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Subcellular localization of docosahexaenoic acid and arachidonic acid ω-hydroxylation activity in the brain, liver and colonic adenocarcinoma

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Abstract

A homogenate of rat brain, rat liver or human colonic well differentiated adenocarcinoma was prepared in 250 m*M* sucrose isoosmolaric buffer (pH 7.6) and fractionated by differential centrifugation at 10^3, 10^4 and 10^5 *g*. Each precipitate or supernatant was incubated with NADPH and docosahexaenoic acid or arachidonic acid as a substrate for 30 min at 37°C under aerobic conditions. ω-Hydroxydocosahexaenoic acid or ω-hydroxyeicosatetraenoic acid from an incubation mixture was detected by reversed-phase high-performance liquid chromatography–thermospray mass spectrometry with selected-ion monitoring. ω-Hydroxy polyunsaturated fatty acids were characterized by high intensity of the molecular ion (MH^+) although common hydroxy polyunsaturated fatty acids were characterized by high intensity of the $MH^+ - H_2O$ ion. For the rat brain, ω-hydroxylation activity (the amount of ω-hydroxy product produced in 30 min) was concentrated to a 10^3 *g* precipitate although the specific activity (the activity per 1 mg of protein) in the 10^3 *g* precipitate did not indicate superiority over other fractions. However, the specific activity of the rat brain increased on addition of a 10^4 or 10^5 *g* precipitate. For the rat liver, although ω-hydroxylation activity was concentrated to a 10^3 *g* precipitate, the specific activity was concentrated to a 10^5 *g* precipitate and the subcellular localization differed from that of rat brain. In the human colonic well differentiated adenocarcinoma, although ω-hydroxylation activity was relatively high in the 10^3 *g* supernatant, the specific activity was relatively high in the 10^3 and 10^5 *g* precipitates. These results suggest that there is a difference regarding subcellular localization of the ω-hydroxylation activity depending on the species of the organs.

Keywords: Liquid chromatography–mass spectrometry; Fatty acids; Arachidonic acid; Eicosapentaenoic acid; Docosahexaenoic acid

1. Introduction

Cytochrome P-450-dependent monooxygenases in microsomes from liver or kidney cortex metabolize polyunsaturated fatty acids (PUFAs) into various products, including ω-hydroxy-PUFAs (ω-HPUFAs) and epoxy-PUFAs (Ep-PUFAs) [1–9]. Liver microsomes from ciprofibrate-treated rats cause an increase in the ω-oxidation of arachidonic acid [20:4(*n* − 6)] [7]. Ethanol treatment of rabbits alters the distribution of P-450 isoforms via the induction of P-450

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2E1 in microscomes from liver and kidney cortex [6], and the P-450 2E1 mainly metabolized 20:4($n-6$) to ($\omega-1$)-hydroxyeicosatetraenoic acid [($\omega-1$)-HETE] and ($\omega-2$)-HETE [6].

ω-HPUFAs derived from eicosapentaenoic acid or docosahexaenoic acid [22:6($n-3$)] by P-450 Ka2 in kidney cortex microsomes of rabbit cannot be detected in significant amount compared with ω-HETE from 20:4($n-6$) [8]. Rat brain or intestine has a low ω-hydroxylation activity of decanoate [10].

We recently developed a method for the simultaneous detection of ω-HPUFAs from each precursor fatty acid in a rat tissue homogenate by high-performance liquid chromatography (HPLC)–thermospray (TSP) mass spectrometry (MS) [11], and found that adult rat brain or large intestine homogenate shows a highly active NADPH-dependent ω-hydroxylation activity of PUFAs, especially at 22:6($n-3$) [11]. We subsequently found that human colonic well differentiated adenocarcinoma homogenate exhibits high NADPH-dependent ω-hydroxylation activity of 22:6($n-3$) or 20:4($n-6$) compared with that of a colonic region remote from the carcinoma [12].

This paper addresses subcellular localization of 22:6($n-3$) and 20:4($n-6$) ω-hydroxylation activity in rat brain, rat liver and human colonic well differentiated adenocarcinoma.

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 Chemical (Ann Arbor, MI, USA), 22:6($n-3$) and 20:4($n-6$) from Nu-Chek Prep. (Elysian, MN, USA), β-NADPH from Oriental Yeast (Osaka, Japan) and a BCA Kit from Pierce (Rockford, IL, USA). Racemic 5,6-dihydroxy eicosatrienoate-1,5-lactone (5,6-DiHETriE-γ-lactone) was obtained from Cascade Biochem (Reading, UK).

2.2. Fatty acid metabolism and extraction from incubation mixture

Male Sprague–Dawley rats (250–300 g) were anaesthetized with diethyl ether and the brain and the liver were perfused with 100 ml of oxygen-saturated physiological saline containing 5 U/ml of heparin. Human colonic well differentiated adenocarcinoma was collected during surgical operation. The brains, livers and the colonic adenocarcinoma were rapidly removed and homogenized in a Polytron (Kinematica, Switzerland) in 250 mM sucrose–50 mM KCl–2 mM $MgCl_2$–20 mM Tris–HCl isoosmolaric buffer (pH 7.6). Each homogenate was fractionated by differential centirfugation at 10^3, 10^4 and 10^5 g as described previously [13]. Aliquots (5 ml) of each precipitate (Pt) suspended in the same isoosmolaric buffer or each supernatant (Sp), a precursor fatty acid [609 nmol of 22:6($n-3$) or 657 nmol of 20:4($n-6$)] and β-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 operated at 120 rpm. The incubation mixture was acidified to ca. pH 4 with 15% formic acid. 15(S)-HETE-d_8 (304 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 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. The amount of the protein in the homogenate were determined using the BCA Kit [14].

2.3. HPLC–TSP-MS

A Shimadzu (Kyoto, Japan) LG-GC-MS-QP-1000S system, 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, v/v/v) at a flow-rate of 1.0 ml/min. The TSP interface temperature was optimized for maximum detection sensitivity in the positive-ion mode under electron-beam-off conditions. The vaporizer control, vaporizer tip, vapour, block and tip heater temperatures were maintained at 162, 287, 321, 345 and 346°C, respectively.

3. Results and discussion

Selected-ion monitoring (SIM) chromatograms of an extract from an incubation mixture of 22:6($n-3$) or 20:4($n-6$) added to the 10^3 *g* Pt or 10^3 *g* Sp fraction obtained from rat brain, rat liver or human colonic adenocarcinoma homogenate with β-NADPH are shown in Figs. 1 and 2. The identification of each peak on the SIM chromatograms was achieved as described previously [11,12,15]. Dihydroxydocosapentaenoic acids (DiHDPEs) appeared as the ion $MH^+ - 2H_2O$ (*m/z* 327), $MH^+ - H_2O$ (*m/z* 345) and quasimolecular ion (*m/z* 362, 367), MH^+ (*m/z* 363), MNH_4^+(*m/z* 380) and MNa^+ (*m/z* 385). Hydroxydocosahexaenoic acids (HDHEs) and epoxydocosapentaenoic acids (EpDPEs) appeared as the ions $MH^+ - H_2O$ (*m/z* 327), MH^+ (*m/z* 345), MNH_4^+(*m/z* 362) and MNa^+ (*m/z* 367). 15(*S*)-HETE-d_8 appeared as the $MH^+ - H_2O$ ion (*m/z* 311). ω-HDHE is characterized

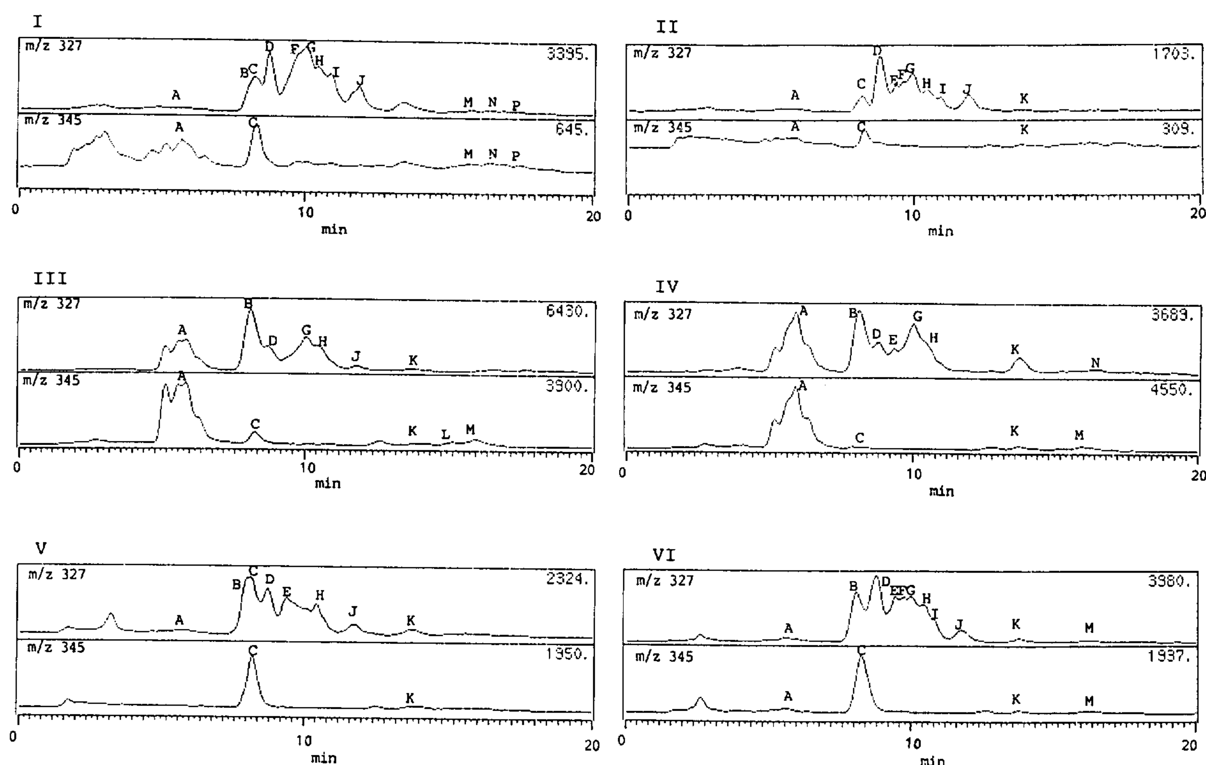

Fig. 1. SIM chromatograms of an extract from an incubation mixture of 22:6($n-3$) additional rat brain 10^3 *g* Pt fraction (I), rat brain 10^3 *g* Sp fraction (II), rat liver 10^3 *g* Pt fraction (III), rat liver 10^3 *g* Sp fraction (IV), human colonic adenocarcinoma 10^3 *g* Pt fraction (V) and human colonic adenocarcinoma 10^3 *g* Sp fraction (VI) with β-NADPH. ω-HDHE (peak C) is characterized by high intensity of the molecular ion (MH^+, *m/z* 345), although common HDHEs (peaks B, D, E, F, G, H, I and J) are characterized by high intensity of the $MH^+ - H_2O$ (*m/z* 327). Other conditions as described under Experimental. The number in the top-right corner of each chromatogram is the ion count.

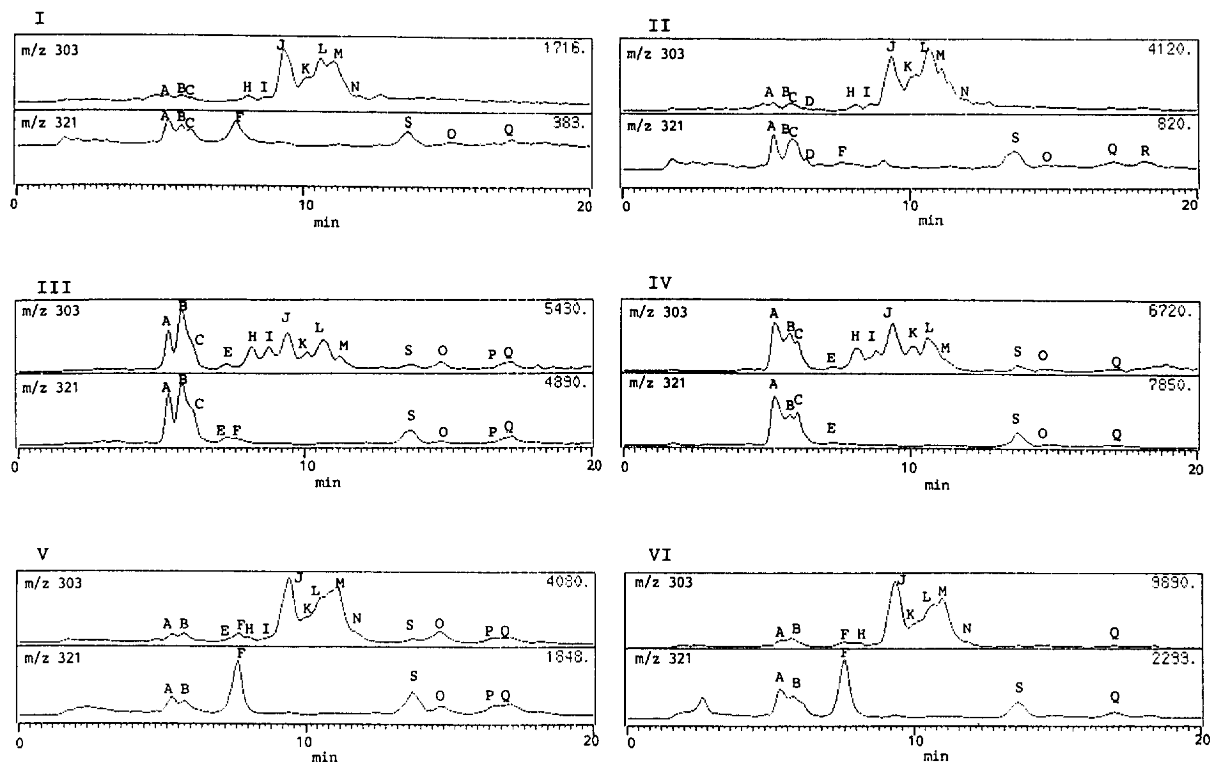

Fig. 2. SIM chromatograms of an extract from an incubation mixture of 20:4($n-6$) additional rat brain 10^3 g Pt fraction (I), rat brain 10^3 g Sp fraction (II), rat liver 10^3 g Pt fraction (III), rat liver 10^3 g Sp fraction (IV), human colonic adenocarcinoma 10^3 g Pt fraction (V) and human colonic adenocarcinoma 10^3 g Sp fraction (VI) with β-NADPH. ω-HETE (peak F) and 5,6-DiHETriE-γ-lactone (peak S) are characterized by high intensity of the molecular ion (MH^+, m/z 321), although common HETEs (peaks E, H, I, J, K, L, M and N) are characterized by high intensity of the $MH^+ - H_2O$ ion (m/z 303). Other conditions as described under Experimental. The number in the top-right corner of each chromatogram is the ion count.

by high intensity of the molecular ion (MH^+, m/z 345) and quasimolecular ion (m/z, 362, 367), although common HDHEs are characterized by high intensity of the $MH^+ - H_2O$ ion (m/z 327). On the basis of m/z 327 or 345, peaks A–P in Fig. 1I–VI were assigned to DiHDPEs (peak A) resulting from hydrolysis of EpDPEs, ($\omega-1$)-HDHE (B). ω-HDHE (C), 20-HDHE (D), 17-HDHE (E), 13-HDHE (F), 14-HDHE (G), 11-HDHE (H), 8-HDHE (I), 4-HDHE (J), 19,20-EpDPE (K), 16,17-EpDPE (L), 13,14-EpDPE (M), 10,11-EpDPE (N), 7,8-EpDPE (O) and 4,5-EpDPE (P).

Dihydroxyeicosatrienoic acids (DiHETriEs) appeared as the ions $MH^+ - 2H_2O$ (m/z 303), $MH^+ - H_2O$ (m/z 321), MH^+ (m/z 339), MNH_4^+ (m/z 356) and MNa^+ (m/z 361). HETEs, epoxyeicosatrienoic acids (EpETriEs) and 5,6-DiHETriE-γ-lactone appeared as the ions $MH^+ - H_2O$ (m/z 303), MH^+ (m/z 321), MNH_4^+ (m/z 338) and MNa^+ (m/z 343). ω-HETE and 5,6-DiHETriE-γ-lactone are characterized by high intensity of the molecular ion (MH^+, m/z 321) and quasimolecular ion (m/z 338, 343) although common HETEs are characterized by high intensity of the $MH^+ - H_2O$ ion (m/z 303). On the basis of m/z 303 or 321, peaks A–S in Fig. 2I–VI were assigned to 14,15-DiHETriE (peak A) resulting from hydrolysis of 14,15-EpETriE, 11,12-DiHETriE (B), 8.9-Di-

HETriE (C), 5,6-DiHETriE (D), ($\omega - 1$)-HETE (E), ω-HETE (F), ($\omega - 3$)-HETE (G), ($\omega - 2$)-HETE (H), ($\omega - 4$)-HETE (I), 15-HETE (J), 11-HETE (K), 12-HETE (L), 9-HETE (M), 5-HETE (N), 14,15-EpETriE (O), 11,12-EpETriE (P), 8,9-EpETriE (Q), 5,6-EpETriE (R) and 5,6-DiHETriE-γ-lactone (S). In the process of incubation and extraction in this study, most 5,6-EpETriE (R) was converted into 5,6-DiHETriE (D), and most 5,6-DiHETriE was converted into 5,6-DiHETriE-γ-lactone (S).

Regarding the rat brain and liver, ω-hydroxylation activity (the amount of ω-HDHE or ω-HETE produced in 30 min) was concentrated to 10^3 *g* Pt (Figs. 1 and 2). In the human colonic well differentiated adenocarcinoma, ω-hydroxylation activity was relatively high in 10^3 *g* Sp. Since the homogenate of rat blood or human myelogenous leukaemia leukocyte did not produce ω-HDHE or ω-HETE in a similar manner as described above (unpublished data), ω-hydroxylation activity in the homogenate of perfused rat brain, perfused liver or non-perfused human colonic adenocarcinoma is independent of blood cell contamination.

The SIM chromatograms of an extract from an incubation mixture of 22:6($n - 3$) or 20:4($n - 6$) added to homogenate or 10^3, 10^4 or 10^5 *g* Pt fraction resulting from rat liver or human colonic well differentiated adenocarcinoma with β-NADPH are shown in Figs. 3 and 4. The peaks in Fig. 3I–VI are identical with the corre-

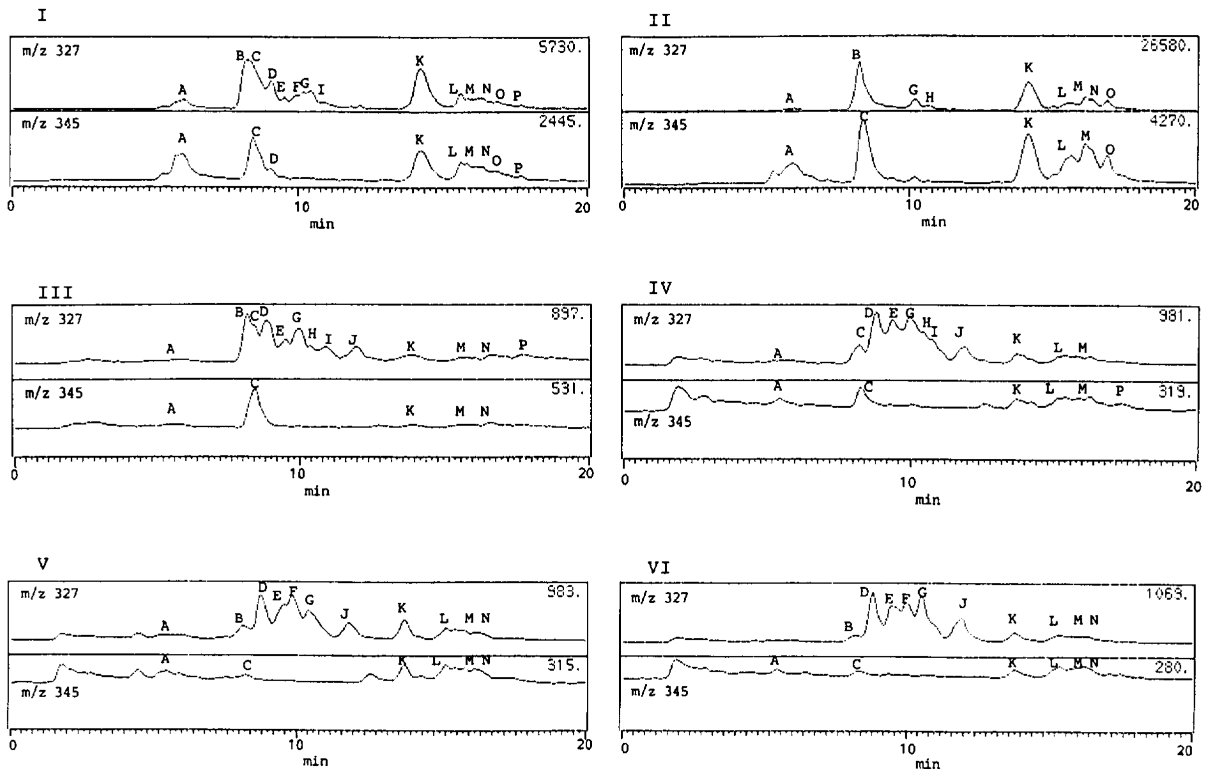

Fig. 3. SIM chromatograms of an extract from an incubation mixture of 22:6($n - 3$) additional rat liver 10^4 *g* Pt fraction (I), rat liver 10^5 *g* Pt fraction (II), human colonic adenocarcinoma homogenate (III), human colonic adenocarcinoma 10^3 *g* Pt fraction (IV), human colonic adenocarcinoma 10^4 *g* Pt fraction (V) and human colonic adenocarcinoma 10^5 *g* Pt fraction (VI) with β-NADPH. Other conditions as described under Experimental. The number in the top-right corner of each chromatogram is the ion count.

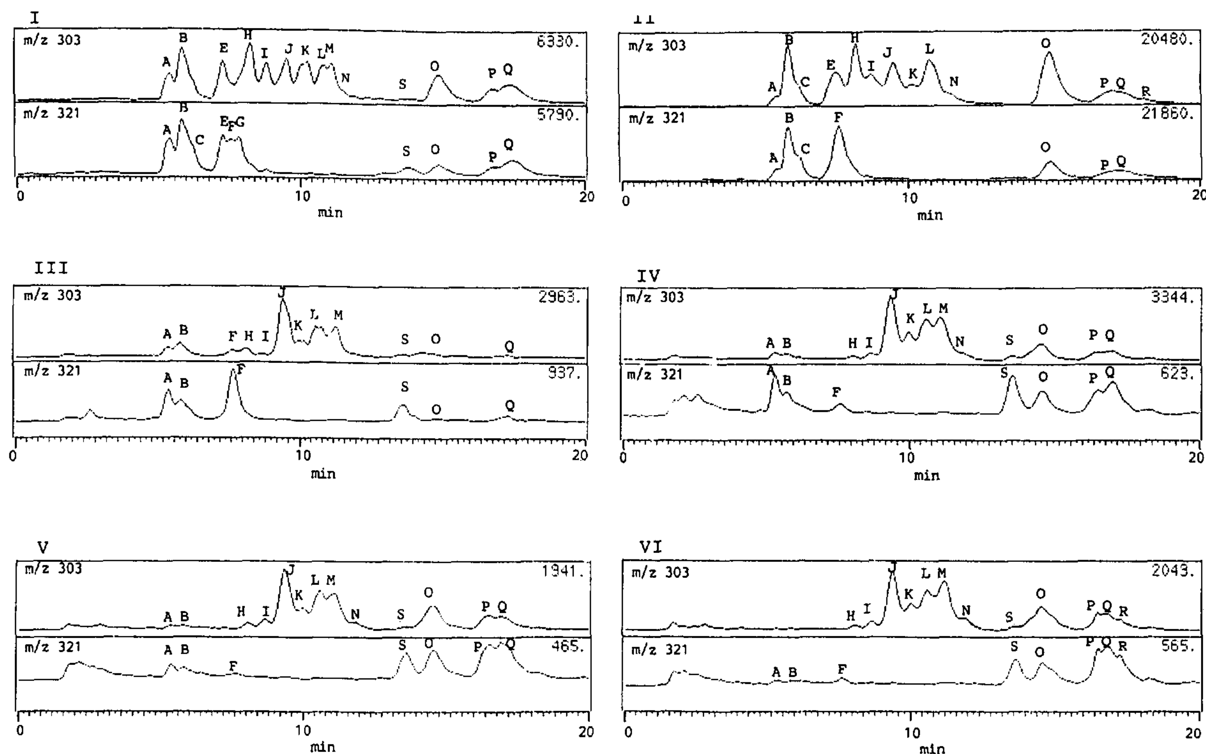

Fig. 4. SIM chromatograms of an extract from an incubation mixture of 20:4($n-6$) additional rat liver 10^4 *g* Pt fraction (I), rat liver 10^5 *g* Pt fraction (II), human colonic adenocarcinoma homogenate (III), human colonic adenocarcinoma 10^3 *g* Pt fraction (IV), human colonic adenocarcinoma 10^4 *g* Pt fraction (V) and human colonic adenocarcinoma 10^5 *g* Pt fraction (VI) with β-NADPH. Other conditions as described under Experimental. The number in the top-right corner of each chromatogram is the ion count.

sponding peaks in Fig. 1. Similarly, the peaks in Fig. 4I–VI are identical with the corresponding peaks in Fig. 2. In rat liver, ($\omega-1$)- or ω-hydroxylation activity was concentrated to 10^5 *g* Pt, although subcellular localization of ω-hydroxylation activity in the human colonic well differentiated adenocarcinoma is similar to that in rat brain.

The ($\omega-1$)- or ω-hydroxylation specific activities [($\omega-1$)- and ω-hydroxylation activity per 1 mg of protein] in each homogenate and each Pt and Sp fraction resulting from the differential centrifugation are shown in Table 1. For the rat brain, although the ω-hydroxylation specific activity in 10^3 *g* Pt decreased compared with that in the homogenate, the specific activity increased on the addition of the 10^4 or 10^5 *g* Pt fraction. This suggests that the 10^3 *g* Pt fraction of rat brain may be the main field of ω-hydroxylation activity, and the 10^4 or 10^5 *g* Pt fraction functions as a co-factor in the activity. Consequently, ω-hydroxylation enzyme in rat brain may be differentiated from cytochrome P-450-4A in liver or kideny as described previously [6,8]. On the other hand, for the rat liver, the ($\omega-1$)- or ω-hydroxylation specific activity in 10^5 *g* Pt increased compared with that in the homogenate, the 10^3 *g* Sp or 10^4 *g* Pt. This suggests that the 10^5 *g* Pt fraction of rat liver is the main field of ($\omega-1$)- or ω-hydroxylation activity, as described previously [4,7]. These results suggest that there is a difference regarding subcellular localization of the ω-hydroxylation activity depending on the species of the organs.

Previous studies on ω-HETE have reported stimulatory effects on vasoactivity in rat thoracic

Table 1
ω-Hydroxylation of 22:6($n-3$) and 20:4($n-6$) by each subcellular fraction

Tissue	Fraction	ω-Hydroxylation specific activity (pmol/mg protein in 30 min)			
		($\omega-1$)-HDHE	ω-HDHE	($\omega-1$)-HETE	ω-HETE
Rat brain	Homogenate	103	152	0	74
	10^3 g Pt	29	62	0	13
	10^3 g Sp	27	48	0	8
	10^4 g Pt	36	52	–	–
	10^5 g Pt	94	0	–	–
	10^4 g Pt + 10^5 g Pt	25	69	–	–
	10^3 g Pt + 10^4 g Pt	9	82	0	22
	10^3 g Pt + 10^5 g Pt	4	76	0	33
Rat liver	Homogenate	981	174	54	16
	10^3 g Pt	1324	339	82	42
	10^3 g Sp	1015	50	120	5
	10^4 g Pt	3591	3004	1760	906
	10^5 g Pt	12 221	10 562	2867	5163
Human colonic adenocarcinoma	Homogenate	40	164	0	65
	10^3 g Pt	20	66	0	18
	10^3 g Sp	13	87	0	25
	10^4 g Pt	223	31	0	10
	10^5 g Pt	134	49	0	44

Each subcellular fraction was incubated with a precursor fatty acid [22:6($n-3$), 121 μM, or 20:4($n-6$), 131 μM] and β-NADPH (1.1 mM) at 37°C for 30 min under aerobic conditions.

aortic rings [16] and erythropoietin-dependent stem-cell growth in human bone marrow [17], and inhibitory effects on human platelet aggregation [18] and rabbit renal medulla microsomal Na^+/K^+-ATPase [19]. We recently found that each homogenate of adult rat brain, adult rat colonic mucosa or human colonic well differentiated adenocarcinoma shows a highly active NADPH-dependent ω-hydroxylation activity of 22:6($n-3$) or 20:4($n-6$) [11,12]. Although the physiological implications of the ω-hydroxylation activity are not known satisfactorily, the activity may be related to the functions characteristic of differentiated epithelial cells.

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