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Journal of Chromatography B, 678 (1996) 339–343

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

High-performance liquid chromatography–thermospray mass spectrometry of 5,6-dihydroxyeicosatrienoate-1,5-lactone from tissue homogenates

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Received 5 September 1995; revised 13 November 1995; accepted 13 November 1995

Abstract

We have developed a method for the analysis of 5,6-dihydroxyeicosatrienoate-1,5-lactone (5,6-DiHETriE-δ-lactone) in tissue homogenates, supplemented with NADPH and arachidonic acid [20:4(*n*−6)] as a substrate. During the incubation and the extraction, most of the 5,6-epoxyeicosatrienoic acid (5,6-EpETriE) was converted to 5,6-dihydroxyeicosatrienoic acid (5,6-DiHETriE), and most of the 5,6-DiHETriE was converted to 5,6-DiHETriE-δ-lactone. Consequently, the chief degradation product of 5,6-EpETriE and 5,6-DiHETriE in the incubation mixture was 5,6-DiHETriE-δ-lactone. 5,6-DiHETriE-δ-lactone, corresponding to [20:4(*n*−6)], was shown to be characterized by a high intensity of quasimolecular ions (MH^+ and MNH_4^+), using ion analysis obtained by reversed-phase HPLC–thermospray MS. On selected-ion monitoring (SIM) chromatograms of 5,6-DiHETriE-δ-lactone and with deuterium-labeled 15(*S*)-hydroxyeicosatetraenoic acid as the internal standard, the regression equation of the peak-area ratio and the amount of 5,6-DiHETriE-δ-lactone was $y = 12.2x + 0.7$ ($r = 0.9996$). 5,6-Epoxygenase activity was represented as the sum of the amount of 5,6-DiHETriE-δ-lactone, 5,6-EpETriE and 5,6-DiHETriE per mg protein, after 30 min in an incubation mixture. The activity from rat brain homogenate decreased considerably with growth of the rat.

Keywords: 5,6-Dihydroxyeicosatrienoate-1,5-lactone; 5,6-Epoxyeicosatrienoic acid; Arachidonic acid; 5,6-Epoxygenase; Cytochromes

1. Introduction

Cytochrome P-450-dependent monooxygenases metabolize arachidonic acid [20:4(*n*−6)] to various products, such as epoxyeicosatrienoic acids (EpETriEs) and hydroxyeicosatetraenoic acids (HETEs) [1–6]. The cytochrome P-450 epoxygenase reaction metabolizes 20:4(*n*−6) to 5,6-, 8,9-, 11,12- and 14,15-EpETriE. EpETriEs are enzymatically hydrolysed by cytosolic epoxide hydrolases to produce the corresponding dihydroxyeicosatrienoic acids (DiHETriEs) [7,8]. EpETriEs, or DiHETriEs, have numerous biological activities, including effects on vascular tissue [9–11], modulation of membrane ion flux [12,13], stimulation of peptide hormone release [14,15], inhibition of cyclooxygenase activity and of platelet aggregation [16].

EpETriEs, especially 5,6-EpETriE, are difficult to measure since they undergo chemical change during isolation and sample work-up [17,18]. Although 5,6-

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0378-4347/96/$15.00
SSDI 0378-4347(95)00519-6

EpETriE has been reported to be a potent vasodilator agent [10,11,18,19], it is the most labile of the epoxides and is rapidly degraded to form 5,6-dihydroxyeicosatrienoate-1,5-lactone(5,6-DiHETriE-δ-lactone) via 5,6-DiHETriE, in an acidic non-polar solvent [17]. Cytochrome P-450 epoxygenase products of the fatty acids, including 5,6-DiHETriE-δ-lactone, contain no conjugated double bonds, making the highly selective detection of this compound by HPLC with UV detection difficult. Although the electron ionization GC–MS spectra of silyl-derivatives of 5,6-DiHETriE-δ-lactone provide structural information, the prominent fragment ions are the same as those of 5,6-dihydroxyeicosatetraenoate-1,5-lactone, derived from eicosapentaenoic acid [20].

We recently developed a method for the simultaneous detection of hydroxypolyunsaturated fatty acids (HPUFAs) and epoxypolyunsaturated fatty acids (EpPUFAs), including dihydroxy derivatives from each precursor fatty acid, in rat tissue homogenates using HPLC–thermospray (TSP) MS [3,4]. Adult rat brain and well-differentiated human colonic adenocarcinoma homogenates were found to show highly active NADPH-dependent ω-hydroxylation activity of docosahexaenoic acid [22:6($n-3$)] or 20:4($n-6$) [4–6]. ω-Hydroxylation activity in rat brain homogenate was concentrated in the 1000 g precipitate fraction (crude nuclei), instead of in the mitochondrial or microsomal fractions [6], and was found to associate with cytochrome P-450 [21]. This activity in rat brain homogenate increases considerably, whereas that in rat liver homogenate decreases significantly, with growth [21].

5,6-EpETriE undergoes enzymatic, or chemical change, to 5,6-DiHETriE-δ-lactone during incubation, isolation and sample work-up [17,18]. As a result, we have developed a method for the detection of 5,6-DiHETriE-δ-lactone corresponding to precursor 20:4($n-6$) in various tissue homogenates using HPLC–TSP-MS. The features of the 5,6-epoxygenase system were determined.

2. Experimental

2.1. Standards and reagents

15(S)-[5,6,8,9,11,12,14,15-$^2H_8$]-HETE[15(S)-HETE-d_8] was obtained from Cayman Chemicals (Ann Arbor, MI, USA). Racemic 5,6-DiHETriE-δ-lactone was obtained from Cascade Biochem (Reading, UK). 20:4($n-6$) was from Nu-Chek Prep. (Elysian, MN, USA). NADPH was from Oriental Yeast (Osaka, Japan). The BCA kit for protein measurement was obtained from Pierce (Rockford, IL, USA).

2.2. Incubation in tissue homogenates and extraction

Sprague–Dawley (SD) rats (1–55 days old) were anesthetized with ethyl-ether and killed by decapitation. The brains and livers were immediately excised at low temperature. Tissue was cut into pieces of ca. 3 × 3 mm and homogenized using a Polytron (Kinematica, Switzerland) in 12 ml of 50 mM Tris–HCl buffer (pH 7.5). The homogenates (5 ml each), a precursor fatty acid [657 nmol of 20:4($n-6$)] and NADPH (5 mg) were homogenized in a vortex-mixer, to disperse the substrate. Each mixture was incubated at 37°C for 30 min under aerobic conditions in a shaker set at 120 rpm. Each incubation mixture was acidified to ca. pH 4 with 15% (v/v) formic acid. 15(S)-HETE-d_8(608 pmol) was then added as the internal standard and the system was extracted twice with ethyl acetate. The ethyl acetate layer was washed twice with water, dried under anhydrous sodium sulfate for 5 min and filtered using No. 5A filter paper (Toyo Roshi, Tokyo, Japan). The filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in 200 μl of acetonitrile and 20-μl aliquots were subjected to HPLC–TSP-MS. The amount of protein in each homogenate was determined using a BCA kit [22].

2.3. HPLC–TSP-MS

A Shimadzu (Kyoto, Japan) LC-GC-MS-QP-1000S, equipped with a Vestec (Houston, TX, USA) Model 750B HPLC–TSP-MS interface, a Shimadzu LC-9A-HPLC pump and a Rheodyne injector, fitted with a 20-μl loop, was used. RP-HPLC separation was carried out using a Nucleosil 100 5C_{18} column (5 μm particle size, 150 × 4.6 mm I.D.; Macherey Nagel, Duren, Germany), with a mobile phase of 0.1 M ammonium formate–0.1 M formic acid–acetonitrile (8:2:15, v/v/v) at a flow-rate of 1.0 ml/min. The TSP interface temperature was optimized for

maximal detection sensitivity in the positive-ion mode under electron-beam-off conditions. The vaporizer control, vaporizer tip, vapour, block and tip heater temperature were maintained at 162, 287, 321, 345 and 346°C, respectively.

3. Results

A 5,6-DiHETriE-δ-lactone standard was shown to be characterized by a high intensity of quasimolecular ions (MH^+, m/z 321 and MNH_4^+, m/z 338), by ion analysis obtained by HPLC–TSP-MS, as shown in Fig. 1. Contamination with Na^+ in a mobile-phase yields a small amount of MNa^+ ion (m/z 343) from the lactone, on ion analysis. The ionic features of 5,6-DiHETriE-δ-lactone in HPLC–TSP–MS were similar to those of ω-HETE [4], although other common HETEs showed high intensities of base ion of MH^+-H_2O (m/z 303), as described previously [3]. A comparison of the sum of the peak areas corresponding to 5,6-DiHETriE-δ-lactone on each SIM chromatogram of MH^+-H_2O, MH^+, MNH_4^+ and MNa^+ ion, with that of the peak areas corresponding to 15(S)−HETE-d_8 as the internal standard on a SIM chromatogram of MH^+-H_2O (m/z 311), indicated an approximately linear relationship between the peak area ratio [5,6-DiHETriE-δ-lactone/15(S)-HETE-d_8] and the amount of 5,6-DiHETriE-δ-lactone. The regression equation was $y = 12.2x + 0.7$ ($r = 0.9996$).

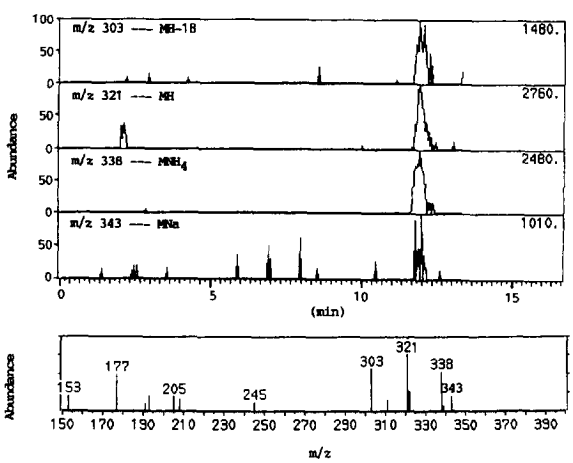

Fig. 1. Mass chromatogram profiles for each m/z number and mass spectrum obtained from 5,6-DiHETriE-δ-lactone. HPLC and TSP conditions are described in Section 2.3. The scan speed used was 1.0 scan/s from m/z 150 to 400. The spectral pattern corresponding to the main peak on each mass chromatogram is shown below. The number in the upper right-hand corner of each chromatogram is the ion count.

SIM chromatograms of an extract from an incubation mixture of 20:4($n-6$), added to brain and liver homogenates from a nineteen-day-old rat, along with NADPH, are shown in Fig. 2. Identification of each peak on the SIM chromatograms was done as described above and previously [3–6,24]. DiHETriEs appeared as MH^+-$2H_2O$ ion (m/z 303), MH^+-H_2O ion (m/z 321), MH^+ ion (m/z 339) and MNH_4^+ ion (m/z 356) as described previously [3]. HETEs, EpETriEs and 5,6-DiHETriE-δ-lactone appeared as the MH^+-H_2O ion (m/z 303), MH^+ ion (m/z 321), MNH_4^+ ion (m/z 338) and as the MNa^+ ion (m/z 343), as described above and previously [3,4,6]. Based on these findings, peaks A–R in Fig. 2-I and 2-II were assigned to: 14,15-DiHETriE (peak A); 11,12-DiHETriE (peak B); 8,9-DiHETriE (peak C); (ω-1)-HETE or 13-HETE (peak D); ω-HETE (peak E); (ω-2)-HETE or 10-HETE (peak F); (ω-4)-HETE (peak G); 15-HETE or 7-HETE (peak H); 11-HETE (peak I); 12-HETE (peak J); 9-HETE (peak K); 5-HETE (peak L); 5,6-DiHETriE-δ-lactone (peak M); 14,15-EpETriE (peak N); 11,12-EpETriE (peak O); 8,9-EpETriE (peak P); 5,6-EpETriE (peak Q) and 15(S)-HETE-d_8 (peak R) as the internal standard, respectively.

The amounts of 5,6-EpETriE and 5,6-DiHETriE were calculated from the sum of peak areas on m/z 303, 321, 338, 339, 343 and 356 as described previously [3]. Thus, 5,6-epoxygenase activity was represented as the sum of the amount of 5,6-DiHETriE-δ-lactone, 5,6-EpETriE and 5,6-DiHETriE per mg protein, after 30 min in an incubation mixture. The activity of rat brain homogenate decreased considerably with growth for up to 55 days, as shown in Fig. 3. In an incubation mixture of rat liver homogenate, it decreased significantly over the following nineteen days.

4. Discussion

Since most tissue homogenates express considerable cytosolic epoxide hydrolase activity [7,8] epoxides generated in an incubation mixture of the homogenate are converted rapidly to the corre-

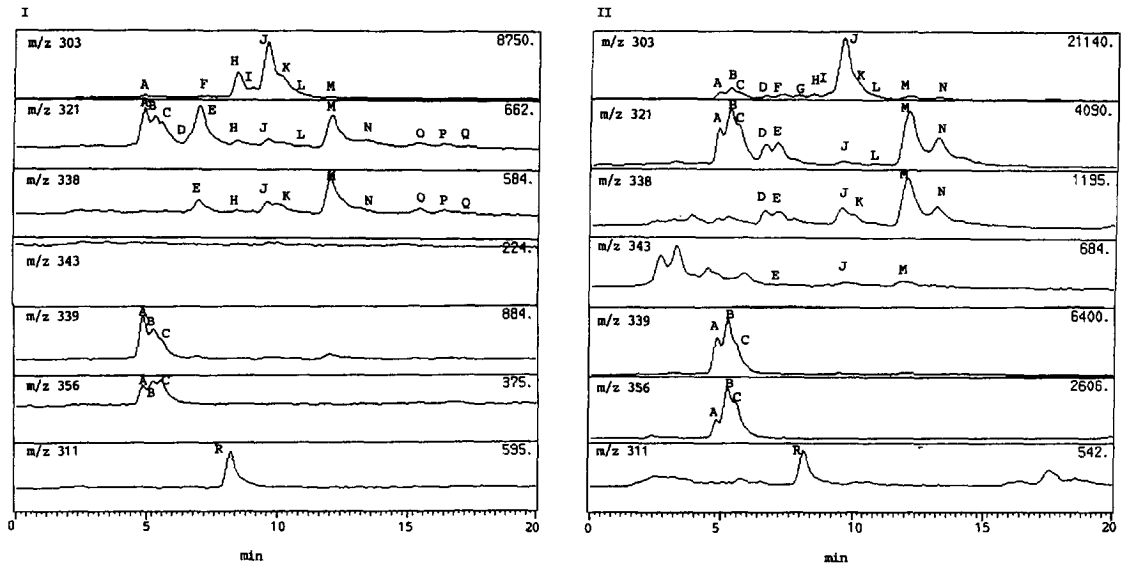

Fig. 2. SIM chromatograms of an extract from an incubation mixture of 20:4($n-6$), containing NADPH and rat brain homogenate (panel I) or rat liver homogenate (panel II) following nineteen days of growth. ω-HETE (peak E), 5,6-DiHETriE-δ-lactone (peak M) and EpETriEs (Peaks N, O, P and Q) show high intensities of MH^+ ion (m/z 321) and MNH_4^+ ion (m/z 338), although common HETEs (peaks D, F, G, H, I, J, K and L) show a high intensity of the MH^+-H_2O ion (m/z 303). DiHETriEs (peaks A, B and C) show a high intensity of the MH^+-2H_2O ion (m/z 303), MH^+-H_2O ion (m/z 321), MH^+ ion (m/z 339) and the MNH_4^+ ion (m/z 356). 15(S)-HETE-d_8 (peak R) as the internal standard shows a high intensity of the MH^+-H_2O ion (m/z 311). Other conditions are as described in Section 2. The number in the upper right-hand corner of each chromatogram is the ion count.

sponding dihydroxy derivatives. In a non-polar solvent, including the acids used in this study, 5,6-DiHETriE was converted rapidly to 5,6-DiHETriE-δ-lactone. The chief degradation product of 5,6-EpETriE and 5,6-DiHETriE, in an extract with a non-polar solvent, including acids from the incubation mixture of the homogenate, is thus 5,6-DiHETriE-δ-lactone, as shown in Fig. 2.

5,6-Epoxygenase activity in rat brain homogenate decreased appreciably with growth for up to 55 days (Fig. 3). However, a considerable amount of 5,6-EpETriE was detected in an incubation mixture of neonatal rat brain homogenate compared to that from nineteen day-old rat brain. However, DiHETriEs in a neonatal rat brain homogenate could hardly be detected. Thus, cytosolic epoxide hydrolase activity may be reduced in neonatal rat brain. Since 5,6-EpETriE was virtually absent from rat liver homogenate throughout this study, rat liver may have high hydrolase activity. The decrease in the 5,6-epoxygenase activity of 20:4($n-6$) (Fig. 3) and the increase in the ω-hydroxylation activity of 20:4($n-6$), or 22:6($n-3$), in rat brain with growth [21], may be related to brain function. On the other hand, since 5,6-EpETriE is a potent vasodilator agent [10,11,18,19], the highly active 5,6-epoxygenase activity in a neonatal rat brain may be related to the blood flow in a neonatal rat brain, and the decrease in the 5,6-epoxygenase activity of rat liver over the following nineteen days may be related to a decrease in the blood flow of rat liver/kg body weight following growth [23]. We recently found that 5,6-epoxygenase activity in rat brain is concentrated in cerebral microvessels and that the activity in rat liver is concentrated in the mitochondrial, or microsomal fraction, but not in the microvessels (data not shown). Our attention has been directed to the relationship between the epoxygenase/ω-hydroxylation activity and senescence/differentiation/apoptosis in the cells.

Although 5,6-EpETriE is the most potent vasodilator agent in cytochrome P-450 products of 20:4($n-6$) [10,11,18,19], the 5,6-EpETriE content is difficult to measure due to its lability [17,18]. Thus, the

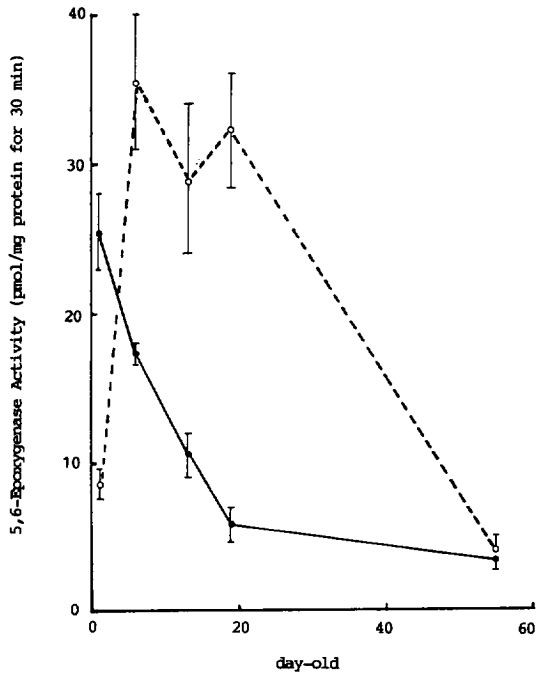

Fig. 3. Influence of growth on the 5,6-epoxygenase activity of 20:4($n-6$) in rat brain and rat liver homogenates, respectively. The amounts of 5,6-EpETriE and 5,6-DiHETriE were calculated from the sum of the peak areas on m/z 303, 321, 338, 339, 343 and 356, as described previously [3]. Thus, 5,6-epoxygenase activity was represented as the sum of the amount of 5,6-DiHETriE-δ-lactone, 5,6-EpETriE and 5,6-DiHETriE per mg protein, after 30 min in an incubation mixture. ● = 5,6-epoxygenase activity in rat brain homogenate; ○ = 5,6-epoxygenase activity in rat liver homogenate. Results are expressed as the mean ± S.E.M. (n = 4). Other conditions are as described in Section 2.

present method should prove quite useful for indirectly measuring of 5,6-EpETriE, in incubation mixtures of various tissue homogenates.

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