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Short Communication

Enantioselective separation of some polyunsaturated epoxy fatty acids by high-performance liquid chromatography on a cellulose phenylcarbamate (Chiralcel OC) stationary phase

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ABSTRACT

cis-Epoxides of linoleic acid, α-linolenic acid, arachidonic acid and the ω3-epoxide of eicosapentaenoic acid were chromatographed on a cellulose trisphenylcarbamate (Chiralcel OC) stationary phase in the normal-phase mode. The *R,S* and *S,R* enantiomers of methyl-14(15)epoxyeicosatrienoate, methyl-9(10)epoxyoctadecadienoate and methyl-9(10)epoxyoctadecenoate could be partly resolved. The *R,S* enantiomer of methyl-14(15)epoxyeicosatrienoate eluted before the *S,R* enantiomer. [^{14}C]14(15)Epoxyeicosatrienoic acid was isolated from an incubation of [^{14}C]20:4*n* − 6 with microsomes of rabbit kidney cortex and the *S,R* enantiomer was found to predominate (about 2:1).

INTRODUCTION

Linoleic and arachidonic acids are oxygenated by P450 to *cis*-epoxides in the liver and the renal cortex [1,2]. 9*R*-Hydroxyoctadecadienoic acid (9*R*-HODE), 13*R*-HODE, 12*R*-hydroxyeicosatetraenoic acid (12*R*-HETE) and other *cis–trans* conjugated hydroxy fatty acids are also formed by P450 [1–5]. The biosynthesis of some of these epoxides and *cis–trans* conjugated hydroxy fatty acids occurs with stereoselectivity [5–8].

Chiral high-performance liquid chromatography (HPLC) is now widely used to resolve enantiomers of *cis–trans* conjugated hydroxy fatty acids [9,10]. Progress in chiral HPLC for separation of enantiomeric fatty acid epoxides has been slow. Recently, Hammonds *et al.* [11] showed that derivatized cellulose (Chiralcel OB; Chiralcel OD) could be used for separation of enantiomers of epoxides formed from arachidonic acid. Two of these epoxides, 14(15)epoxyeicosatrienoic [14(15)EpETrE] methyl ester and 5(6)-EpETrE pentafluorobenzyl ester, were resolved on Chiralcel OB, while the other two, 8(9)-EpETrE pentafluorobenzyl ester and 11(12)-EpETrE pentafluorobenzyl ester, were resolved on Chiralcel OD. Epoxides of other fatty acids were not investigated.

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Many chiral phases with derivatized cellulose are now commercially available [12], but most are very expensive. The usefulness of these chiral phases can only be determined experimentally. In the present report, a cellulose trisphenylcarbamate stationary phase (Chiralcel OC) was evaluated for separation of enantiomers of monoepoxides of different polyunsaturated fatty acids (18:2$n-6$, 18:3$n-3$, 20:4$n-6$ and 20:5$n-3$).

EXPERIMENTAL

Materials

18:2$n-6$, 18:3$n-3$ and 20:4$n-6$ (99%) were from Sigma (St. Louis, MO, USA). 20:5$n-3$, 15*S*-HETE and 15*R*-HETE were from Cascade Biochem (Reading, UK). *cis*-Epoxides of the fatty acids were prepared by oxidation with *m*-chloroperoxybenzoic acid in chloroform as previously described [3,13] or by selective methods [14]. The epoxides were purified by HPLC and by thin-layer chromatography (TLC) [3,13–15]. The structures were confirmed by gas chromatography–mass specrometry (GC–MS) [16]. 14(15) EpETrE was chemically converted to 15-HETE by treatment with methylmagnesium bromide and isopropylcyclohexylamine (1:1) in tetrahydrofuran as previously described [17]. Methyl esters were prepared with ethereal diazomethane, ethyl esters with ethanol–diethoxypropane–hydrochloric acid (15:20:5, v/v) and pentafluorobenzyl esters by treatment with 7.5% pentafluorobenzyl bromide in acetonitrile–diisopropylamine (5:1, v/v). Solvents for HPLC were from Merck.

Microsomes of renal cortex were prepared from male white New Zealand rabbits (2–3 kg) as previously described [18] and incubated with 0.1 m*M* [^{14}C]20:4$n-6$ (4 Ci/mol; Amersham International, Amersham, UK), 1 m*M* NADPH and an epoxide hydrolase inhibitor [1 m*M* 1(2) epoxy-3,3,3-trichloropropane; Sigma] for 20 min at 37°C [3]. After extractive isolation, [^{14}C]14(15) EpETrE was purified by reversed-phase HPLC [3], combined with unlabelled racemic 14(15) EpETrE (0.1 mg), methylated and analysed by chiral HPLC.

Chromatograhy

The HPLC apparatus consisted of a pump (Milton Roy 4000), an injector (Rheodyne 7125), a variable-wavelength UV detector (Kratos Spectroflow 757) and an integrator (Hitachi D-2500) or a diode-array detector (Waters 991). The chiral HPLC column [250 mm × 4.6 mm I.D.; cellulose trisphenylcarbamate bound to silica gel (Chiralcel OC)] was from Diacel (Tokyo, Japan). The column was eluted isocratically (0.8–1.0 ml/min) at room temperature with a mobile phase containing 0.5% 2-propanol in hexane (normal-phase mode). The epoxy fatty acid methyl esters (100–300 μg) were dissolved in the mobile phase before injection. The elution of epoxides was followed by UV analysis (210 nm). Peaks of eluting compounds were evaporated to dryness and checked by TLC (using a specific colour reaction for epoxy fatty acids [19]) or by GC–MS [3,16]. If the enantiomers were partially separated, the epoxides were also chromatographed with a less polar mobile phase (0.25% 2-propanol in hexane). Radioactivity was measured by liquid scintillation (Beckman LS2800). Methyl-15-HETE was analysed by chiral HPLC [250 mm × 4.6 mm I.D.; (*R*)-(−)-N-3,5-dinitrobenzoyl-α-phenylglycine ionically bound to 5-μm modified Spherisorb, Pirkle type 1-A] and UV detection (235 nm) as previously described [9].

RESULTS

Epoxides of 20:4$n-6$ and 20:5$n-3$

The enantiomers of 5(6)EpETrE, 8(9)EpETrE and 11(12)EpETrE methyl ester were not resolved, while the enantiomers of 14(15)EpETrE methyl ester were partly resolved by chiral HPLC as shown in Fig. 1A. The absolute configuration of the enantiomers of 14(15)EpETrE was determined by chemical transformation to 15-HETE [17]. This procedure converted 14*R*(15*S*)EpETrE to 15*S*-HETE and 14*S*(15*R*)EpETrE to 15*R*-HETE. The first-eluting enantiomer of 14(15)-EpETrE yielded 15*S*-HETE, as shown in Fig. 1B, while the other enantiomer was converted to 15*R*-HETE (Fig. 1C). The elution order of the enantiomers of 14(15)EpETrE on Chiralcel OC was therefore *R,S* before *S,R*.

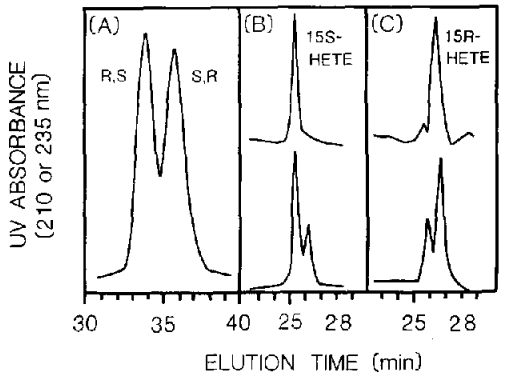

Fig. 1. (A) Partial separation of enantiomers of 14(15)epoxyeicosatrienoic acid methyl ester by chiral HPLC (Chiralcel OC) in the normal-phase mode (eluted with 0.25% 2-propanol in hexane at 1.0 ml/min). The first- and second-eluting enantiomers were converted to 15-HETE methyl ester [17], *i.e.* 14*R*(15*S*)-EpETrE was converted to 15*S*-HETE and 14*S*(15*R*)EpETrE to 15*R*-HETE. The formed enantiomers of 15-HETE were analysed by chiral HPLC (Pirkle 1-A) in (B) and (C) according to Kühn *et al.* [9]. The top chromatograms in (B) and (C) show elution of reference substances, 15*S*-HETE methyl ester and 15*R*-HETE methyl ester, respectively. The first-eluting enantiomer of 14(15)EpETrE in (A) was thus converted to 15*S*-HETE methyl ester and this enantiomer was identified as 14*R*(15*S*)EpETrE.

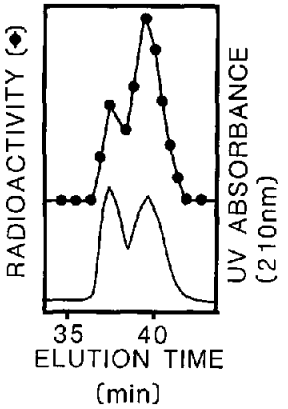

Fig. 2. Partial separation by chiral HPLC of enantiomers of methyl [^{14}C]14(15)epoxyeicosatrienoate, which was obtained by incubating microsomes of rabbit renal cortex with [^{14}C]arachidonic acid, NADPH and an epoxide hydrolase inhibitor. The biosynthetic epoxide was combined with racemic, unlabelled epoxide. The top curve shows elution of radioactivity, while the bottom curve shows the elution of UV-absorbing material at 210 nm; chromatographic conditions as in Fig. 1A.

[^{14}C]14(15)EpETrE was isolated from an incubation of rabbit kidney microsomes with [^{14}C]20:4$n-6$ and combined with unlabelled racemic 14(15)EpETrE. Chiral separation (Fig. 2) showed that about 30% of the radiolabelled metabolite eluted in the first peak (baseline to valley) and 70% in the second peak (valley to baseline), indicating that P450 of rabbit renal cortex preferentially formed the *S,R* enantiomer. The ω3 epoxide of 20:5$n-3$, 17(18)epoxyeicosatetraenoic acid, could be resolved neither as the methyl ester nor as the ethyl ester or the pentafluorobenzyl ester derivative. Attempts to separate the 11(12)EpETrE pentafluorobenzyl ester were also unsuccessful.

Epoxides of 18:2n−6 and 18:3n−3

Methyl 15(16)epoxyoctadecadienoic acid [15(16)EpODE] and methyl 9(10)EpODE had almost the same R_F values on TLC [14,20]. The two epoxides were separated by chiral HPLC (Fig. 3A and C), but only the enantiomers of 9(10)-EpODE could be resolved. The enantiomers of the third epoxide of methyl linolenoate, methyl 12(12)EpODE, were not resolved (Fig. 3B). Ethyl

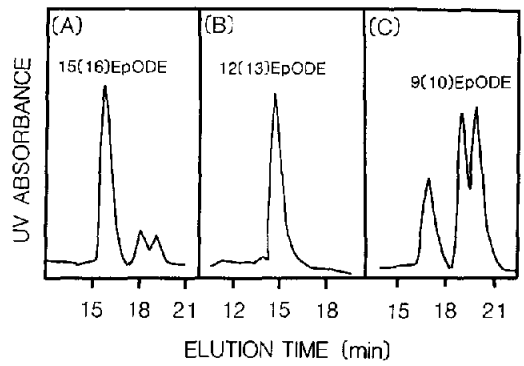

Fig. 3. Separation of monoepoxides of α-linolenic acid (18:3n − 3) by chiral HPLC (Chiralcel OC) with 0.5% 2-propanol in hexane (1.0 ml/min). (A) Chromatography of the ω3-epoxide of 18:3$n-3$, 15(16)epoxyoctadecadienoic acid methyl ester [15(16)-EpODE]. This sample also contained small amounts of 9(10)-EpODE methyl ester, which eluted with partial separation of the enatiomers after 18–20 min. (B) Chromatography of the ω6-epoxide of 18:3$n-3$, methyl 12(12)epoxyoctadecadienoate. (C) Partial separation of the enantiomers of methyl 9(10)epoxyoctadecadienoate. This sample also contained methyl 15(16)epoxyoctadecadienoate, which eluted between 16 and 18 min.

ester derivatives of these three epoxides chromatographed essentially as the methyl esters.

The antipodes of methyl 12(13)epoxyoctadecenoic acid [12(13)EpOME] were not resolved by chromatography on Chiralcel OC, while the enantiomers of 9(10)EpOME were partly resolved to the same extent as the two enantiomers of methyl 9(10)EpODE (see Fig. 3C).

DISCUSSION

The present report shows that the enantiomers of the ω6-epoxide of arachidonic acid and the ω9-epoxides of linoleic and α-linolenic acid could be separated as methyl esters by HPLC on a Chiralcel OC column with isopropanol–hexane as mobile phase, *i.e.* in normal-phase mode. This finding is of interest since 14(15)EpETrE is the main epoxide formed by hepatic and renal P450 [3,18], while 9(10)EpOME (coronaric acid, leucotoxin) is formed by plants, cytochrome P450 and by leukocytes [21–23]. Columns of derivatized cellulose can also be eluted with ethanol–water (reversed-phase mode), but this may lead to rapid loss of column performance [11]. Only the normal-phase mode was therefore evaluated.

Hammonds *et al.* [11] found that the enantiomers of the methyl ester of the ω6-epoxide of arachidonic acid were resolved by Chiralcel OB (cellulose derivatized with –CO–phenyl). The elution order of the *R,S* and *S,R* enantiomers of 14(15)EpETrE methyl ester was the same on Chiralcel OB and on Chiralcel OC (cellulose derivatized with –CO–NH–phenyl) as determined here. Hammonds *et al.* [11] also found that the enantiomers of ω9- and ω12-epoxides of arachidonic acid could be resolved as pentafluorobenzyl ester derivatives by Chiralcel OD (cellulose with –CO–NH–dimethylphenyl). Chiralcel OC appeared to be inferior to Chiralcel OD in this respect, since the pentafluorobenzyl ester of 11(12)EpETrE was not resolved.

Enantioselective analysis of [^{14}C]14(15)-EpETrE, isolated after incubating [^{14}C]arachidonic acid with rabbit kidney microsomes, indicated that the *S,R* enantiomer was formed with some stereoselectivity (about 70%) by the renal ω6-epoxygenase. Microsomes of monkey seminal vesicles contain an ω3-epoxygenase, which selectively (>90%) forms the *S,R* enantiomer of 17(18)EpETE [24]. In rat liver, however, the *R,S* enantiomer of 14(15)EpETrE predominates [25].

Enantioselective separation of ω3-epoxides of polyunsaturated fatty acids by HPLC has not been successful thus far. These epoxides are prominent P450 metabolites in some tissues [15,24,26]. Attempts were made to separate the optical isomers of the ω3-epoxides of α-linolenic and eicosapentenoic acids as methyl ester, ethyl ester or pentafluorobenzyl ester derivatives, but partial resolution could not be achieved on Chiralcel OC. Nevertheless, Chiralcel OC was useful for the separation of regioisomeric epoxides, *e.g.* the ω3- and ω9-epoxides of α-linolenic acid, which otherwise requires several purifications by TLC (Fig. 2).

The absolute configuration of fatty acid epoxides, which cannot be resolved by chiral HPLC, can be determined after chemical conversion to allylic alcohols [8,17]. The latter can then be analysed in different ways, *e.g.* by HPLC on a chiral column [8,9], by HPLC of diastereomeric derivatives [7] or by gas chromatography after ozonolysis of diastereomeric (−)-menthoxycarbonyl derivatives of the allylic alcohol [27]. However, the large number of steps makes these processes unsuitable for routine steric analysis of small amounts of biological products. Further studies on chiral phases for enantioselective separation of fatty acid epoxides are therefore needed.

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