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Separation of hydroxylated polyenoic fatty acid enantiomers on Pirkle-type chiral phase high-performance liquid chromatographic columns

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ABSTRACT

Enantiomers of monohydroxy polyenoic fatty acids containing a conjugated diene system can be separated on an (*R*)-(−)-N-(3,5-dinitrobenzoyl)-α-phenylglycine (DNBPG) column. For all hydroxy fatty acids tested the *S*-isomer is earlier eluted than the *R*-isomer. Columns with ionically linked chiral phases show a better enantiomer resolution than those with covalently linked phases. Derivatization of the hydroxy polyenoic fatty acids to their corresponding benzoyl or naphthoyl derivatives strongly improved the enantiomer resolution, but reversed the elution order. Various Pirkle-type chiral stationary phases and several derivatization procedures were tested to optimize the enantiomer resolution. The best resolutions were achieved for the separation of the naphthoyl esters on a DNBPG column. There are considerable differences in the enantiomer resolution between columns obtained from different manufacturers. Some racemic diastereomers of dihydroxy polyenoic fatty acids which cannot be separated by reversed- and normal-phase high-performance liquid chromatography were separated on (*R*)-DNBPG columns.

INTRODUCTION

Chiral phase high-performance liquid chromatography (HPLC) is increasingly the method of choice for the analytical and preparative separation of optical isomers [1,2]. For a variety of compounds, in particular those containing aromatic residues, enantiomer resolution on Pirkle-type chiral stationary phases has been reported [3,4]. Two years ago we introduced chiral phase HPLC on an ionically linked (*R*)-(−)-N-(3,5-dinitrobenzyl)-α-phenylglycine (DNBPG) column (Baker, Phillipsbourgh, NJ, U.S.A.) as a convenient method for the determination of the enantiomer composition of lipoxygenase-derived hydroxylated polyenoic fatty acid methyl esters [5]. This was the first report on an enantiomer separation of non-aromatic compounds on this type of chiral stationary phase. In comparison with the methods used previously for structure elucidation of hydroxylated polyenoic fatty acids, based on the chromatographic separation of diastereomers formed by derivatization with a chiral resolving agent [6–8], chiral phase HPLC offers several advantages: simplicity, high sensitivity and no derivatization procedure. However, the preparative application of this method on the milligram scale has been restricted by the fact that the underivatized hydroxy polyenoic fatty acid methyl esters are only partly separated with resolution values ranging

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from 0.8 to 1.2. Therefore, we modified this method by a simple derivatization procedure. Introduction of an aromatic ester residue at the OH group of the hydroxy polyenoic fatty acid methyl ester (benzoylation or naphthoylation) strongly improved the enantiomer resolution on DNBPG columns [9].

In recent studies we tested various chiral stationary phases of the Pirkle type and some commercially available DNBPG columns for their suitability to separate the enantiomers of derivatized and underivatized hydroxylated polyenoic fatty acid methyl esters. Further, the suitability of DNBPG columns for the separation of racemic diastereomeric dihydroxy fatty acids was tested.

EXPERIMENTAL

Materials

Linoleic acid, linolenic acid, arachidonic acid, bis(trimethylsilyl)trifluoroacetamide and α-tocopherol (D/L) were obtained from Serva (Heidelberg, F.R.G.). Soybean lipoxygenase I was purchased from Sigma (St. Louis, MO, U.S.A.). Benzoyl chloride, 1-naphthoyl chloride, triphenylphosphine and (−)-menthyl chloroformate were obtained from Aldrich (Milwaukee, WI, U.S.A.). Sodium borohydride was purchased from Ferak (Berlin, F.R.G.). All solvents were of analytical reagent grade and distilled prior to use.

Chiral phase HPLC columns

The following commercially available chiral columns were tested: DNBPG column (250 × 5 mm I.D., 5 μm particle size; ionically linked phase) from Baker, DNBPG column (250 × 5 mm I.D., 5 μm particle size, ionically linked phase) from Regis (Morton Grove, IL, U.S.A), DNBPG column (250 × 5 mm I.D., 5 μm particle size, covalently linked phase) from Regis, DNBPG column (250 × 5 mm I.D., 5 μm particle size, covalently linked phase) from Baker, DNBPG column (250 × 5 mm I.D., 5 μm particle size, irregular, covalently linked phase) from Serva, DNBPG column (125 × 5 mm I.D., 5 μm particle size, spherical, covalently linked phase) from Serva, DNBPG column (250 × 5 mm I.D., 5 μm particle size; ionically linked phase) from Serva, DNB-Leu column(250 × 5 mm I.D., 5 μm particle size, covalently linked phase) from Baker. The chiral columns listed in Table I were prepared in the laboratory of Professor W. H. Pirkle (School of Chemical Sciences, University of Illinois, Urbana-Champaign, IL, U.S.A.). The resolution values were calculated according to the equation $R_s = (t_2 - t_1)/0.5(t_{w_2} - t_{w_1})$, where t_1 and t_2 are the retention times of the enantiomers (apex of the peaks) and t_{w_2} and t_{w_1} the band widths of the peaks. The R_s values of the enantiomers were generally determined from the separation of a racemic mixture.

Instruments

HPLC analysis and preparations were carried out on a Shimadzu HPLC system coupled with a Hewlett-Packard model 1040 A diode-array detector which was connected to an HP ChemStation. Gas chromatographic–mass spectrometric (GC–MS) analyses were performed on a Hewlett-Packard 5995 GC–MS system equipped with an SE-54 column (25 m × 0.24 mm I.D.). IR spectra were recorded with a Digilab FTS-15C Fourier transform spectrophotometer and UV spectra with a Specord M 40

spectrophotometer (Carl Zeiss, Jena, G.D.R.) in methanol. Circular dichroism (CD) measurements were performed with a Jouan Russel 185 CD photometer in methanol.

Preparations

A mixture of all positional and optical isomers of hydroxyarachidonic acid (HETE) and hydroxylinoleic acid (HODE) was prepared by vitamin E-controlled autoxidation of the fatty acid methyl esters [10].[a] Briefly, 100 mg of the fatty acid methyl ester were mixed with 20 mg of α-tocopherol and incubated for 72 h at 40°C under an oxygen atmosphere. The mechanism of the oxygenation involves hydrogen removal, double-bond conjugation with *cis–trans* isomerization of one double bond and the introduction of molecular oxygen forming the hydroperoxy fatty acid methyl ester (Fig. 1). Vitamin E was used to prevent the hydroperoxy fatty acids from radical-mediated decomposition via hydroperoxidase reactions [10]. The formation of the *cis,trans*-conjugated diene chromophore with its absorbance maximum at 235 nm can be followed over time as an indicator of the oxygenation reaction. When the absorbance at 235 nm did not increase further, the products were dissolved in 5 ml of methanol–water (8:2, v/v) and reduced by addition of a molar excess of sodium borohydride. The different positional isomers of the hydroxy polyenoic fatty acids were subsequently separated by reversed-phase (RP) and normal-phase (NP) HPLC. According to the mechanism of the autoxidation, one would expect for a fatty acid with n double bonds $2(n-1)$ positional isomers of hydroperoxy derivatives containing a conjugated diene system each of which consists of two enantiomers. Therefore, from linoleic acid with two double bonds four product isomers, from linolenic acid eight product isomers and from arachidonic acid twelve product isomers are formed.

The different isomers of (8*R*/*S*,15*S*)-DiHETE and (8*R*/*S*,15*R*/*S*)-DiHETE were prepared by the same method except that (15*S*)-HETE methyl ester and (15*R*/*S*)-HETE methyl ester, respectively, were used as substrate. Authentic standards of chiral (15*S*)-HETE, (13*S*)-HODE and (8*S*,15*S*)-DiHETE were prepared from arachidonic acid and linoleic acid, respectively, with soybean lipoxygenase [11,12]. (9*S*)-HODE was obtained by the reaction of tomato fruit lipoxygenase with linoleic acid

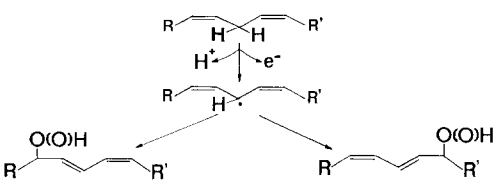


Fig. 1. Autoxidation of polyenoic fatty acids.

[a] The following abbreviations are used: 15-HETE = 15-hydroxy-(5*Z*,8*Z*,11*Z*,13*E*)-eicosatetraenoic acid; 12-HETE = 12-hydroxy-(5*Z*,8*Z*,10*E*,14*Z*)-eicosatetraenoic acid; 11-HETE = 11-hydroxy-(5*Z*,8*Z*,12*E*,14*Z*)-eicosatetraenoic acid; 9-HETE = 9-hydroxy-(5*Z*,7*E*,11*Z*,14*Z*)-eicosatetraenoic acid; 8-HETE = 8-hydroxy-(5*Z*,9*E*,11*Z*,14*Z*)-eicosatetraenoic acid; 5-HETE = 5-hydroxy-(6*E*,8*Z*,11*Z*,14*Z*)-eicosatetraenoic acid; 13-HODE = 13-hydroxy-(9*Z*,11*E*)-octadecadienoic acid; 9-HODE = 9-hydroxy-(10*E*,12*Z*)-octadecadienoic acid; 8,15-DiHETE = 8,15-dihydroxy-[5*Z*,9*E*,11*Z*(*E*),13*E*]-eicosatetraenoic acid; DNBPG = (*R*)-(−)-N-(3,5 dinitrobenzoyl)-α-phenylglycine.

[13]. All compounds were identified by UV spectrometry, co-chromatography with authentic standards and/or by GC–MS. For (13*S*)-HODE, (15*S*)-HETE and (8*S*,15*S*)-DiHETE the absolute configuration of the chiral centres was determined by converting the compounds to their menthoxycarbonyl derivatives, oxidative ozonolysis and subsequent GC separation of the diastereomeric fragments [14].

Derivatization

Fatty acids were methylated with diazomethane in diethyl ether. Alkaline hydrolysis was achieved by incubating the methyl esters in methanol–40% KOH (7:1, v/v) for 30 min at 60°C under argon. Menthoxycarbonyl derivatives of the hydroxy fatty acids were obtained by the reaction with (−)-menthylchloroformate (15 min at 60°C) in toluene–pyridine (4:1, v/v). Hydroxy groups were silylated by reaction with BSTFA (15 min at 60°C) in the presence of 10% pyridine.

Benzoyl and naphthoyl derivatives of the hydroxy fatty acid methyl esters were obtained by reaction with a 10-fold molar excess of benzoyl chloride and 1-naphthoyl chloride, respectively, in pyridine [9]. After 20 min at 60°C under argon the solvent was evaporated, the residue was dissolved in methanol–water (95:5, v/v) and the derivatives were purified by RP-HPLC on a Zorbax ODS column (250 × 4.6 mm I.D., 5 μm particle size) with methanol–water (95:5, v/v) as eluent. The retention times were 15 min for the benzoyl esters and 25 min for the naphthoyl derivatives.

Urethane derivatives were prepared by reaction of the hydroxy fatty acid methyl esters with a 4-fold molar excess of dinitrobenzoyl isocyanate in toluene–pyridine (9:1, v/v) at 60°C for 1 h under argon [15]. The urethane derivatives were purified by RP-HPLC with methanol–water (90:10, v/v) as eluent.

RESULTS

Separation of enantiomers of various monohydroxy polyenoic fatty acids

The positional isomers of the hydroxy polyenoic fatty acids formed via vitamin E-controlled autoxidation can be separated by NP- and/or RP-HPLC. In Fig. 2 a representative NP-HPLC trace for a mixture of the autoxidation products of linoleic acid and arachidonic acid is shown. It can be seen that all positional isomers of hydroxylinoleic acid (HODEs) and all positional isomers of hydroxyarachidonic acid (HETEs) are well separated from each other. All the compounds detected were characterized by the typical conjugated diene chromophore. However, there are some spectral difference, as the λ_{max} values for certain isomers are different [16]. Because of these differences one can differentiate, for instance, between 8-HETE and 9-HODE (inset in Fig. 2), which are not well resolved in NP-HPLC.

The optical isomers of hydroxylated polyenoic fatty acid methyl esters which cannot be separated by NP- and RP-HPLC can be resolved by HPLC on a DNBPG column. As shown in Fig. 3, a racemic mixture of 13- and 9-HODE methyl esters is split into two pairs of compounds (Ia and Ib, IIa and IIb). The IR, UV and mass spectra of Ia and Ib and those of IIa and IIb were identical. CD measurements showed an inverse Cotton effect. These data indicate that the compounds separated are optical isomers. Co-injections of the racemic mixture with chiral standards indicated that the *S*-isomer is eluted first. This behaviour has been shown for 13- and 9-HODE and 12-, 15-, 8- and 5-HETE. The elution order *S* before *R* is, of course, not

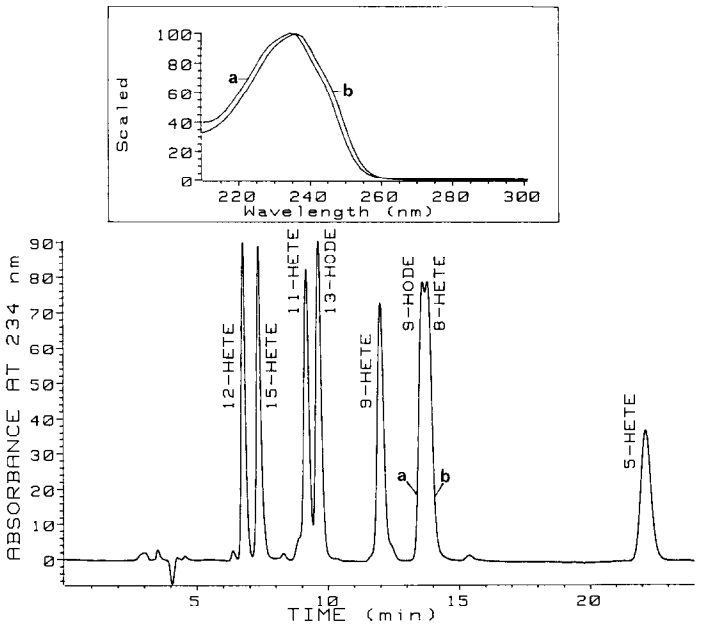


Fig. 2. NP-HPLC of a mixture of all positional isomers of hydroxylinoleic acid (HODE) and hydroxyarachidonic acid (HETE). The hydroxy fatty acid methyl esters were prepared as described under Experimental. The free hydroxy fatty acids were recovered from the methyl esters by alkaline hydrolysis and analysed by NP-HPLC on a Zorbax SIL column (250 × 4.6 mm I.D., 5 μm particle size) with hexane–2-propanol–acetic acid (100:2:0.1, v/v/v) as eluent at a flow-rate of 1 ml/min. UV spectra were measured with a Hewlett-Packard diode-array detector.

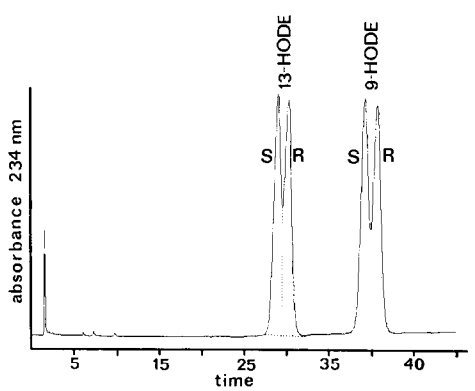


Fig. 3. Chiral phase HPLC of (13*R*/*S*)- and (9*R*/*S*)-HODE on a DNBPG column. Methyl esters of (13*R*/*S*)- and (9*R*/*S*)-HODE were prepared by autoxidation of methyl linoleate [10] and subsequent purification by NP-HPLC. Chiral phase HPLC was carried out on a Bakerbond DNBPG column (250 × 5 mm I.D., 5 μm particle size, covalently linked chiral phase) with hexane–2-propanol (100:0.5, v/v), as eluent at a flow-rate of 1 ml/min. Time in min.

trivial. For the structure elucidation of an unknown compound, a co-injection with a chiral standard is necessarry. It should be stressed, however, that for all the hydroxy polyenoic fatty acids tested so far the *S*-isomer was eluted first.

The chromatographic resolution of the underivatized enantiomers was incomplete. Variation of the temperature or flow-rates did not lead to a better resolution (not shown). In order to improve the resolution we introduced a simple derivatization procedure, converting the hydroxy polyenoic fatty acid methyl esters into their corresponding benzoyl or 1-naphthoyl derivatives. In these derivatives the aromatic residue is linked via an ester bond to the chiral centre. It can be seen from Fig. 4 that the naphthoyl derivatives (B) show the best enantiomer resolution with resolution values (R_s) higher than 2, followed by the benzoyl derivatives (A) and the underivatized methyl esters (C). As shown in Fig. 4, the elution order of the enantiomers changes after derivatization, with the *S*-isomer now being retained more strongly on the column.

The enantiomers of the free hydroxy fatty acids can also be separated on a DNBPG column (not shown). However, their resolution values are lower than those of the methyl esters. For the separation of the free acids a solvent system of *n*-hexane containing 2% 2-propanol and 0.01% acetic acid was used. In our experience the presence of acetic acid in the eluent decreases the lifetime of the column, particularly if ionically linked chiral phases are used.

In order to optimize the enantiomer resolution further, we tested a selection of Pirkle-type stationary chiral phases for their suitability for the separation of the enantiomers of hydroxy fatty acid methyl esters. Further, some derivatization procedures were checked for a possible improvement in the enantiomer separation. The results shown in Table I can be summarized as follows. Introduction of a π-base (benzoyl residue) increased the enantiomer resolution on stationary phases containing a π-acid (DNBPG). Introduction of a π-acid (DNB ester or carbamate) increased the enantiomer resolution with stationary phases containing a π-base (naphthoyl residue). If the π-base is to large, steric hindrance might be the reason for the lack of

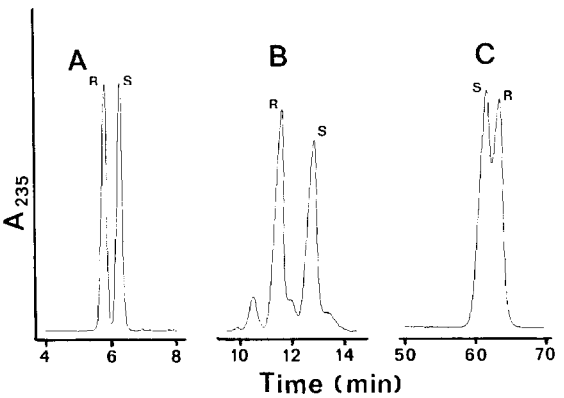
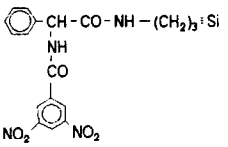
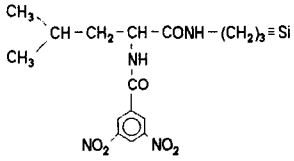
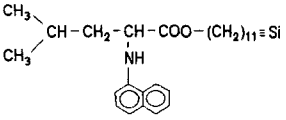
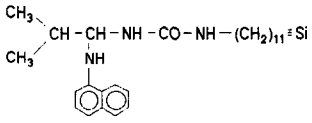
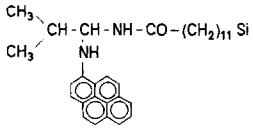


Fig. 4. Enantiomer resolution of various aromatic derivatives of (13*R*/*S*)-HODE methyl ester. The aromatic derivatives of the 13-HODE were obtained as described under Experimental. Chiral phase HPLC was carried out as described in Fig. 2 with the exception that the eluent contained 0.25% of 2-propanol. A = Benzoyl derivatives, B = naphthoyl derivatives and C = underivatized esters.

TABLE I

SEPARATION OF ENANTIOMERS OF HYDROXY FATTY ACID DERIVATIVES ON VARIOUS PIRKLE-TYPE CHIRAL STATIONARY PHASES

Eluent: hexane–isopropanol (100:1, v/v).

Chiral phase	Analyte	Resolution
	5-HETE 5-HETE benzoyl ester	0.8 2.1
	5-HETE	No separation
	5-HETE dinitrophenyl ester 5-HETE dinitrophenyl carbamate	2.1 2.0
	5-HETE dinitrophenyl carbamate	1.3
	5-HETE dinitrophenyl carbamate	No separation

enantiomer resolution. The enantiomer resolution of hydroxy fatty acids does not differ between the dinitrophenyl carbamate and the dinitrophenyl ester.

It is of particular importance for the chiral recognition mechanism that the enantiomers of hydroxy fatty acid methyl esters are separated on DNB-phenylglycine columns but that there is only a poor separation on a DNB-leucine column (Table I). This behaviour has been shown for all positional isomers tested.

During our work on the enantiomer separation of hydroxy fatty acids, we noticed that there are considerable differences in the resolving power of DNBPG columns obtained from different manufacturers. Therefore, we decided to systematically investigate this problem. The results summarized in Table II indicate that the columns obtained from Baker and Regis behave very similarly with respect to their separation properties. The columns obtained from Serva did only show a poor if any enantiomer resolution of all positional isomers tested.

TABLE II

SUITABILITY OF VARIOUS COMMERCIALLY AVAILABLE DNBPG COLUMNS FOR THE SEPARATION OF HYDROXY FATTY ACIDS ENANTIOMERS

The columns used were described under Experimental section. Chiral phase HPLC was performed with hexane–2-propanol (100:1, v/v) as eluent. The R_s values calculated from the separation of a racemic mixture are given.

Analyte	Ionic columns			Covalent columns	
	Regis	Baker	Serva	Baker	Serva
13-HODE	0.9	1.2	No separation	0.8	No separation
15-HETE	0.8	1.0	No separation	0.8	No separation
9-HODE	Not tested	0.9	No separation	0.9	No separation

Separation of racemic diastereomers of dihydroxy polyenoic fatty acids

Dihydroxy polyenoic fatty acids such as LT B_4, 8,15-DiHETE and 14,15-DiHETE are biosynthesized in a variety of cellular systems [17,18] and by pure lipoxygenases [12,19]. The relative configuration of the chiral centres of the DiHETEs (diastereomer separation) can be easily determined by RP- or NP-HPLC [20]. The determination of the absolute configuration of the chiral centres requires a complex analytical procedure including gas–liquid chromatography of the ozonolysis fragments of the methoxycarbonyl derivatives [14,20]. We tested the suitability of chiral phase HPLC on a DNBPG column for the determination of the absolute configuration of DiHETE isomers. By autoxidation of (15*R*/*S*)-HETE methyl ester all optical isomers of 8,15-dihydroxy-[5*Z*,9*E*,11(*E*,*Z*),13*E*]-eicosatetraenoate methyl ester were prepared. As standard compounds we used (8*S*/R,15*S*)-DiHETE methyl ester prepared by autoxidation of (15*S*)-HETE methyl ester [10] and (8*S*,15*S*)-DiHETE methyl ester obtained by the oxygenation of arachidonic acid by soybean lipoxygenase I [12]. Separation of the 8,15-DiHETE methyl ester diastereomers obtained by autoxidation of (15*S*)-HETE methyl ester and (15*R*/*S*)-HETE methyl ester by NP-HPLC gave an identical chromatogram (not shown).

In Fig. 5A a chromatogram of the 8,15-DiHETE isomers formed via autoxidation of (15*S*)-HETE methyl ester on a DNBPG column is shown. It looks very similar to that obtained on a silica gel column with the exception that the resolution values for the different isomers are higher on the chiral column (not shown). The structure of compounds I–IV was deduced from the elution order in NP-HPLC and from the UV and mass spectra. When the 8,15-DiHETE methyl ester isomers obtained by autoxidation of (15*R*/*S*)-HETE methyl ester were chromatographed on the same column (Fig. 5B), peaks I and IV were each split into two. The identical UV spectra suggest that the compounds separated are optical isomers. Co-injection with authentic standards of (8*S*,15*S*)-DiHETE indicates the structure of Ia and IVa to be (8*S*,15*S*)-DiHETE-(5*Z*,9*E*,11*Z*,13*E*) and (8*S*,15*S*)-DiHETE (5*Z*,9*E*,11*E*,13*E*). According to the elution order of hydroxy fatty acids (*S* before *R*), one can postulate that the late-eluting products are the corresponding (8*R*,15*R*)-DiHETEs.

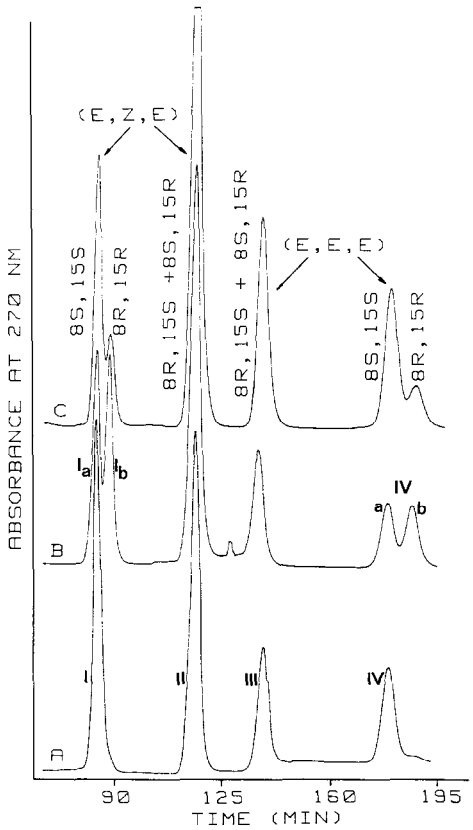


Fig. 5. Chiral phase HPLC of various 8,15-DiHETE isomers. Chiral phase-HPLC of (8*R*/*S*,15*R*/*S*)-DiHETE methyl ester and (8*R*/*S*,15*S*)-DiHETE was carried out as described in Fig. 2. (A) (15*S*)-HETE autoxidation products; (B) (15*R*/*S*)-HETE autoxidation products; (C) co-injection of A and B. I = (8*S*,15*S*)-dihydroxy-(5*Z*,9*E*,11*Z*,13*E*)-eicosatetraenoic acid; II = (8*R*,15*S*)-dihydroxy-(5*Z*,9*E*,11*Z*,13*E*)-eicosatetraenoic acid; III = (8*R*,15*S*)-dihydroxy-(5*Z*,9*E*,11*E*,13*E*)-eicosatetraenoic acid; IV = (8*S*,15*S*)-dihydroxy-(5*Z*,9*E*,11*E*,13*E*)-eicosatetraenoic acid.

DISCUSSION

Chiral phase HPLC on DNBPG columns can be used for the separation of enantiomers of hydroxylated polyenoic fatty acids. This result was unexpected as DNBPG columns were assumed to separate only enantiomers that contain aromatic residues. In fact, the hydroxylated polyenoic fatty acid methyl esters are the first family of non-aromatic compounds the enantiomers of which can be resolved on this type of chiral column. Introduction of an aromatic (benzoyl or naphthoyl) ligand improves the enantiomer resolution on DNBPG columns. In contrast, the enantiomers of the dinitrophenyl derivatives (esters or carbamates) are separated on naphthyl columns.

The mechanism of the chiral recognition for the hydroxy fatty acids has not been studied in detail. However, some mechanistic conclusions can be drawn from the

separation behaviour on different chiral phases. From theoretical considerations one would expect that the chiral recognition of the hydroxy fatty acid methyl esters involves a π–π interaction between the electron-deficient dinitrobenzoyl residue of the chiral phase with the conjugated diene system of the analyte, which is proposed to act as π-base because of the positive mesomeric effect of the OH group. In the case of the aromatic derivatives the benzoyl or naphthoyl residues are proposed to act as π-bases in the π–π interactions. The conjugated diene system is likely no longer to participate in the chiral recognition process. These changes in the binding forces might be the reason for the inverse elution order of the enantiomers of the underivatized hydroxy fatty acid methyl esters and their aromatic derivatives. The observation that the enantiomers are well resolved on DNB-phenylglycine columns but not on DNB-leucine columns suggests that the phenyl residue is also involved in the chiral phase–analyte interaction. On a naphthyl column the enantiomer resolution of the dinitrophenyl esters is comparable to that of the dinitrophenyl carbamates. These results suggest that the hydrogen bonding between the carbamate nitrogen of the analyte and the carboxylic oxygen of the chiral phase is likely not to be involved in the stereoselective retention of the enantiomers.

The reciprocal concept of the interaction of chiral stationary phases with the analyte requires three simultaneous interactions, at least one of which should be stereochemically dependent [3–5]. For the retention of hydroxy fatty acid methyl esters a π–π interaction of the conjugated diene is most probable; the other two binding forces are unknown. It might be speculated that the OH group at the hydroxy fatty acid methyl esters might form a hydrogen bond with a carbonyl function of the chiral stationary phase. Further, a steric repulsive effect of the long fatty acid chain or hydrophobic interactions with the C_{11} spacer used for coupling the chiral phase to the silica matrix may also be involved.

It is of particular interest for the user that DNBPG columns from different manufacturers show different separation properties for hydroxy polyenoic fatty acids even if the enantiomers of other compounds are comparably well resolved. In other words, a good enantiomer resolution of 1-(9-anthracyl)-2,2,2-trifluorethanol, which is usually used to test commercially available columns, does not mean that these columns are suitable for the enantiomer separation of hydroxy fatty acids. Therefore, the analyst should establish whether a particular column is suitable for that purpose before purchase.

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