)		S.D.
1.0	0	0	0.0	($n = 4$)	2.83
0.82	0.42	33.9	30.6	($n = 4$)	0.26
0.41	0.84	67.2	65.1	($n = 4$)	1.69
0	1.0	100	96.3	($n = 7$)	2.07

[a] Each standard solution was prepared by mixing the racemate and 3-monopalmitoyl-*sn*-glycerol in the appropriate ratio, and their optical purity was calculated from the ratio of the racemate and 3-monopalmitoyl-*sn*-glycerol contents [% e.e. before derivatization = *sn*-3/(racemate + *sn*-3) · 100].

[b] The HPLC peak areas of di-(*S*)-TBMB-carbonyl-monoacylglycerol derivatives (*sn*-1 and *sn*-3) derived from each standard monoacylglycerol mixture were used directly to determine the optical purity of the mixture of monoacylglycerols without correction [% e.e. after derivatization = (peak area of *sn*-3 − peak area of *sn*-1)/(peak area of *sn*-1 + peak area of *sn*-3) · 100].

sn-3-monoacyl enantiomers and the *sn*-2-isomer, in addition to the corresponding diacylglycerols, were simultaneously separated from each other within 80 min, and the analysis could be performed with less than 1 pmol of mono- and diacylglycerols, taking advantage of the strong fluorescence of the (*S*)-TBMB-carbonyl group.

We shall apply this approach to study the stereoselectivities of lipase reactions producing mono- and diacylglycerols and to clarify the separation mechanism of the enantiomeric mono- and diacylglycerols with this agent.

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