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Short communication

High-performance liquid chromatograpy–thermospray mass spectrometry of ω-hydroxy polyunsaturated fatty acids from rat brain homogenate

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Abstract

A method for the analysis of ω-hydroxy polyunsaturated fatty acids (ω-HPUFAs) in rat tissue homogenate, supplemented with NADPH and homo-γ-linolenic acid [20:3($n-6$)], arachidonic acid [20:4($n-6$)], eicosapentaenoic acid [20:5($n-3$)] or docosahexaenoic acid [22:6($n-3$)] as a substrate was developed. By ion analysis of chromatograms obtained with reversed-phase HPLC–thermospray MS, many ω-HPUFAs corresponding to each precursor fatty acid could be characterized by the high intensity of the molecular ion (MH^+) and quasimolecular ion (MNH_4^+, MNa^+), while other common HPUFAs were characterized by the high intensity of the base ion of $MH^+ - H_2O$. On a selected-ion monitoring chromatogram of rat brain homogenate, significant amounts of ω-HPUFA from each precursor fatty acid, especially from 22:6($n-3$), were detected compared with the amounts found in rat large intestine homogenate. Based on these results, a highly active NADPH-dependent ω-oxidation system is suggested for rat brain homogenate resulting in extensive oxidation of 22:6($n-3$).

1. Introduction

Cytochrome P-450 dependent monooxygenases metabolize arachidonic acid [20:4($n-6$)] to several products such as epoxy eicosatrienoic acids (EpETriEs) [1,2] and hydroxy eicosatetraenoic acids (HETEs) including 16-, 17-, 18-, 19- and 20-HETEs [3–6]. These EpETriEs and ω-hydroxylated HETEs such as ω-, (ω-1)- and (ω-2)-HETE do not have conjugated double bonds, and thus the highly selective detection of these compound by HPLC with UV detection is difficult. Although GC–MS of ω-HETE separated by an HPLC method with radioactive detection is the most reliable method at present [7–9], an ion characteristic for only ω-HETE has not been found on the MS pattern. Consequently, the selective detection of ω-HETE overlapping with other HETEs by selected-ion monitoring (SIM) is impossible.

Cytochrome P-450 dependent monooxygenases metabolize homo-γ-linolenic acid [20:3($n-6$)], eicosapentaenoic acid [20:5($n-3$)] or docosahexaenoic acid [22:6($n-3$)] to several products such as epoxy polyunsaturated fatty acids (EpPUFAs) [10] and ω-hydroxy polyunsaturated fatty acid (ω-HPUFA) [9,11]. ω-HPUFA derived from 20:5($n-3$) or 22:6($n-3$)

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by cytochrome P-450 dependent monooxygenases could not be found in significant amounts as compared with ω-HETE derived from 20:4 ($n-6$) [9].

We have developed a method for the simultaneous detection of ω-HPUFAs corresponding to each precursor fatty acid in rat tissue homogenates by HPLC–thermospray(TSP) MS. The features of the ω-oxidation system in rat brain homogenate were determined.

2. Experimental[1]

2.1. Standards and reagents

(5*Z*, 8*Z*, 11*Z*, 14*Z*)-20-Hydroxy eicosatetraenoic acid (ω-HETE) and 20:5($n-3$) were obtained from Cascade Biochem (Reading, UK). 12(*S*)-[5, 6, 8, 9, 11, 12, 14, 15-$^2H_8$]-HETE [12(*S*)-HETE-d_8] was obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). (±)14, 15-Epoxy eicosatrienoic acid-d_8 (14, 15-EpETriE-d_8) was obtained from Biomol Laboratories (Plymouth Meeting, PA, USA); 20:3($n-6$), 20:4($n-6$) and 22:6($n-3$) were obtained from Nu-Chek Prep. (Elysian, MN, USA). β-NADPH was obtained from Oriental Yeast Co. (Osaka, Japan). Other solvents and reagents were of analytical-reagent grade.

2.2. Extraction from rat tissue homogenate

A male Wistar rat (400 g, 11 month old) was killed by decapitation, and the brain (2 g) and large intestine (2 g) were immediately excised at low temperature. The tissue was cut into pieces (*ca.* 2 × 2 mm) and washed twice by decantation with 5 ml of 8.5 g/l sodium chloride. The pieces were suspended in 20 ml of 50 m*M* Tris-HCl buffer (pH 7.5) and homogenized in a Polytron (Kinematica, Switzerland) homogenizer. Aliquots of 20:3($n-6$), 20:4($n-6$), 20:5($n-3$) or 22:6($n-3$) containing *ca.* 600 nmol in ethanol, were evaporated to dryness in incubation tubes under reduced pressure. To the dry residue, rat brain or intestine homogenate (5 ml each) and β-NADPH (4.6 mg) were added, and the mixture was 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 operated at 120 rpm. The incubation mixture was acidified to *ca.* pH 3 with 15% formic acid, 12(*S*)-HETE-d_8 or 14, 15-EpETriE-d_8 was added as the internal standard, and the system was extracted twice with ethyl acetate. The ethyl acetate layer was washed with water until the aqueous layer had a pH of *ca.* 4, dried under anhydrous sodium sulfate for 5 min and filtered with 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.

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, Düren, Germany), with a mobile phase of 0.1 *M* ammonium formate–0.1 *M* formic acid–acetonitrile (8:2:15 or 4:1:5, v/v) at a flow-rate of 1.0 ml/min. TSP interface temperature was optimized for maximum detection sensitivity in the positive-ion mode under electron-beam-off conditions. The vaporizer control, vaporizer tip,

[1] Abbreviations: arachidonic acid [20:4($n-6$)], epoxy eicosatrienoic acid (EpETriE), hydroxy eicosatetraenoic acid (HETE), selected-ion monitoring (SIM), homo-γ-linolenic acid [20:3($n-6$)], eicosapentaenoic acid [20:5($n-3$)], docosahexaenoic acid [22:6($n-3$)], epoxy polyunsaturated fatty acid (EpPUFA), ω-hydroxy polyunsaturated fatty acid (ω-HPUFA), thermospray (TSP), $^2H_8$ (d_8), dihydroxy eicosatetraenoic acid (DiHETE), epoxy eicosatetraenoic acid (EpETE), hydroxy eicosapentaenoic acid (HEPE), hydroxy eicosatrienoic acid (HETriE), hydroxy docosahexaenoic acid (HDHE), epoxy eicosadienoic acid (EpEDE), dihydroxy eicosatrienoic acid (DiHETriE), epoxy docosapentaenoic acid (EpDPE).

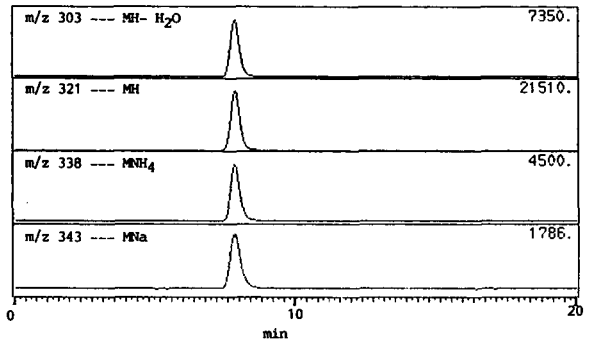

Fig. 1. SIM chromatograms of authentic ω-HETE (ca. 3 nmol). 0.1 *M* Ammonium formate–0.1 *M* formic acid–acetonitrile (8:2:15, v/v) was used as mobile phase. Other conditions as described in Experimental. The number in the upper right-hand corner of each chromatogram is the ion count.

vapour, block and tip heater temperatures were maintained at 151, 263, 316, 344 and 343°C, respectively.

3. Results and discussion

By ion analysis of chromatograms obtained with HPLC–TSP-MS, a ω-HETE standard was characterized by the high intensity of the molecular ion (MH^+) and quasimolecular ion (MNH_4^+, MNa^+) as shown in Fig. 1. Other common HETEs were characterized by the high intensity of the base ion of $MH^+ - H_2O$ as described previously [10].

SIM chromatograms of an extract from an incubation of 20:5($n-3$) added to rat brain homogenate with or without β-NADPH are shown in Fig. 2. On the basis of *m/z* 319, 301 or 336 peak C in Fig. 2 was assigned to 20-hydroxy eicosapentaenoic acid (ω-HEPE) by comparison with the chromatographic behaviour and the ion pattern characteristic of ω-HETE. On the basis of *m/z* 301 or 319 peaks A and B were assigned to dihydroxy eicosatetraenoic acids (DiHETEs) resulting from hydrolysis of epoxy eicosatetraenoic acids (EpETEs) as described previously [10]. On the basis of *m/z* 301 or 336 peaks D, E, F and G were assigned to 18-HEPE, 15-HEPE, 12-HEPE and 17, 18-EpETE, respectively, as described previously [10]. On the basis of *m/z* 311 peak H was the ionic peak of 14, 15-EpETriE-d_8 used as the internal standard. Addition of β-NADPH increased the amount of ω-HEPE (peak C) and similarly addition of β-NADPH to an incubation mixture of the precursor fatty acid added to rat brain homogenate increased the content of ω-hydroxy eicosatrienoic acid (ω-HETriE), ω-HETE or ω-hydroxy docosahexaenoic acid (ω-HDHE) (data not shown). Rat brain homogenate would thus appear to express enzymic ω-oxidative activity (i.e. cytochrome P-450 system).

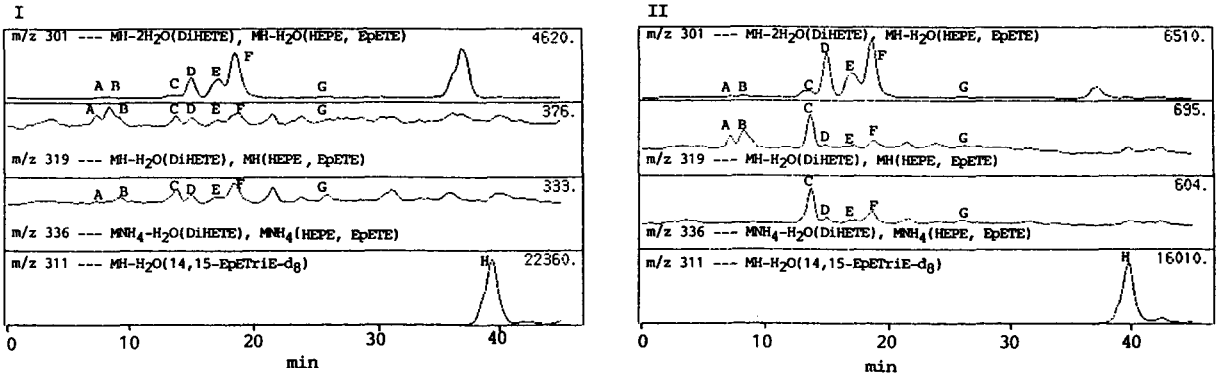

Fig. 2. SIM chromatograms of an extract from an incubation mixture of 20:5($n-3$) with rat brain homogenate with (panel II) or without (panel I) β-NADPH. 0.1 *M* Ammonium formate–0.1 *M* formic acid–acetonitrile (4:1:5, v/v) was used as mobile phase. Other conditions as described in Experimental. The number in the upper right-hand corner of each chromatogram is the ion count.

SIM chromatograms of an extract from an incubation mixture of 20:3($n-6$), 20:4($n-6$), 20:5($n-3$) 22:6($n-3$) added to rat brain homogenate along with β-NADPH are shown in Fig. 3. On the basis of m/z 305, 323, 340 or 345 peak C in Fig. 3I was assigned to ω-HETriE by comparison with the chromatographic behaviour and the ion pattern characteristic for ω-HETE or ω-HEPE. The shoulder B of peak C can be regarded as (ω-1)-HETriE by comparison with its chromatographic behaviour [3–5], but additional analysis was not performed. On the basis of m/z 305 or 323 peak A was assigned to dihydroxy eicosadienoic acids derived from hydrolysis of epoxy eicosadienoic acids (EpEDEs) as described previously [10]. On the basis of m/z 305 or 345 peaks D, E, F and G were assigned to isomers of HETriE as described previously [10]. On the basis of m/z 323, 340 or 345 peaks H, I and J were assigned to 14, 15-EpEDE, 11, 12-EpEDE and 8, 9-EpEDE, respectively, as shown previously [10].

On the basis of m/z 303, 321, 338 or 343 peak E in Fig. 3II was assigned to ω-HETE by comparison with the chromatographic behaviour and the ion pattern characteristic of authentic ω-HETE (Fig. 1) described above. Peak F can be regarded as (ω-2)-HETE by comparison with its chromatographic behaviour [3–5] and by the high intensity of the base ion of $MH^+ - H_2O$, but no additional analysis was performed. On the basis of m/z 303 or 321 peaks A, B, C and D were assigned to 14, 15-dihydroxy eicosatrienoic acid (14, 15-DiHETriE), 11, 12-DiHETriE, 8, 9-DiHETriE and 5, 6-DiHETriE respectively, as described previously [10]. On the basis of m/z 303 or 343 peaks G, H, I and J were assigned to 15-HETE, 11-HETE, 12-HETE and 9-HETE, respectively, as described previously [10].

Peaks A, B, C, D, E, F and G in Fig. 3III are

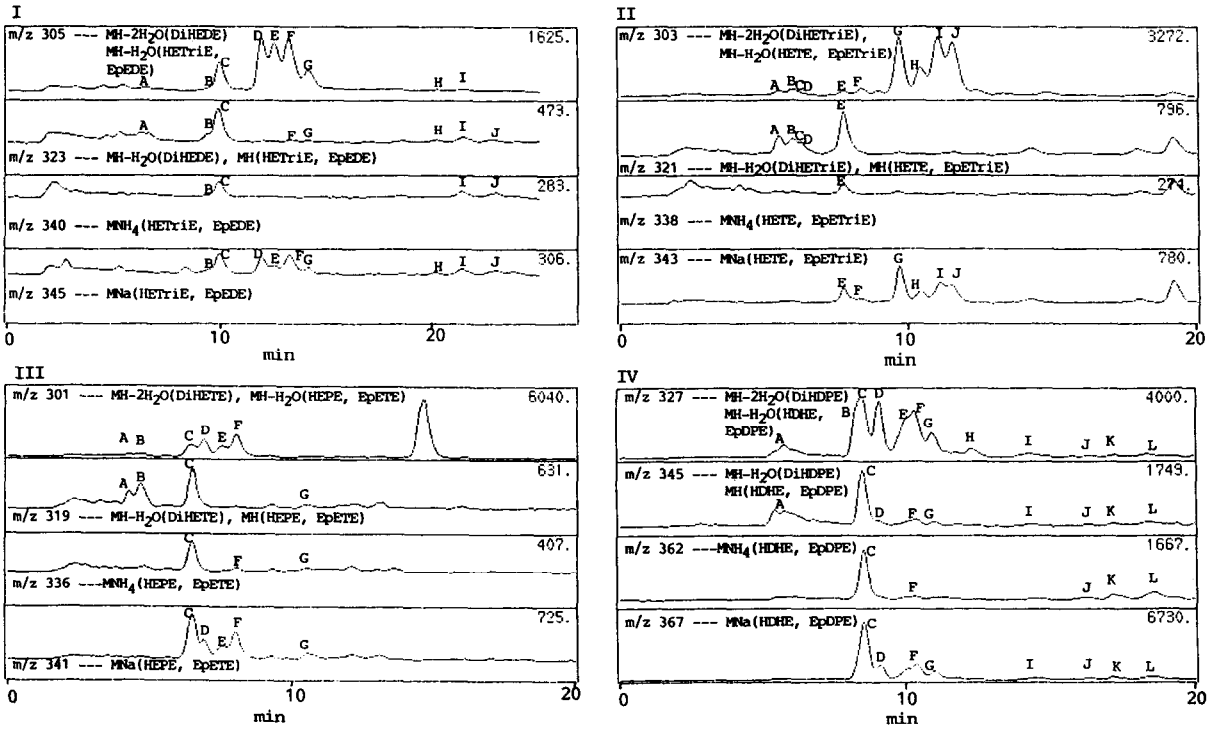

Fig. 3. SIM chromatograms of an extract from an incubation mixture of 20:3($n-6$) (panel I), 20:4($n-6$) (panel II), 20:5($n-3$) (panel III) or 22:6($n-3$) (panel IV) with rat brain homogenate with β-NADPH. 0.1 *M* Ammonium formate–0.1 *M* formic acid–acetonitrile (8:2:15, v/v) was used as mobile phase. Other conditions as described in Experimental. The number in the upper right-hand corner of each chromatogram is the ion count.

identical to the corresponding peaks in Fig. 2. The late emerging peak under m/z 301 in Fig. 3III and Fig. 2 shows a base-ion of m/z 279 and a sub-ion of m/z 301 as described previously [10]. While this is assigned as a byproduct resulting from the organic solvents, identification and effective removal were not performed.

On the basis of m/z 327, 345, 362 or 367 peak C in Fig. 3IV was assigned to ω-HDHE by comparison with chromatographic behaviour and the ion pattern characteristic of ω-HETriE, ω-HETE or ω-HEPE as described above. The shoulder B of peak C on m/z 327 can be regarded to be due to (ω-1)-HDHE by comparison with its chromatographic behaviour and by the high intensity of the base ion of MH^+-H_2O, but additional analysis was not done. Peaks A, D, E, F, G, H, I, J, K and L were assigned to dihydroxy docosapentaenoic acids resulting from hydrolysis of epoxy docosapentaenoic acids (EpDPEs), 20-HDHE, 17-HDHE, 14-HDHE, 11-HDHE, 4-HDHE, 19, 20-EpDPE, 13, 14-EpDPE, 10, 11-EpDPE and 7,8-EpDPE, respectively, as described previously [10].

A standard curve for ω-HETE is shown in Fig. 4. When the sum of the peak areas corresponding to ω-HETE on each SIM chromatogram of $MH^+ - H_2O$, MH^+, MNH_4^+ and MNa^+ ion is compared with the sum of the peak areas corresponding to 12(S)-HETE-d_8 as the internal standard on each SIM chromatogram of MH^+-H_2O (m/z 311) and MNa^+ (m/z 351), an approximately linear relationship exists between the peak area ratio [ω-HETE/12(S)-HETE-d_8] and the amount of ω-HETE. Also for other ω-HPUFAs such a relationship was found. As shown in Table 1, the fatty acids of the n-3 system, particularly 22:6($n-3$), were extensively converted to ω-HPUFAs in rat brain homogenate compared with those of the $n-6$ system. Rat brain homogenate expressed considerable ω-hydroxylation activity compared with the rat large intestine homogenate. Rat brain thus appears to exhibit high NADPH-dependent ω-hydroxylation activity, and this ω-hydroxylation system may be the major metabolic pathway of 22:6($n-3$) in rat brain. A low ω-hydroxylation activity of decanoate in rat brain or intestine [12] and a low ω-hydroxylation activity of 22:6($n-3$) in P-450 Ka-2 [9] have been demonstrated and thus, rat brain may possess a 22:6($n-3$) specific NADPH dependent ω-hydroxylation enzyme.

Previous studies have reported: The stimulatory effect of (ω-1)-HETE on rat renal cortex microsomal Na^+/K^+-ATPase [13] and on vascular activity in rat kidney [14], the effect of (ω-2)-HETE on contraction activity of guinea pig lung strips and relaxation activity of guinea pig arteries [15], as well as the effect of ω-HETE on vasoactivity [4], inhibitor effect on rabbit renal medulla microsomal Na^+/K^+-ATPase [8], stimulatory effect on erythropoietin-dependent stem cell growth in human bone marrow [16] and inhibitor effect on platelet aggregation [17].

The physiological activity of ω-HDHE or ω-HEPE is clearly of interest.

4. Conclusions

Since ω-HPUFA was characterized by the high intensity of the molecular and quasimolecular

Table 1
Conversion of each precursor fatty acid to ω-HPUFA using rat brain or rat large intestine homogenate

Tissue homogenate	Conversion of each precursor fatty acid to ω-HPFUA(%)[a]			
	20:3($n-6$)	20:4($n-6$)	20:5($n-3$)	22:6($n-3$)
Rat brain	0.42	0.39	0.90	2.06
Rat large intestine	0.08	0.06[b]	0.11	0.87

[a] Percentage conversion determined from amount of ω-HPUFA measured with 12(S)-HETE-d_8 as the internal standard and amount of precursor fatty acid.
[b] ω-HETE was not detected, and thus percentage was determined as conversion to (ω-2)-HETE.

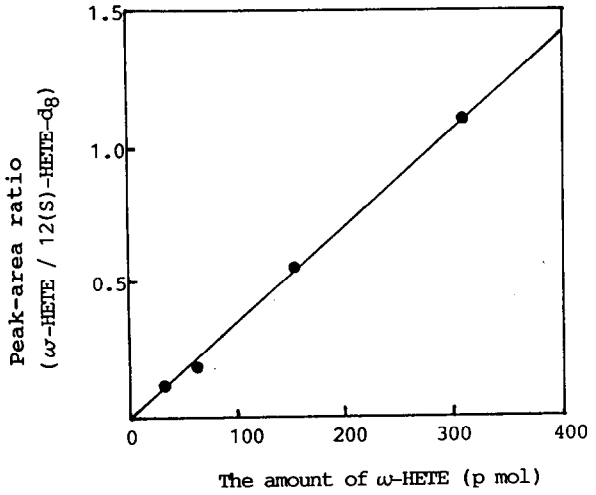

Fig. 4. A standard curve of ω-HETE. A fixed aliquot (304 pmol) of 12(*S*)-HETE-d_8 as an internal standard was mixed with various known quantities of ω-HETE from 31 to 312 pmol, and analyzed by HPLC–TSP-MS in the SIM mode. The sum of the peak areas corresponding to ω-HETE on each SIM chromatogram of $MH^+ - H_2O$, MH^+, MNH_4^+ and MNa^+ ion was compared with the sum of the peak areas corresponding to 12(*S*)-HETE-d_8 as the internal standard on each SIM chromatogram of $MH^+ - H_2O$ (*m/z* 311) and MNa^+ (*m/z* 351).

ions (MH^+, MNH_4^+ and MNa^+) in HPLC–TSP-MS analysis, SIM chromatograms using these ions could be used to selectively detect ω-HPUFA from other common HPUFAs. Significant amounts of ω-HPUFA from each precursor fatty acid, especially from 22:6($n-3$) were detected in rat brain homogenate. Rat brain thus appears to exhibit a high NADPH-dependent ω-hydroxlation activity, and this ω-hydroxylation system may be the major metabolic pathway of 22:6($n-3$) in rat brain.

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