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Journal of Chromatography B, 657 (1994) 23–29

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Direct resolution of epoxyeicosatrienoic acid enantiomers by chiral-phase high-performance liquid chromatography

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(First received July 14th, 1993; revised manuscript received March 10th, 1994)

Abstract

A chromatographic method was developed to separate enantiomers of four regioisomeric epoxyeicosatrienoic acids (EETs), which are cytochrome P-450 monooxygenase products of arachidonic acid. Enantiomers of individual epoxyeicosatrienoic acids were separated by chiral-phase HPLC using Chiralcel OD columns. In contrast to the pre-derivatization of EETs, the method is simple and convenient. It can be readily used in analytical studies and in preparative applications. The method can also be applied to the separation of methyl ester derivatives of epoxyeicosatrienoic acid enantiomers.

1. Introduction

Arachidonic acid is metabolized by cytochrome P-450, cyclooxygenase and lipoxygenase to form a series of potent autocoids that possess diverse biological activities. Cytochrome P450-mediated oxidation results in the formation of four regioisomeric *cis*-epoxyeicosatrienoic acids (EETs), 5,6-, 8,9-, 11,12-, and 14,15-EET, each of which consists of an enantiomeric pair [1,2]. It has been demonstrated that EETs can stimulate peptide hormone secretion [3], inhibit chloride transport in renal tubules [3], inhibit Na^+-K^+-ATPase [4], relax blood vessels [5] and inhibit platelet aggregation [2]. The excretion of EETs in human urine was shown to increase dramatically during pregnancy-induced hypertension [6]. Enantioselective formation and metabolism of EETs has been observed [7,8] and the biological activity of EETs can also be enantioselective. For example, 14(*R*),15(*S*)-EET inhibits cyclooxygenase [5] and 8(*S*),9(*R*)-EET is a potent renal vasoconstrictor, an activity that is abolished in the presence of cyclooxygenase inhibitors [8].

In order to further investigate the physiological activity and enantioselective metabolism of EETs, a simple and rapid chromatographic method was needed to separate enantiomers of each EET regioisomer. Hammonds *et al.* [9] have developed a method to separate methyl and pentafluorobenzyl (PFB) esters of EETs by chiral-phase chromatography. However, several disadvantages exist with this method. Firstly, the solvent systems that are required can degrade the chiral column. Secondly, the method is not suitable for the preparation of non-esterified EETs for use in biological experiments.

We report an efficient chromatographic method to directly separate EET free acids and their

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0378-4347/94/$07.00
SSDI 0378-4347(94)00172-2

methyl esters using a chiral-phase HPLC column. We also discuss methods for quantitative analysis of EET enantiomers using gas chromatography–electron capture negative chemical-ionization mass spectrometry (GC–ECNCI-MS).

2. Experimental

2.1. Chemicals

Arachidonic acid was obtained from Sigma (St. Louis, MO, USA). [^{14}C]arachidonic acid (50 mCi/mmol) was purchased from NEN Research Products (Boston, MA, USA). *m*-Chloroperoxybenzoic acid, pentafluorobenzyl bromide (PFBBr) and N,N-diisopropylethylamine were purchased from Aldrich (Milwaukee, WI, USA). All organic solvents were of HPLC grade and were obtained from Fisher (Pittsburgh, PA, USA). Authentic samples of EET enantiomers were a kind gift of Dr. J.R. Falck (Southwestern Medical Center, Dallas, TX, USA).

2.2. Preparation of racemic EETs

EETs and [^{14}C]EETs were prepared by epoxidation of arachidonic acid and [^{14}C]arachidonic acid (50 mCi/mmol), respectively using *m*-chloroperoxybenzoic acid according to a previously published method [10]. Products were separated by reversed-phase HPLC using a C_{18} Econosphere column (250 × 4.6 mm I.D., 5 μm; Alltech, Deerfield, IL, USA). A linear gradient was used from water–acetonitrile–acetic acid (50:49.95:0.05, v/v) to acetonitrile–acetic acid (99.95:0.05, v/v) over 40 min at a flow-rate of 1.0 ml/min. 14,15-EET eluted as a single peak at 25 min. 5,6-, 8,9-, and 11,12-EET eluted between 26–29 min and were collected as a mixture. These three regioisomers were then separated from each other using an Econosphere silica column (250 × 4.6 mm I.D., 5 μm; Alltech). The mobile phase was hexane–isopropanol–acetic acid (98.45:1.5:0.05, v/v) and the flow-rate was 1.5 ml/min. Using this system, the 11,12-EET enantiomers eluted at 10 min, the 8,9-EET enantiomers eluted at 13 min and the 5,6-EET enantiomers eluted at 25 min. UV absorbance was monitored at 210 nm. Tetradeuterated EETs were prepared by reaction of *m*-chloroperoxybenzoic acid with 17,17,18,18-[$^{2}H_4$]arachidonic acid which had been synthesized by a modification of a previously described method [11]. Individual regioisomers were separated as described above except that different HPLC columns were used in order to prevent contamination with unlabelled material.

2.3. Preparation of methyl esters

The EET (50 μg) in methanol (0.2 ml) was treated with a freshly distilled solution of ethereal diazomethane (1 ml). After allowing the solution to stand for 15 min the solvent was evaporated under nitrogen. The residue was used without further purification.

2.4. Preparation of pentafluorobenzyl esters

This was carried out by a modification of a procedure described previously [12]. The EET (50 μg) was dissolved in acetonitrile (100 μl). To this solution was added 200 μl of PFBBr in acetonitrile (1:9, v/v) and 50 μl of diisopropylethylamine in acetonitrile (1:19, v/v). The solution was stored under nitrogen for 30 min at room temperature and then evaporated under nitrogen. The residue was suspended in water (0.5 ml) and extracted with 1 ml of ethyl acetate–hexane (1:4, v/v). A second extraction with 0.5 ml ethyl acetate–hexane was then carried out. The combined organic extracts were evaporated and the residue used without further purification.

2.5. Separation of enantiomers

Enantiomers of each of the racemic regioisomeric EET free acids and their methyl esters were resolved using a Chiralcel OD HPLC column (250 × 4.6 mm I.D., 5 μm; J.T. Baker, Phillipsburg, NJ, USA) using the solvent systems shown in Table 1. The recovery of each pair of [^{14}C]EETs was quantified by triplicate injections of each regioisomer, collection of individual

Table 1
Chromatographic conditions for HPLC resolution of the four regioisomeric EETs

EET regioisomer	Mobile phase	Flow-rate (ml/min)	Retention time (min)	
			R,S	*S,R*
5,6-EET	isopropanol–hexane–acetic acid (0.3:99.65:0.05)	2.0	69	74
8,9-EET	isopropanol–hexane–acetic acid (0.9:99.05:0.05)	1.5	44	50
11,12-EET	isopropanol–hexane–acetic acid (1.2:98.75:0.05)	1.0	24	20
14,15-EET	isopropanol–hexane–acetic acid (0.4:99.55:0.05)	1.5	57	51
5,6-EET-ME	isopropanol–hexane (0.2:99.8)	1.0	29	27
8,9-EET-ME	isopropanol–hexane (0.2:99.8)	0.7	41	48
11,12-EET-ME	isopropanol–hexane (0.25:99.75)	1.0	25	20
14,15-EET-ME	isopropanol–hexane (0.06:99.94)	1.5	49	44

enantiomers and counting the radioactivity in a 1219 Rackbeta LKB Wallac liquid scintillation counter (Wallac Oy, Turku, Finland). Each regioisomer was separated into its individual enantiomers in separate HPLC analyses. Variability in retention time between injections for a particular enantiomer was less than 30 s. Absolute configurations were assigned by chromatographic comparisons with enantiomerically pure standards that were prepared by Dr. Falck (Southwestern Medical Center, Dallas, TX) using total asymmetric synthesis according to published procedures [13–15].

2.6. Gas chromatography–mass spectrometry

Helium was used as the carrier gas and methane as the reagent gas. EET methyl esters were analyzed by GC–positive chemical-ionization (PCI)-MS using a Finnigan-MAT INCOS 50B quadrupole mass spectrometer interfaced with a Hewlett-Packard 5890 gas chromatograph. GC was carried out using a DB-5 fused-silica column (15 m × 0.25 mm I.D, 0.25 μm coating thickness; J and W Scientific, Folsom, CA, USA). The oven temperature was held at 150°C for 1 min and linearly programmed to 300°C at a rate of 15°/min. The injector and transfer lines were maintained at 250°C, and the source temperature was 120°C. PFB derivatives were analyzed by GC–ECNCI-MS using a Nermag R-1010C mass spectrometer interfaced with a Varian Vista 6000 gas chromatograph. Chromatography was carried out on an SPB-1 fused-silica capillary column (15 m × 0.32 mm I.D., 0.25 μm coating thickness; Supelco, Bellefonte, PA, USA). The injector, transfer line and ion source were each maintained at 260°C. The GC oven temperature was maintained at 190°C for 0.5 min and then linearly programmed to 320°C at a rate of 20°/min. It was then held for 3 min at this temperature.

3. Results and discussion

In their studies of hepatic cytochrome P-450-mediated arachidonic acid metabolism, Hammonds *et al.* developed a chiral HPLC method to analyze the EET enantiomers that were formed [9]. The methodology involved chemical derivatization and chiral HPLC analysis on Chiralcel columns. There are several disadvantages of this method. Firstly, the solvent system of ethanol–water (70:30, v/v) required to separate the 5,6-EET methyl ester enantiomers is viscous and can over-pressurize the column bed and lead to degradation of the Chiralcel OB column. Secondly, the methodology is not convenient. The methyl esters of 5,6-EET and 14,15-EET were separated using the OB column, while the PFB esters of 8,9-EET and 11,12-EET were resolved using the OD column. If the free EETs are required for biological experiments, they must

then be saponified to their free acids. If high sensitivity analysis of 5,6-EET or 14,15-EET has to be carried out, saponification followed by derivatization to PFB esters is required prior to GC–ECNCI-MS analysis. This makes the method tedious for routine analyses. Chiral stationary-phase columns have been used previously for the enantioselective analysis of arachidonic acid metabolites without the need for prior derivatization [16]. The Chiralcel column stationary phase consists of a silica matrix coated with cellulose benzoates (OB), phenyl carbamates (OC) or 3,5-dimethylphenyl carbamates (OD). According to the manufacturer (J.T. Baker, Phillipsburg, NJ), the cellulose 3,5-dimethylphenyl carbamate (OD) stationary phase is stable in the presence of 0.1% (v/v) acid. Okamoto *et al.* reported resolution of carboxylic enantiomers by a Chiralcel OD column using hexane–isopropanol in the presence of a small amount of trifluoroacetic acid [17]. In view of this report, it seemed likely that Chiralcel OD columns would separate the EET free acid enantiomers. The EETs were unstable in the presence of trifluoroacetic acid and so acetic acid was used instead. In the presence of 0.05% (v/v) acetic acid, efficient resolution of enantiomers of the four regioisomeric free acid forms of the EETs was possible on the Chiralcel OD column (Fig. 1, Table 1).

The 11,12-EET enantiomers were the most easily resolved among the four regioisomers. These were separated using a solvent system of isopropanol–hexane–acetic acid (1.2:98.75:0.05, v/v), where 11(*S*),12(*R*)-EET and 11(*R*),12(*S*)-EET eluted at 20 and 24 min, respectively. The 8,9-EET enantiomers were separated using a less polar solvent of isopropanol–hexane–acetic acid (0.9:99.05:0.05, v/v) solvent system at 1.5 ml/min. The 14,15-EET enantiomers required an even less polar solvent of isopropanol–hexane–acetic acid (0.4:99.55:0.05, v/v) for resolution. Baseline resolution could not be achieved for the 5,6-EET enantiomers, although several solvent systems were tried. Results presented in Fig. 1 demonstrate that greater resolution was achieved when the epoxide functionality was located in the middle of the chain, such as 8,9-EET and 11,12-EET. When the epoxide functionality was located near the terminus of the chain, such as

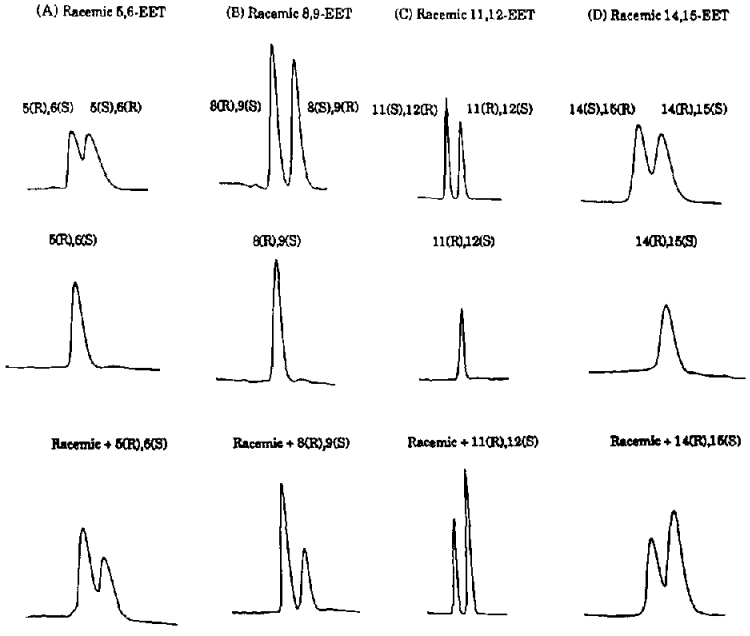

Fig. 1. Partial UV chromatograms of enantiomers of enantiomerically pure (25 μg) and racemic mixtures of four regioisomeric epoxyeicosatrienoic acids (50 μg) separated using a Chiralcel OD column. UV absorbance was monitored at 210 nm.

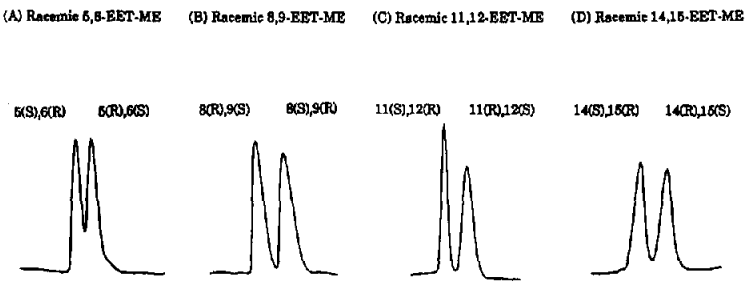

Fig. 2. Partial UV chromatograms of enantiomers of four regioisomeric epoxyeicosatrienoic acid methyl esters (50 μg) separated using a Chiralcel OD column. UV absorbance was monitored at 210 nm.

5,6-EET and 14,15-EET, the free acid enantiomers were not so well resolved. We also found that satisfactory resolution of EET methyl ester derivatives could be achieved with Chiralcel OD columns operated in the normal-phase mode. The OD column resolved the EET methyl esters more completely than the OB column. Thus, the four regioisomeric EET methyl esters were separated using the OD column (Fig. 2, Table 1). The 8,9-EET methyl ester diastereomers were not resolved on the OB column (data not shown).

Optimal HPLC conditions for resolution of free EETs and their methyl esters were established utilizing a racemic mixture of each synthetic EET regioisomer. After separation using a Chiralcel OD column, the EETs were collected, methylated with diazomethane and analyzed by GC–PCI-MS. Results presented in Fig. 3 show the PCI mass spectrum of 11(R),12(S)-EET methyl ester. A protonated molecular ion appeared at m/z 335. Fragment ions present at m/z 317, m/z 303 and m/z 285 are thought to arise from loss of H_2O, CH_3OH and [H_2O +

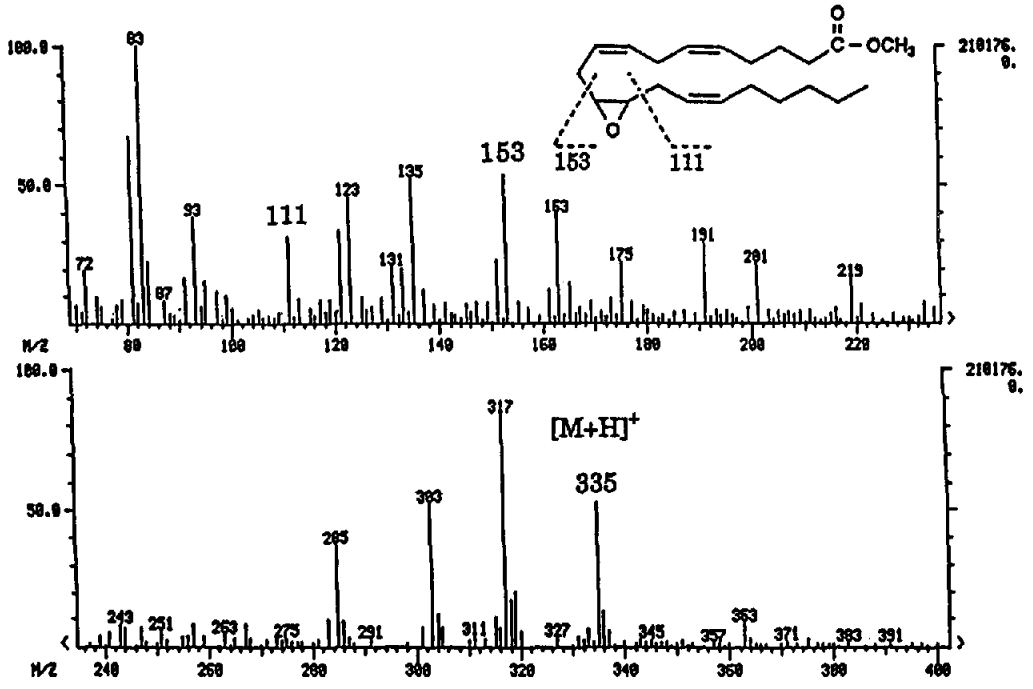

Fig. 3. Positive chemical-ionization mass spectrum of 11(R),12(S)-epoxyeicosatrienoic acid methyl ester.

CH_3OH], respectively. Low-mass fragments at m/z 153 and m/z 111 resulted from an α-cleavage of the epoxide moiety, which confirmed that the epoxide was located at the C11–C12 position. Each isomeric EET pair was quantified according to the recovery of injected [^{14}C]EET. In each case, liquid scintillation counting of the resolved enantiomers showed that the racemic standard contained a 1:1 enantiomeric ratio, and the recovered yield in each case was >90%. Absolute configurations were assigned based upon chromatographic comparisons of the racemic samples with co-injected enantiomerically pure standards (Fig. 1).

In combination with GC–ECNCI-MS, this enantioselective separation methodology can be applied to quantitative analysis of EETs in biological fluids such as urine and plasma. High sensitivity can be attained using the PFB ester derivative which is widely used in eicosanoid analysis because of its efficient electron capturing characteristics [12]. Tetradeuterated EETs were prepared by *m*-chloroperoxybenzoic acid oxidation of tetradeuterated arachidonic acid that was synthesized by a modification of a previously described method [11]. The deuterated EETs were then used as internal standards in GC–MS assays. Each enantiomeric EET pair was separated using a Chiralcel OD column and derivatized to the PFB ester for GC–ECNCI-MS analysis. A selected-ion current profile of an authentic standard 11(*R*),12(*S*)-EET-PFB derivative and its corresponding 11(*R*),12(*S*)-[$^2H_4$]EET-PFB derivative is shown in Fig. 4. Ions at m/z 319 and m/z 323 correspond to loss of the PFB moiety from the molecular radical anions of the EET-PFB ester and the [$^2H_4$]EET-PFB ester, respectively [12]. EET concentrations can be determined from the ratio of the peak intensities or peak areas of m/z 319 to m/z 323.

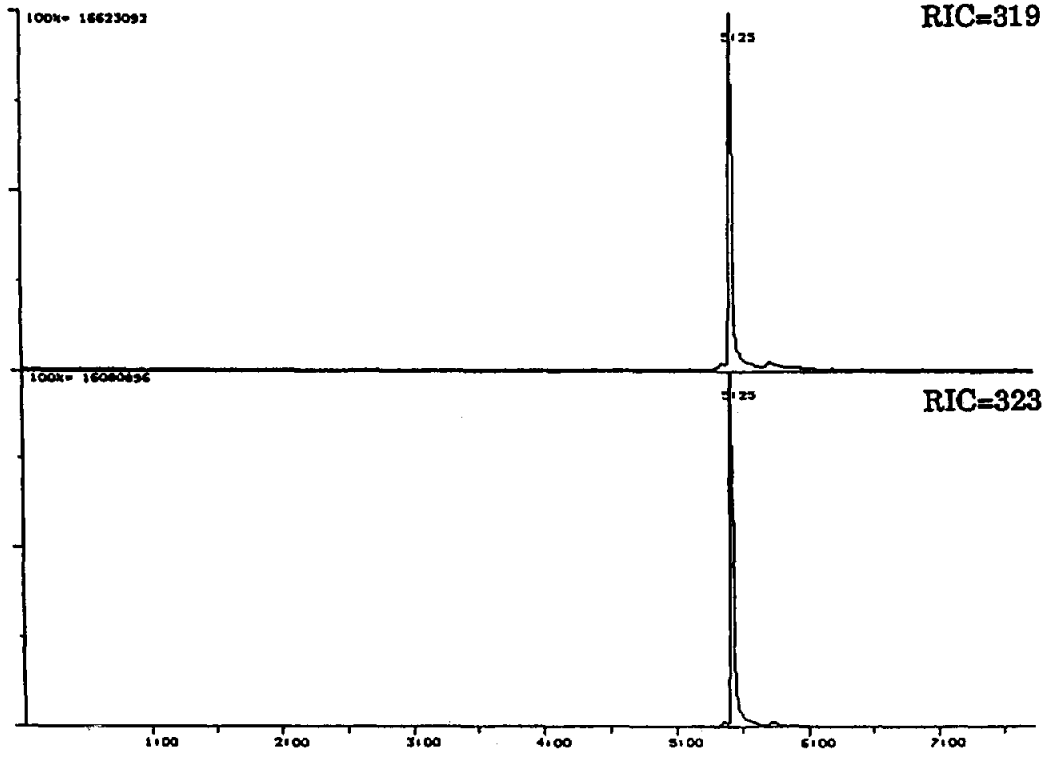

Fig. 4. Selected-ion current profiles for GC–ECNCI-MS analysis of 11(*R*),12(*S*)-epoxyeicosatrienoic acid pentafluorobenzyl ester and its internal standard, 11(*R*),12(*S*)-[$^2H_4$]epoxyeicosatrienoic acid pentafluorobenzyl ester.

4. Conclusions

A chiral chromatographic method was developed to directly separate the enantiomers of four regioisomeric EETs. This procedure offers significant advantages over other chiral separation methods. It is simple, efficient and provides high recoveries of pure EET enantiomers. No derivatization is necessary and the EETs are not exposed to reagents that could react with the epoxide moiety. Consequently, this method can be used for preparation of enantiomerically pure EETs as their free acids. We have successfully used this method to obtain 8,9-EET enantiomers for studies of cyclooxygenase-mediated enantioselective metabolism of 8,9-EET [18]. A combination of chiral HPLC with subsequent PFB derivatization and GC–ECNCI-MS analysis provides a useful tool for investigating the metabolism of arachidonic acid in biological systems. The availability of this methodology will allow the physiological significance of cytochrome P-450-mediated metabolism of arachidonic acid to be more fully investigated. The chiral HPLC methodology can potentially also be used to separate other biologically active lipid-epoxides, such as long chain epoxy-acids and epoxy-alcohols.

Acknowledgements

The authors thank Charles A. Goldthwaite, Jr. for his assistance in the preparation of this manuscript. This work was supported by a research grant from the National Institute of Health (DK 38226).

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