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Capillary Electrophoresis of Cytochrome P-450 Epoxygenase Metabolites of Arachidonic Acid. 2. Resolution of Stereoisomers

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Each of the four regioisomers of epoxyeicosatrienoic acids (EETs) is a candidate for being an endothelial-dependent hyperpolarizing factor (EDHF). One regioisomer, 14,15-EET, stereospecifically blocks cyclooxygenases from converting arachidonic acid to prostaglandins and stereospecifically binds to cellular receptors. Both stereospecific actions emphasize the need to establish the tissue availability of the 14,15-EET enantiomers. The present work describes a method to quantitate picogram amounts of 14,15-EET enantiomers by capillary electrophoresis. The 14,15-EET enantiomers were baseline resolved ($R = 1.3$) using unsubstituted β-cyclodextrin and 32% acetonitrile (v/v). When absorption at 194 nm was monitored using a photodiode array detector, 8 and 1 pg of underivatized 14,15-EET were readily quantitated and detected, respectively. Capillary electrophoresis accurately assessed chiral excesses up to 97:3 for either 14,15-EET enantiomer. Moreover, capillary electrophoresis with a photodiode array detector was sufficiently sensitive to detect and measure 14,15-EET enantiomers from murine liver. Thus, unlike chiral-phase high-performance liquid chromatography, capillary electrophoresis can be used to directly assess the chirality of trace amounts of underivatized eicosanoids.

Cytochrome P-450 epoxygenases metabolize the essential fatty acid, arachidonic acid, to four regioisomers of epoxyeicosatrienoic acids (EETs). The EETs exhibit a wide range of physiological effects, some of which are regioisomer specific (part 1).[1] Recently, there has been intense interest in clarifying the role of EETs as endothelial-dependent hyperpolarizing factors (EDHFs).[2] One regioisomer 14,15-EET, characterized by the epoxide ring between the 14th and 15th carbon (Figure 1 of part 1), potently dilates arterioles[3] and inhibits platelet aggregration.[4,5] By activating potassium channels and hyperpolarizing cell membranes, 14,15-EET also inhibits calcium influx into vascular smooth muscle cells.[3,6] In addition, the 14,15-EET regioisomer stimulates tyrosine phosphorylation and induces mitogenesis in renal epithelial cells.[7] In contrast to other EET regioisomers, 14,15-EET appears to be concentrated in phosphatidylinositol of endothelial cell membranes, where it is available for rapid release by physiological agonists.[8] Thus, arachidonic acid is converted by cytochrome P-450 epoxygenases to a 14,15-EET regioisomer, which has multiple biological actions.

Along with regiospecificity, EETs often demonstrate stereospecificity in their physiological effects.[9−11] The 14(*R*),15(*S*)-EET but not its antipode inhibits platelet thromboxane A_2 synthesis.[4] Moreover, 14(*R*),15(*S*)-EET may have a specific receptor; the *R*,*S* enantiomer but not the *S*,*R* antipode binds to plasma membranes of monocytes and appears to stimulate cAMP formation.[12] Therefore, methods for determining tissue levels of EET enantiomers would establish the availability of active enantiomeric species. Furthermore, defining EET chiral excesses also helps elucidate their mechanism of formation;[13] tissue enrichment with a single enantiomer indicates enzymatic (cytochrome P-450 epoxygenase) formation, whereas racemic accumulations suggest free radical mechanisms (autoxidation), which may occur in vivo or during sample processing.[14,15] Therefore, a method for directly determining tissue enantiomer levels is important for defining the physiologically relevant concentrations and mechanism of formation of 14,15-EETs.

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EET chirality has traditionally been assayed by converting nanogram to microgram amounts of EETs to various esters, separating them by chiral HPLC, saponifying, and converting them to pentafluorobenzyl esters for quantitation by gas chromatography/mass spectrometry (GC/MS).[16,17]The combination of multiple derivations, expensive chiral columns, and subsequent GC/MS analyses makes this approach arduous and expensive. Moreover, because multiple steps and derivatizations are required, recoveries are typically only 5−15%.[16] Sensitivity becomes even more critical in cardiovascular tissues where EET levels appear to be lower than found in solid tissues such as liver and kidney.[18] For instance, the concentration of total unesterified EETs in human plasma is only ∼1.0 nM; thus, it is not surprising that the chirality of these potent autacoids has yet to be established.[19]

Capillary electrophoresis (CE) coupled with UV absorbance detection is an inexpensive yet highly effective alternative to chiral-phase HPLC and GC/MS. Part 1 of our studies demonstrated that CE with a substituted β-cyclodextrin resolved underivatized EET regioisomers. In the present work, CE with unsubstituted β-cyclodextrin was used to resolve and quantitate picogram quantities of underivatized 14,15-EET stereoisomers. The method was further used to determine the chiral excess and concentration of 14,15-EET enantiomers in murine liver.

EXPERIMENTAL SECTION

Materials. Chiral standards of 14,15-EET were purchased from Cascade Biochemicals (Reading, England; independent measurements revealed that ∼2% of each enantiomer standard was contaminated by its antipode). Unlabeled 8,9-, 11,12-, and 14,15-EET as well as [1-^{14}C]-14,15-EET tracers were synthesized as before.[20] Individual cyclodextrins (β-cyclodextrins (Astec, Whippany, NJ) and substituted (hydroxypropyl, hydroxyethyl, and di- and trimethyl) β-cyclodextrins and hydroxypropyl- α- and γ-cyclodextrins (Astec, Aldrich) were made up in running buffer in a Teflon vial, filtered (0.22 μm) and sonicated for 40 s before use.

Capillary Electrophoresis. After being suspended in sample buffer, EETs were transferred to plastic vials (Beckman-Coulter, Fullerton, CA) for analysis or stored in screw-capped Teflon vials. To minimize evaporative effects, each suspension of EETs was prepared using Hamilton syringes just prior to injection. Separations were carried out using a 5500 P/ACE system with a photodiode array detector (Beckman). Fused-silica capillaries with 75- or 50-μm-i.d. and 200- or 150-μm extended light paths, respectively, were employed (Agilent Technologies). To permit correct positioning of the light path in the Beckman cartridges, interfering column collars were removed by soaking them in acetone for several hours. The columns were cut to length (87 or 97 cm = L_t) and the extended paths positioned over the cartridge slits (80 or 90 cm = L_{eff}) using a microscope. To eliminate carryover between samples and reduce baseline noise, 3−4 mm of the polyimide covering was removed from each end of the columns using a resistive heating stripper (Scientific Resources, Inc., Eatontown, NJ). The running buffer was composed of 50 mM sodium phosphate (pH 7.0), 15 mM cyclodextrin, and 0−40% CH_3CN (v/v). Samples were made up in a low ionic strength ("stacking") buffer (5 mM sodium phosphate, pH 8.0). By applying 0.5 psig pressure for 5 or 25 s, comparable injections into the 75- or 50-μm (i.d.) capillaries, respectively, were achieved. Separations were done at 20 °C and at 20 kV (normal polarity).

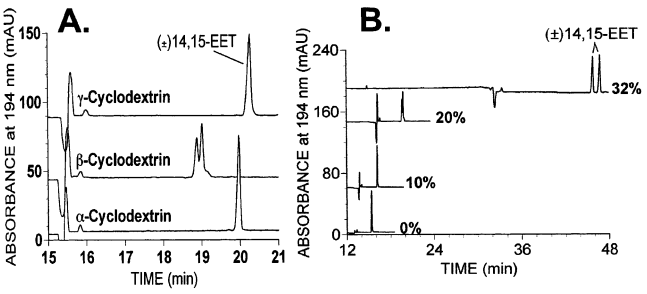

Figure 1. Effects of (A) α-, β-, and γ-cyclodextrins plus (B) CH_3CN addition on 14,15-EET enantiomer resolution. Racemic 14,15-EET was suspended in 5 mM sodium phosphate (pH 8.0) and injected by pressurization for 25 s. Electrophoresis was carried out at 20 kV (230 V/cm, normal polarity, 50-μm-i.d., 87-cm capillary length), at 20 °C with 15 mM cyclodextrin in 50 mM sodium phosphate (pH 7.0). In (A), 20% CH_3CN (v/v) was used and traces were corrected for slight variations in EOF. In (B), 0−20% CH_3CN concentrations were used with the conditions described in (A); however, trace labeled "32%" was generated with 32% CH_3CN (v/v) and a 97-cm capillary (205 V/cm). Absorbance was monitored at 194 nm.

RESULTS AND DISCUSSION

Resolution of 14,15-EET Enantiomers. A suite of nine cyclodextrins (α-, β-, and γ-cyclodextrins plus hydroxypropyl-, hydroxyethyl-, di- and trimethyl-substituted β-cyclodextrins, hydroxypropyl- α- and γ-cyclodextrins) at 15 mM concentrations was screened using a range of ionic strengths up to 200 mM sodium phosphate (pH 7) and CH_3CN concentrations up to 40% (v/v). Only the unsubstituted β-cyclodextrin showed any resolution of the 14,15-EET enantiomers (Figure 1A). Overall, increasing the CH_3CN concentrations improved resolution and slowed the electroosmotic flow (EOF) (Figure 1B). Under an optimized separation condition of 32% CH_3CN (v/v) and by use of a 97-cm capillary, (±)14,15-EET was baseline resolved ($R \sim 2$) (Figure 1B). Thus, by use of inexpensive and commonplace reagents, CE completely resolved underivatized 14,15-EET enantiomers. In contrast, the use of multiple expensive and proprietary chiral high-performance liquid chromatography (HPLC) columns does not completely resolve 14,15-EET enantiomers, with or without prior derivatization.[21,22]

The presence of other regioisomers in the sample may interfere with determinations of 14,15-EET chirality. To test this possibility, EET standards were injected with (±)14,15-EET after 14(*S*),15(*R*)EET was shown to migrate before 14(*R*),15(*S*)EET. Under these conditions, (±)8,9-EET comigrated with 14(*S*),15(*R*)-EET, and (±)11,12- EET comigrated with 14(*R*),15(*S*)EET

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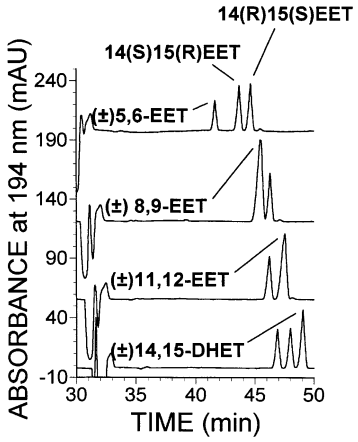

Figure 2. Comigration of (±)EET regioisomers with 14,15-EET enantiomers. Standards of (±)11,12-EET (33 ng/μL), (±)8,9-EET (35 ng/μL), (±)5,6-EET (10 ng/μL) or (±)14,15-DHET (24 ng/μL) were mixed with (±)14,15-EET (25 ng/μL per enantiomer) and separated by CE using 32% CH_3CN as described in Figure 1B.

(Figure 2). Therefore, the 14,15-EET enantiomers isolated from biological samples must first be freed of the 8,9-EET and 11,12-EET regioisomers before chirality is measured. Fortunately, (±)-14,15-EET is readily isolated by reversed-phase HPLC; in contrast, the other three EET regioisomers are poorly resolved from each other by this method.[23] Interestingly, both 14,15-EET enantiomers migrated before 14,15-dihydroxyeicosatrienoic acid (DHET); thus, 14,15-EET chirality determinations are unaffected by the presence of 14,15-DHET. However, the complete absence of 14,15-DHET is helpful in establishing that no 14,15-EET has hydrolyzed during storage. Perhaps more importantly, the complete separations and sharp peak shapes of 14,15-EET enantiomers resolved by CE facilitate the detection of disturbances in peak shape. In contrast, such interferences are less readily detected by chiral HPLC, because neither underivatized nor derivatized 14,15-EET enantiomers are completely resolved.[17, 21]

As noted above, the four EETs and 14,15-DHET interacted with β-cyclodextrin, and eluted in the order of 5,6-, 8,9-, 11,12-, and 14,15-EET and 14,15-DHET (Figure 2). Thus, all five eicosanoids elute in order of decreasing hydrophobicity, as determined by reversed-phase HPLC on octadecasilyl columns.[23] Accordingly, one element of 14,15-EET binding involves hydrophobic interactions; i.e., 14,15-EET probably enters into the β-cyclodextrin helix and interacts with the hydrophobic cavity.[24−26] In addition to hydrophobic binding, there was strong enantioselective binding of 14,15-EET antipodes. Weaker enantioselective binding was detected with 8,9-EET antipodes ($R \sim 0.7$), at least under conditions of high CH_3CN concentration (40%, v/v) and high ionic strength (200 mM sodium phosphate). In contrast, the 11,12-EET antipodes were not resolved at any combination of cyclodextrin, ionic strength, and CH_3CN concentration tested. While β-cyclodextrin enantioselectivity was strongest for 14,15-EET and weakest for 11,12-EET, the basis of the interaction is unclear. Defining the precise binding sites of both the hydrophobic and enantioselective interactions will probably require the use of NMR and molecular modeling techniques.

Table 1. Effects of Column Geometry and Slit Length on Apparent Enantiomer Resolution and Signal-to-Noise Ratio[a]

internal diam/ extended light path (μm)	slit length (μm)	resolution ($N = 3$)	noise[b] (mAU)	signal-to-noise ratio ($N = 6-8$)
75/200	200	1.65 ± 0.05	0.14	15.0 ± 0.5
	800	1.70 ± 0.01	0.04	20.8 ± 0.4
50/150	200	1.06 ± 0.06	0.31	9.5 ± 0.4
	800	1.16 ± 0.01	0.04	41.0 ± 1.8

[a] Data represent mean values ± SEM. Resolution and signal-to-noise ratios were determined by injecting 17.5 nL of 280 or 2.8 ng/μL (±)14,15-EET, respectively, into each 90-L_{eff}/97-cm-long column containing β-cyclodextrin (see Experimental Section for details). [b] Noise was assessed as peak-to-peak heights.

Calibration Plots for 14,15-EET Enantiomers. For chiral analyses, sensitivity is a critical issue because the trace amount of regioisomer present in biological samples is divided between two fractions (enantiomers) of varying proportions. One way to enhance sensitivity is to increase the optical path length. Although the 75-μm (i.d.) columns have a 200-μm extended light path, the 50-μm (i.d.) columns with 150-μm extended light paths were more compatible with the constant 100-μm slit width in Beckman cartridges and supported a higher apparent molar extinction coefficient and better signal-to-noise ratio (Table 1). Double bonds have high absorptivity at 192 nm.[27] After the wavelength was varied between 190 and 194 nm and the band-pass adjusted from 1 to 3 nm, 194 ± 2 nm was found to generate the highest signal-to noise ratio. Use of a 800 × 100 μm cartridge aperture reduced noise (0.04 mAU, peak to peak) to one-eight or one-third that seen with the 200 × 100 μm aperture (Table 1), possibly because light transmission was increased 4-fold; unfortunately, light emission from deuterium lamps is characteristically weak below 200 nm. Perhaps more importantly, enantiomer resolution was not impaired by use of an 800-μm-long aperture, probably because several millimeters separated the 14,15-EET enantiomers. Thus, the following studies were done with 50-μm (i.d.) columns containing a 150-μm extended light path positioned over an 800 × 100 μm aperture.

Injections up to 380 pg of each enantiomer were baseline resolved ($R \sim 1.32$). The amounts injected were calculated from the original antipode concentrations and a constant 17.5-nL plug being injected into the capillary when 0.5 psig was applied for 25 s.[28] Migration times for the *SR* and *RS* antipodes were quite consistent at 48.67 min ±1.5% relative standard deviation (RSD) and 49.82 min ± 1.5% RSD ($n = 5$), respectively. Perhaps more significantly, down to 7.6 pg of each 14,15-EET enantiomer was resolved ($R = 1.75 \pm 5.0\%$ RSD) and detected with a signal-to-noise ratio of 24:1, setting the detection limit at ~1 pg (Figure 3A). Thus, because of the presence of three isolated double bonds, each 14,15-EET enantiomer was detected at 194 nm with high sensitivity.[27] In contrast, during chiral-phase HPLC, microgram amounts of 14,15-EET enantiomers are required when absorption

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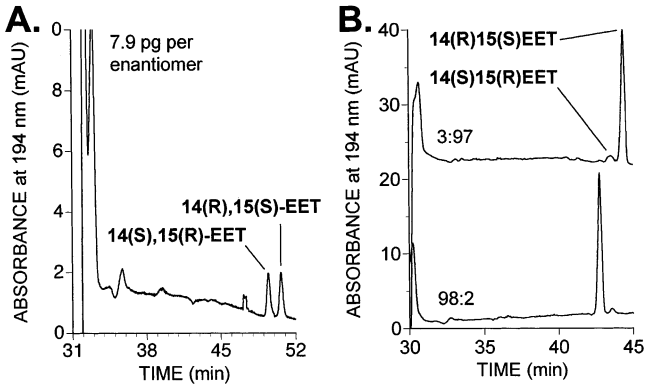

Figure 3. Electropherograms for quantitating 14,15-EET enantiomers. Serial dilutions of racemic [1-^{14}C]-14,15-EET (43.4 ng/μL) or varying ratios of unlabeled 14,15-EET enantiomers at a combined concentration of ~20 ng/μL were subjected to electrophoresis with 32% CH_3CN as described in Figure 1B. In panel A, a signal-to-noise ratio of 24:1 was measured after 15.8 pg of racemic 14,15-EET was injected. In panel B, enantiomers at extreme ratios were baseline separated.

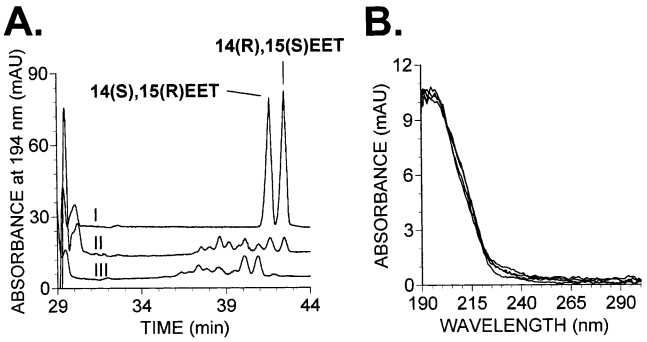

Figure 4. (A) CE of 14,15-EET extracted from murine liver and (B) corresponding UV spectra. Liver phosholipids from four mice were pooled and saponified, and the 14,15-EET released was isolated by HPLC and subjected to CE with 32% CH_3CN as described in Figure 1B. Panel A shows tracings from (I) (±)14,15-EET standard, (II) hepatic 14,15-EET, and (III) hepatic 14,15-EET mixed with enough (±)14,15-EET standard to roughly double peak areas. Panel B shows UV spectra of liver 14,15-EET enantiomers, with or without added (±)14,15-EET standard.

at 210 nm is monitored.[17,21,22] In the present study, peak area responses for the *SR* and *RS* antipodes were linear ($r^2 = 0.9921 \pm 0.6\%$ RSD and $0.9938 \pm 0.6\%$ RSD, respectively; $n = 4$) over an 8−380-pg range. Thus, 14,15-EET enantiomers could be directly quantitated over a dynamic range of 50:1 down to ~ 8 pg; moreover, ~ 1 pg of either enantiomer was readily detectable. Unfortunately, no chiral-phase HPLC calibration curves are available for comparisons, because this method has been exclusively used for preparative purposes; the actual quantitations are done later by GC/MS.[22]

Important for in vivo studies on biological activity, the purity of 14,15-EET enantiomer preparations can be accurately assessed by CE with β-cyclodextrins (Figure 3B). Accurate determinations of chiral excesses require a high degree of resolution of the antipodes; by essentially providing a third phase, large amounts of the early enantiomer tend to reduce the separation of trace amounts of the later antipode. Unfortunately, chiral-phase HPLC is unable to completely separate the 14,15-EET enantiomers, even after derivatization;[17,21,22] thus, current chiral-phase HPLC methods cannot substantiate high degrees of enantiomeric purity. In the present study, determination of an enantiomeric excess of at least 97:3 with either enantiomer was readily achieved **(**Figure 3B**)**. Moreover, the measured chiral excess agreed within 3% of theoretical values. Interestingly, the accuracy of 14,15-EET antipode determinations was not limited by resolution but by the purity of chiral standards. Widely separated by CE, both enantiomer standards were found to be contaminated 1.8−2.0% with antipode. However, because of the high resolution ($R = 1.451$) and linearity ($r^2 = 0.9838$, $n = 2$), CE of 14,15-EET is likely to detect antipode contaminations much less than 1% when appropriate standards become available. Establishing a high degree of purity for 14,15-EET enantiomers is important for in vivo studies where the unwanted side reactions may be due to antipode contaminants.

Quantitation of EET Stereoisomers Isolated from Mouse. A combination of reversed-phase HPLC and CE were used to measure the EET composition of murine liver. Because more than 85% of the EET present in mammalian liver is esterified to cellular phospholipids,[16,18] lipids from murine liver ($n = 4$) were first extractively isolated, separated into neutral and phospholipid fractions by silicic acid column chromatography, and saponified. The 14,15-EET released was completely resolved from other EETs by reversed-phase HPLC (see part 1 for details) and was collected, pooled, and analyzed for chiral excesses. In Figure 4A, the upper trace (I) shows the separation of racemic (±)14,15-EET standards. The middle trace (II) demonstrates that 14,15-EETs isolated from murine liver had an enantiomeric ratio of 48% *SR*: 52% *RS*. The lowest trace (III) shows that murine 14,15-EET mixed with (±)-14,15-EET standard had identical retention times. Moreover, the UV spectra generated from liver 14,15-EETs were unaltered by the addition of (±)14,15-EET standard (Figure 4B). Note that, in the lowest electropherogram, both 14,15-EET and the EOF (indicated by the refractive index changes at ~29 min) appear earlier than in previous runs. In the chiral analysis of murine samples, no correction of the traces with respect to EOF or time was attempted. Instead, the presence of 14,15-EET enantiomers in murine liver was established by matching migration times and UV spectra with those of standards.

In addition to identification, CE with UV detection was used to quantitate the levels of 14,15-EET enantiomers in murine liver. Based on the linear regression curves, the peak areas corresponding to the *SR* and *RS* antipodes (Figure 4A, trace II) represented 9.0 and 7.7 ng/μL, respectively. Based on the 14,15-EET specific radioactivity and liquid scintillation assays, the mass from [1-^{14}C] internal standards was 0.14 ng/μL. Thus, the ratio of liver *SR* or *RS* to internal standard was 64:1 and 55:1, respectively. From the amount of internal standard originally added, the 14(*R*),15(*S*)-EET and 14(*S*),15(*R*)-EET concentrations in mouse liver phospholipids were calculated to be 0.48 and 0.41 μg/g of wet weight liver, respectively. This contrasts with the 0.28 and 0.08 μg/g of wet weight values found for the *RS* and *SR* enantiomers, respectively, in rat liver.[16] Whether the differences represent dietary, species, strain, or individual effects will require more detailed investigations.

It should be emphasized that because UV spectra incompletely differentiate the four EET regioisomers (part 1), measurements of tissue 14,15-EET levels by using absorption at 194 nm need to

be carefully validated by more specific techniques such as mass spectrometry. Except for 14,15-EET, EET regioisomers are incompletely resolved by capillary gas chromatographic techniques.[23] Moreover, to achieve picogram sensitivities, one must resort to negative-ion chemical ionization mass spectrometry, which analyzes EETs as carboxyl anions, requires derivatization, and does not differentiate between regioisomers.[23] If 14,15-EETs are first isolated by chiral HPLC techniques, the selection of an internal standard to normalize for varying recoveries becomes problematical. Commercially available [5,6,8,9,11,12,14,15-D_8]-EETs are "unacceptably separated" from overlapping endogenous EETs during reversed-, normal-, and chiral-phase HPLC.[17] In contrast, [1-^{14}C]-EETs precisely coelute with endogenous EETs and have been successfully used as internal standards for mass spectrometry. However, [1-^{14}C]-EETs have 6−11% 1-^{12}C contaminations and are limited by the natural abundance of [^{18}O]-EETs.[17] In short, no mass spectrometric method currently exists to readily establish high enantioselectivity in tissue levels of 14,15-EETs. For these reasons, a direct coupling of high-resolution chiral separations with mass spectrometry holds great promise.[29,30]

CONCLUSIONS

In the present report, HPLC and CE were used in tandem to identify and quantitate 14,15-EET enantiomers in murine liver. CE with a UV photodiode array detector set at 194 nm was a facile technique and was sufficiently sensitive to measure 8-pg quantities of underivatized enantiomers with a detection limit of ∼1 pg. To our knowledge, this represents the first time that CE has been used to analyze the chirality of trace endogenous eicosanoids. Because of comparable high absorptions at 194 nm,[27] other eicosanoids that are generated enzymatically and by free radical processes, e.g., prostaglandins and isoprostanes, should be amenable to similar CE analyses. Moreover, due to the high resolution (R = 1.32), the present CE method can be used to evaluate the enantiomeric purity of 14,15-EET standards for in vivo tests. Due to the variety of neutral and charged additives now becoming commercially available, we anticipate the CE resolution of all of the other EET enantiomers will soon be accomplished. Such CE methods will help elucidate the roles and mechanisms of EET synthesis in a variety of tissues.

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