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High-performance liquid chromatography–thermospray mass spectrometry of hydroxy-polyunsaturated fatty acid acetyl derivatives

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ABSTRACT

A method for the determination of hydroxy-polyenoic acids has been developed. Hydroxy groups of hydroxy-polyunsaturated fatty acid were acetylated by acetic anhydride, and the mixture was partially purified on a Sep-Pak C_{18} cartridge and analysed by high-performance liquid chromatography combined with thermospray mass spectrometry. Using this method, fifteen kinds of hydroxy-polyunsaturated fatty acid derivative could be detected simultaneously within 30 min on a selected-ion monitoring detection chromatograph without a gradient system. Generally, the base ion, $[M+H-n(60)]^+$, is produced through elimination of acetic acid (n = number of the hydroxy group of hydroxy-polyunsaturated fatty acid). The detection limit for these derivatives was *ca.* 0.2 pmol at the levels of hydroxy-polyenoic acids prior to derivatization. They could be analysed in the range 0.5–25 pmol. The assay was successfully applied to hydroxy-polyunsaturated fatty acids from an incubation mixture of rat brain homogenate to which polyunsaturated fatty acid was added.

INTRODUCTION

Almost all hydroxy-polyunsaturated fatty acids (HPUFAs) are produced via the lipoxygenase pathway from polyunsaturated fatty acids (PUFAs). These compounds show varied and potent biological action and have been implicated in many inflammatory reactions in humans [1–5]. HPUFAs have been assayed by high-performance liquid chromatography (HPLC) and gas chromatography–mass spectrometry (GC–MS). UV detection is generally used with HPLC, and a reversed-phase or normal-phase column is used for the separation of each HPUFA [6–12]. However, 12-hydroxyeicosatetraenoic acid (12(*S*)-HETE) and 14-hydroxydocosahexaenoic acid (14-HDHE), for example, which overlap in the UV detection chromatogram, could not be detected [18]. At present, GC–MS is the most reliable method, although its operation is complicated because the carboxyl and hydroxyl groups must all be derivatized [13–15]. The technique has not proved entirely suitable for all HPUFAs (notably the 5-isomers of hydroxyeicosatetraenoic acids (HETEs)) because of thermal breakdown in the GC column [14].

High-performance liquid chromatography–thermospray mass spectrometry (HPLC–TSP-MS) has recently come to be used for analysis of HPUFAs [16–18]. The technique has great potential for the analysis of labile polar biological samples because the spectrum of an intact molecule can be readily obtained without prior derivatization. However, this procedure is not satisfactory owing to the sensitivity in di- or tri-HPUFAs because fragment ions appeared following elimination of hydroxyl groups [16].

We recently presented a new method for the

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determination of prostaglandin (PG)-related substances [19]. In this method, hydroxyl groups in the PG-related substance were acetylated with acetic anhydride, partially purified on a Sep-Pak C_{18} cartridge and analysed by HPLC–TSP-MS. Generally, the base ion, $[M + H - n(60)]^+$, is produced through elimination of acetic acid (n = number of the hydroxyl group of the PG-related substance).

In this study, the hydroxyl groups of various HPUFAs were acetylated and the products were partially purified and analysed by HPLC–TSP-MS in a similar manner. This technique was successfully applied to HPUFAs from an incubation mixture of PUFAs added to rat brain homogenate.

EXPERIMENTAL

Standards and reagents

HPUFA standards (5(*S*)-HETE, 9(*S*)-HETE, 12(*S*)-HETE, 15(*S*)-HETE, 5(*S*)-hydroxyeicosapentaenoic acid (5(*S*)-HEPE), 9(*S*)-HEPE, 15(*S*)-HEPE, 5(*S*),6(*S*)-dihydroxyeicosatetraenoic acid (5(*S*),6(*S*)-DiHETE), leukotriene-B_4 (LTB_4), 6-*trans*-12-epi-LTB_4 (5(*S*),12(*S*)-DiHETE), 8(*S*),15(*S*)-dihydroxyeicosatetraenoic acid (8(*S*),15(*S*)-DiHETE), 5(*S*),6(*R*),15(*S*)-trihydroxyeicosatetraenoic acid (lipoxin-A_4) and 5(*S*),14(*R*),15(*S*)-trihydroxyeicosatetraenoic acid (lipoxin-B_4)) were obtained from Cascade Biochem (Berkshire, UK). [3,3,4,4-$^2H_2$]$PGF_{2\alpha}$ ($PGF_{2\alpha}$-d_4), [3,3,4,4-$^2H_2$]PGA_2 (PGA_2-d_4) and 12(*S*)-hydroxyheptadecatrienoic acid (12(*S*)-HHT) were obtained from Cayman (Ann Arbor, MI, USA). (±)5-Hydroxyeicosatrienoic acid ((±)5-HETriE) and 15(*S*)-hydroxyeicosatrienoic acid (15(*S*)-HETriE) were obtained from Biomol Research Lab. (Plymouth Meeting, PA, USA). *cis*-8,11,14-Eicosatrienoic acid (homo-γ-linolenic acid, 20:3(n − 6)), 5,8,11,14-eicosatetraenoic acid (arachidonic acid, 20:4(n − 6)), 5,8,11,14,17-eicosapentaenoic acid (20:5(n − 3)), *cis*-4,7,10,13,16,19-docosahexaenoic acid (22:6(n − 3)) and indomethacin were obtained from Sigma (St. Louis, MO, USA). 17-Hydroxydocosapentaenoic acid (17-HDPE), 17-hydroperoxydocosapentaenoic acid (17-HPDPE), 17-HDHE, 17-hydroperoxydocosahexaenoic acid (17-HPDHE) and 5,15-dihydroxyeicosapentaenoic acid (5,15-DiHEPE) were prepared from 22:5(n − 3), 22:6(n − 3) and 5(*S*)-HEPE by the method of Hamberg and Samuelsson [20] by using soybean lipoxygenase, respectively, and purified by using the HPLC–UV system [21]. The water for the HPLC eluent was of Milli-Q grade (Waters Assoc., Milford, MA, USA), that for preparing 15% ethanol, 5% acetonitrile, 2 *M* hydrochloric acid and 10% acetic acid, and for washing and equilibrating the Sep-Pak C_{18} cartridge (Waters Assoc.) was prepurified by a Sep-Pak C_{18} cartridge. The other solvents and reagents were of analytical-reagent or chromatographic grade.

Extraction from biological sample

One rat (male Wistar rat weighing 150 g) was killed by decapitation, and the brian was immediately excised at a low temperature. The tissue (1.5 g of brain) was cut into pieces of *ca.* 2 × 2 mm with a razor blade, and washed twice by decantation with 5 ml of 0.85% sodium chloride. The washed tissue pieces were suspended in 22 ml of 52.6 m*M* Tris–HCl buffer (pH 7.52). The suspension was set on a nitrogen cavitation homogenizer (Parr Instrument, Moline, IL, USA) and homogenized after being subjected to 100 kg/cm_2 nitrogen gas pressure for 20 min. Aliquots of 20:3(n − 6), 20:4(n − 6), 20:5(n–3) or 22:6(n–3) containing at *ca.* 400 nmol in chloroform were evaporated to dryness in each incubation tube under reduced pressure. Rat brain homogenate (13 ml) and 0.55 ml of indomethacin solution (7 mg/100 ml, 1 m*M* Na_2CO_3) or 0.55 ml of 1 m*M* Na_2CO_3 solution were added, and 2.5-ml portions of the mixture were homogenized to disperse the substrate in each incubation tube as described above by a vortex mixer. Each mixture was incubated at 37°C for 60 min on a shaker operated at 120 rpm. $PGF_{2\alpha}$-d_4 (278 pmol), as the internal standard (I.S.), 50 μl of 2 *M* hydrochloric acid and 0.43 ml of ethanol were added. Each mixture was then centrifuged at 1500 *g* for 10 min at 4°C to remove excess tissue, and each supernatant was applied to a Sep-Pak C_{18} car-

tridge equilibrated with water. The cartridge was washed successively with 5 ml of 5% acetonitrile and 10 ml of water. HPUFAs and PG-related substances in the cartridge were eluted with 6 ml of acetonitrile, and the eluent was evaporated to dryness under reduced pressure.

Derivatization

HPUFAs and PG-related substances were derivatized using acetic anhydride in pyridine to obtain the acetic ester. HPUFAs and PG-related substances were dissolved in 0.2 ml of pyridine, and 0.08 ml of acetic anhydride was added. The reaction mixture was left overnight under argon at 5°C. The mixture was acidified to pH 4–5 by the addition of 3.5 ml of 10% acetic acid and 0.5 ml of acetonitrile. The mixture was then immediately applied to a Sep-Pak C_{18} cartridge equilibrated with water. The cartridge was washed successively with 3 ml of 5% acetonitrile and 10 ml of water, and the acetyl derivatives of HPUFAs and PG-related substances in the cartridge were eluted with 5 ml of acetonitrile. The eluent containing the HPUFAs and PG-related substances was stored at −20°C until analysis by HPLC–TSP-MS.

HPLC–TSP-MS

A Shimadzu (Kyoto, Japan) LC/GC/MS-QP1000S, 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. HPLC separation was carried out using a Nucleosil 100-5C_{18} (5 μm particle size, Macherey Nagel, Düren, Germany) 150 mm × 4.6 mm I.D. column, with a mobile phase of 0.1 *M* ammonium formate–0.1 *M* formic acid–acetonitrile (8:2:15,v/v) at a flow-rate of 1.0 ml/min.

The TSP interface temperature was optimized for maximum detection sensitivity for the acetyl derivatives. In the positive-ion mode, the optimal vaporizer control, vaporizer tip, vapour, block and tip heater temperatures were maintained at 146, 280, 323, 346 and 348°C, respectively, under electron beam-off or electrical discharge-off conditions.

RESULTS AND DISCUSSION

MS patterns of the derivatives showed a characteristic base ion (Fig. 1). As shown in Table I, the base ion was $[M + H - n(60)]+$, based on the elimination of acetic acid (60 mass units) from the molecular ion. Chemical ionization due to an electric beam (filament on 150 μA) or electrical discharge caused no increase in the ion intensity of the acetyl derivatives. A peak appearing at a retention time of 13–14 min in the total ion chromatogram (TIC) of Fig. 1 gave a base ion of *m/z* 279. As the peak was obtained even in blank experiments, it was assigned as an unidentified component unrelated to HPUFAs. Apart from this *m/z* 279 peak, since the peaks corresponding to unreactive HPUFAs are very few in the TIC of Fig. 1, the applied conditions are adequate for complete acetylation of each HPUFA. However, the sub-ion of *m/z* 279 component appeared in the area of *m/z* 301, as shown in Fig. 1C, Fig. 1D, Fig. 1E and Fig. 2.

Most acetyl derivatives of HPUFAs could be easily detected on the selected-ion monitoring (SIM) chromatogram at *ca.* 5 pmol of HPUFAs prior to derivatization (Fig. 2). The bi-phasic peak of lipoxin B_4 in the region of *m/z* 299 in Fig. 2 was assumed to be an isomer of lipoxin B_4, although this was not confirmed. The derivatives of DiHETE showed lower sensitivity than the others, possibly owing to the low yields of their acetylation. The relationship between the peak area on SIM detection and the times for acetylation under 40°C is shown in Fig. 3. The yields of acetyl-HPUFAs containing DiHETE did not increase with increasing acetylation time or increased heating, rather those conditions seemed to accelerate their degradation. As degradation occurred with the acetylation, we performed the acetylation overnight under argon at 5°C.

Fig. 4 shows an SIM chromatogram of HPUFAs prior to derivatization of *ca.* 0.5 pmol. The limit of detection was considered to be *ca.* 0.2 pmol. The relationship between the peak area on the SIM chromatogram and the amount of HPUFAs prior to derivatization is given in Fig. 5. Determination was carried out from 0.5 to 25

pmol of HPUFAs prior to derivatization. Since the ratio of the total amount of ions yielded to the amount of applied material is constant in LC–TSP-MS analysis under the constant temperature conditions of TSP, Fig. 5 shows that the yield of each acetylated HPUFA is constant under the acetylation conditions described above.

SIM chromatograms of the acetyl derivatives of extracts from an incubation mixture of PUFA additional rat brain homogenate with or without

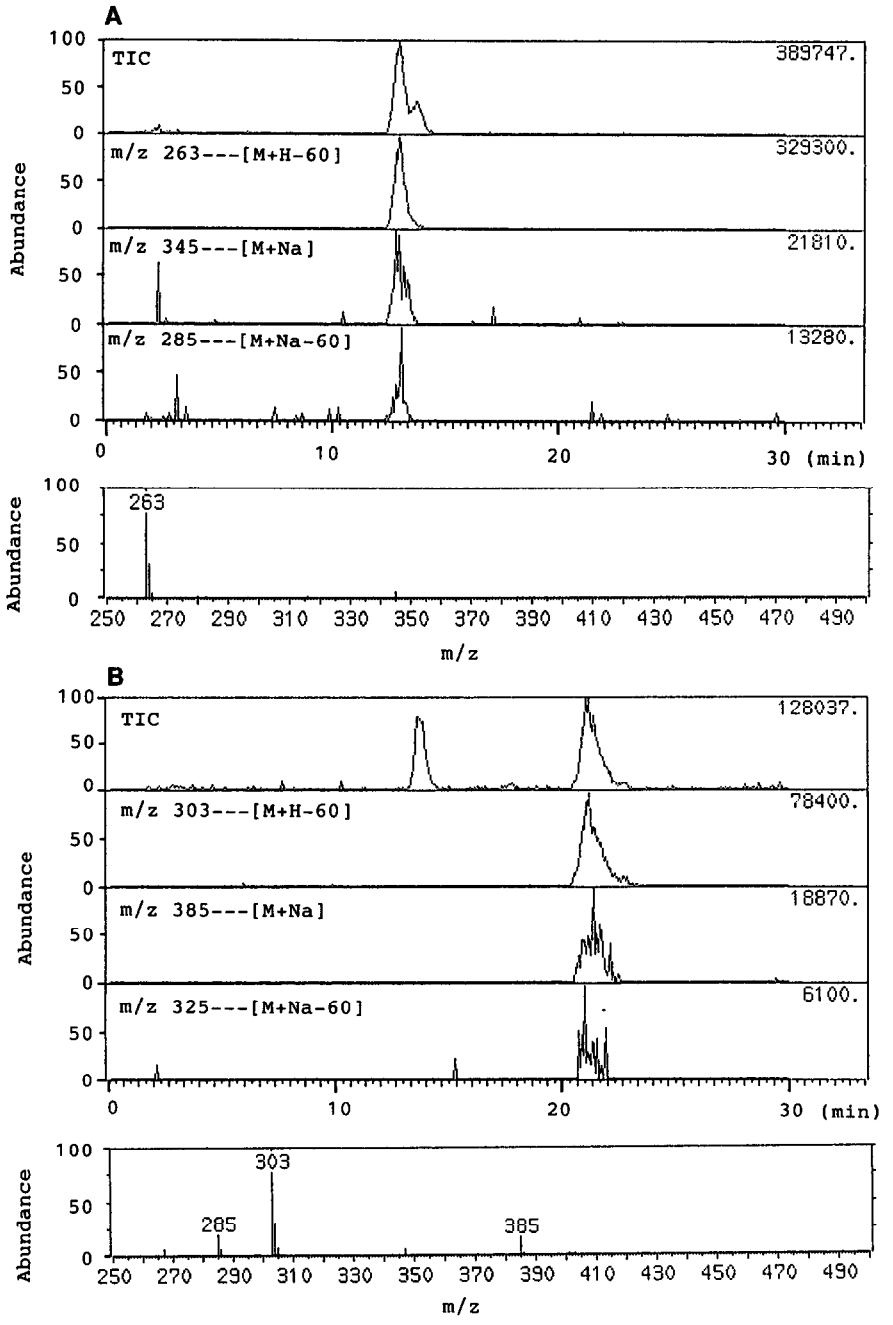

Fig. 1.

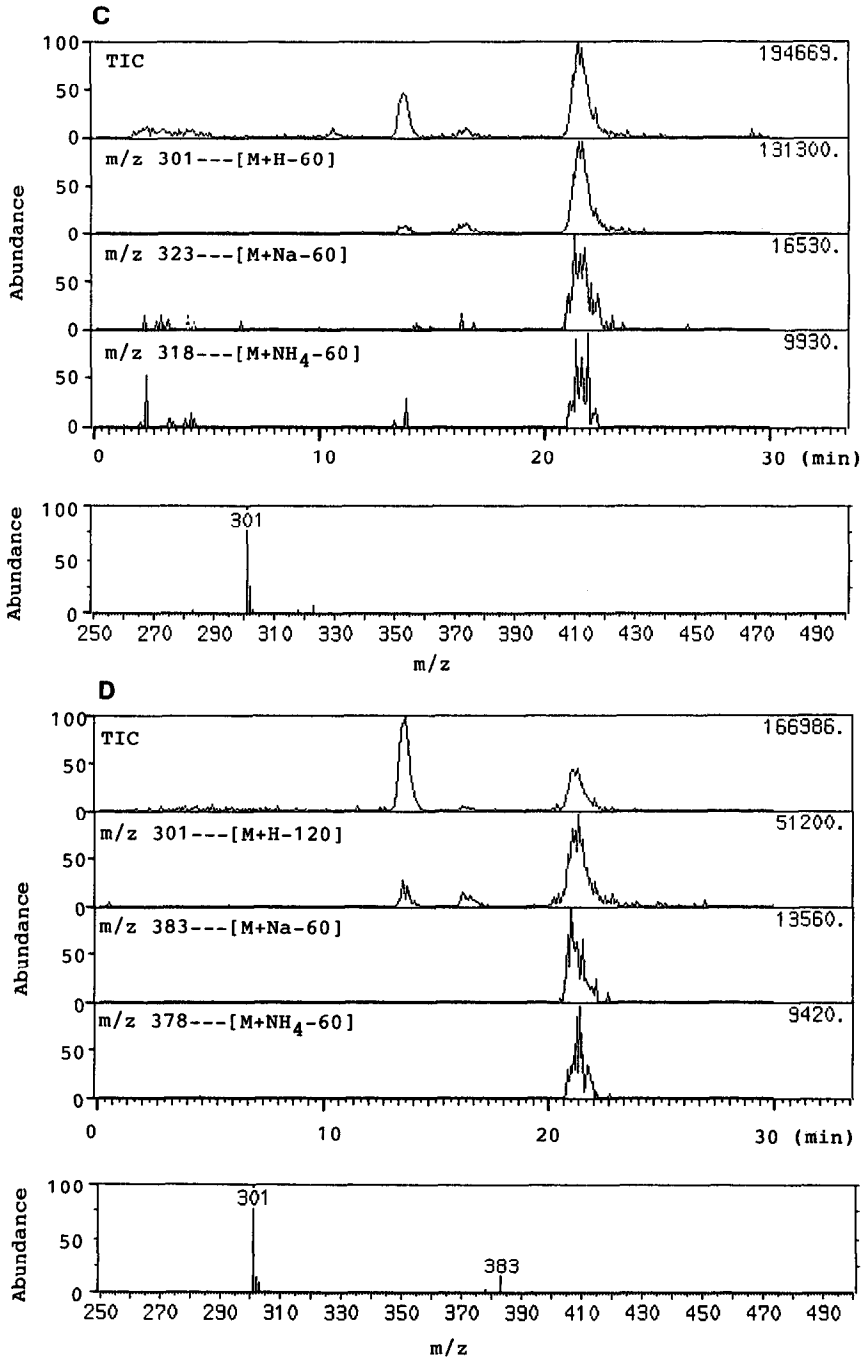

Fig. 1.

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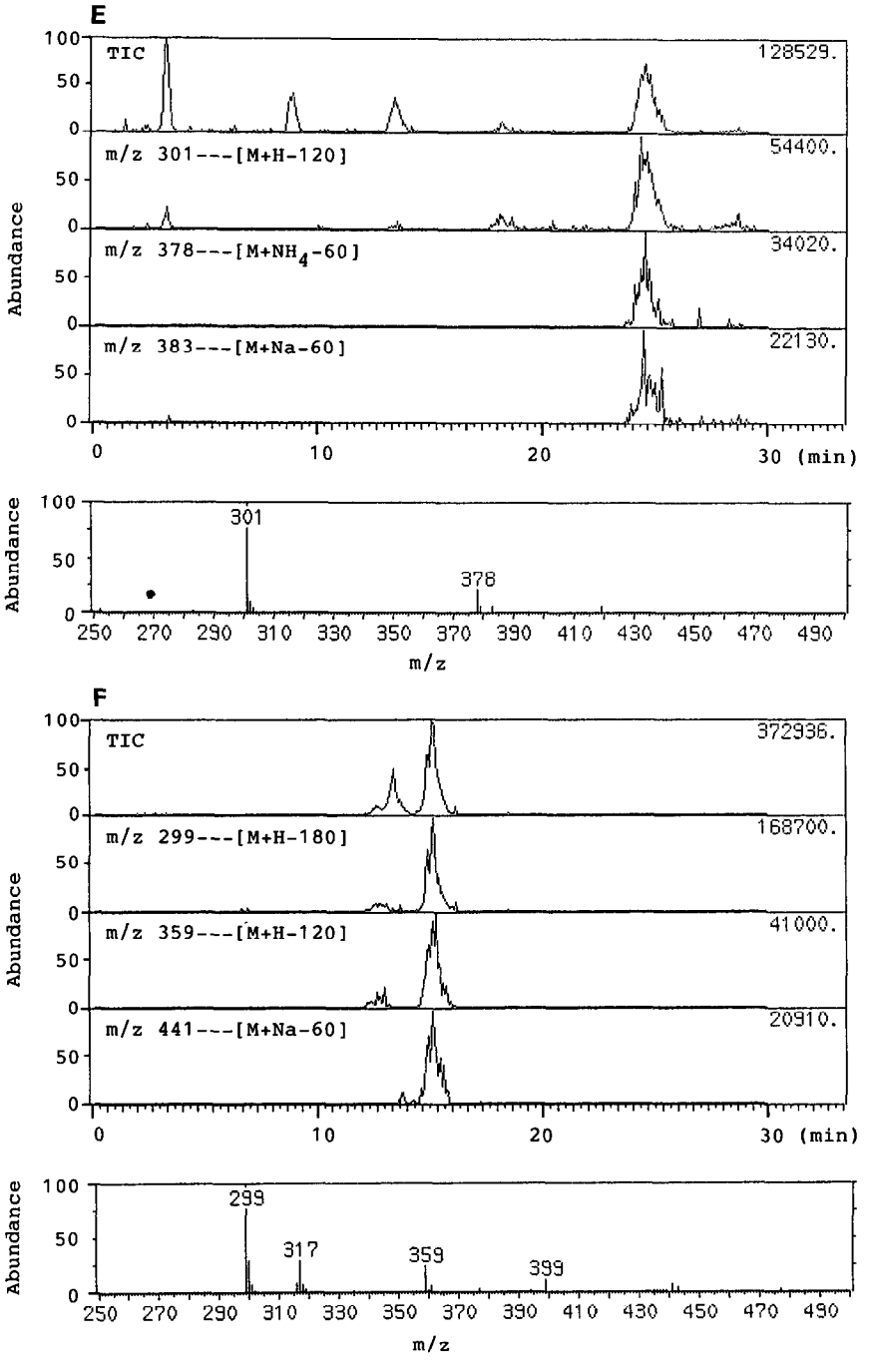

Fig. 1. Total ion chromatogram (TIC) profile, mass chromatogram profile on each *m/z* number and mass spectra obtained from HPUFA acetyl derivatives. HPLC and TSP conditions as described in Experimental. Scan speed, 1.0 scans/s from *m/z* 250 to 500. Filament off. Spectral patterns corresponding to the main peak on the TIC are shown below. (A) 12(*S*)-HHT acetyl derivative; (B) 15(*S*)-HETE acetyl derivative; (C) 5(*S*)-HEPE acetyl derivative; (D) LTB_4 acetyl derivative; (E) 5(*S*),6(*S*)-DiHETE acetyl derivative; (F) lipoxin-A_4 acetyl derivative. The number in the upper right-hand corner of each chromatogram is ion count.

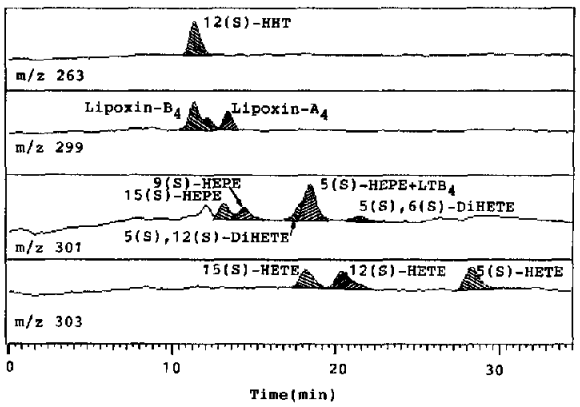

Fig. 2. SIM chromatogram of the acetyl derivatives of HPUFAs at *ca.* 5 pmol of HPUFAs prior to derivatization. HPLC and TSP conditions as described in Experimental.

indomethacin are shown in Figs. 6–10. On the chromatograms from the 20:4(n − 6) additional homogenate with indomethacin, peaks due to the derivatives of 5(S)-HETE, 12(S)-HETE, 15(S)-HETE, 5(S),12(S)-DiHETE, $PGF_{2\alpha}$, dihydroxydocosahexaenoic acid (DiHDHE) and HDHE could be seen (Fig. 6). Similarly, 5(S)-HETE, 12(S)-HETE, 15-(S)-HETE, HETriE and HDHE from the 20:3(n − 6) additional homogenate with indomethacin (Fig. 7); 5,15-DiHEPE, 5(S)-HEPE, 9(S)-HEPE, 15(S)-HEPE, 5(S)-HETE, 12(S)-HETE, 15(S)-HETE and HDHE from the 20:5(n − 3) additional homogenate with indomethacin (Fig. 8); and 5(S)-HETE, 12(S)-HETE, 15(S)-HETE, 17-HPDHE, DiHDHE, HDHE, dihydroxydocosapentaenoic acid (DiHDPE), HPDPE and HDPE from the 22:6(n − 3) additional homogenate with indomethacin were noted (Fig. 9).

However, 5,15-DiHEPE was assumed from $[M + H - 120]^+$ ion (m/z 299) and $[M + Na - 60]^+$ ion (m/z 381); HDPE was assumed from $[M + H - 60]^+$ ion (m/z 329) and $[M + Na]^+$ ion (m/z 411); HDHE was assumed from $[M + H - 60]^+$ ion (m/z 327), $[M + Na]^+$ ion (m/z 409) and $[M + NH_4 - 60]^+$ ion (m/z 344); DiHDPE was assumed from $[M + H - 120]^+$ ion (m/z 327), $(M + Na - 60]^+$ ion (m/z 409) and $[M + NH_4 - 60]^+$ ion (m/z 404); DiHDHE was assumed from $[M + H - 120]^+$ ion (m/z 325), $[M + Na - 60]^+$ ion (m/z 407) and $[M + NH_4 - 60]^+$ ion (m/z 402); HPDPE was assumed from $[M + H - 60 - H_2O]^+$ ion (m/z 327), $[M + H - 60]^+$ ion (m/z 345), $[M + NH_4 - H_2O]^+$ ion (m/z

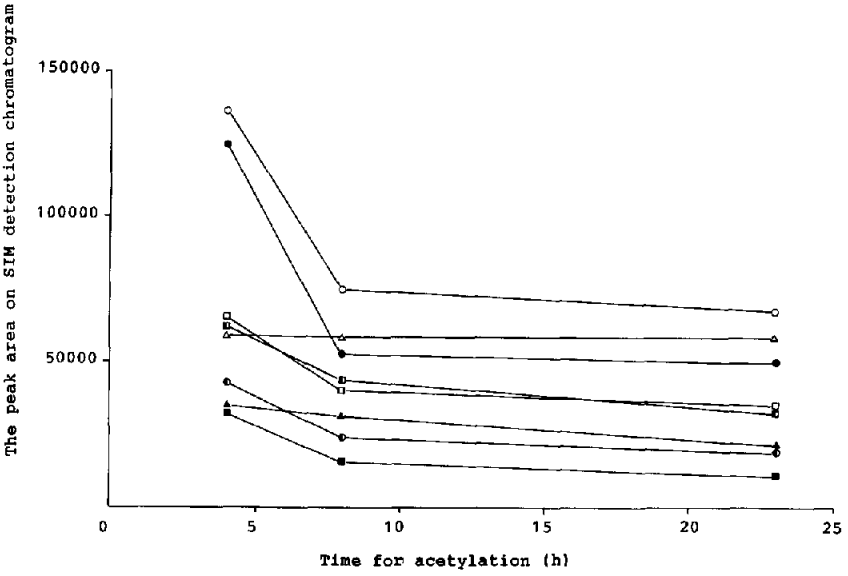

Fig. 3. Relationship between the peak area on the SIM chromatogram and the acetylation time. The conditions of the acetylation were as described in Experimental, except reaction temperature (40°C) and reaction time. (●) 12(S)-HHT; (○) 15(S)-HEPE; (◐) PGA_2-d_4; (△) LTB_4; (▲) 5(S),6(S)-DiHETE; (□) lipoxin B_4; (■) lipoxin A_4; (◧) $PGF_{2\alpha}$-d_4.

TABLE I

RELATIVE ABUNDANCES OF IONS IN THE TSP SPECTRA OF HPUFA ACETYL DERIVATIVES

HPUFA	Acetyl group	M	[M + Na]	[M + NH_4]	[M + H]	[M + Na − 60]	[M + NH_4 − 60]
12(*S*)-HHT	1	322	6	–	–	4	7
(±)5-HETriE	1	364	–	–	–	–	–
15(*S*)-HETriE	1	364	24	–	7	–	–
5(*S*)-HETE	1	362	–	–	–	14	14
9(*S*)-HETE	1	362	12	–	–	5	2
12(*S*)-HETE	1	362	–	–	–	–	13
15(*S*)-HETE	1	362	24	–	–	7	3
5(*S*)-HEPE	1	360	–	–	–	12	7
9(*S*)-HEPE	1	360	4	–	–	4	4
15(*S*)-HEPE	1	360	9	–	–	3	6
5(*S*),6(*S*)-DiHETE	2	420	–	–	–	40	62
5(*S*),12(*S*)-DiHETE	2	420	–	–	–	4	2
8(*S*),15(*S*)-DiHETE	2	420	18	13	–	4	–
LTB_4	2	420	–	–	–	26	18
Lipoxin-A_4	3	478	–	–	2	12	9
Lipoxin-B_4	3	478	–	–	–	17	2

404) and [M + Na − H_2O]$^+$ ion (*m/z* 409); HPDHE was assumed from [M + H − 60 − H_2O]$^+$ ion (*m/z* 325); [M + H − 60]$^+$ ion (*m/z* 343), [M + NH_4 − H_2O]$^+$ ion (*m/z* 402) and [M + Na − H_2O]$^+$ ion (*m/z* 407). The bi-phasic peaks of HETriE, DiHDPE, HDHE and DiHDHE from rat brain homogenate in Figs. 6–9 were assumed to be isomers, based on the bond structure of the hydroxyl groups, but this was not confirmed. Moreover, the peaks appearing as tailing peaks in Fig. 6–9 were assumed to be caused by contact with small amounts of each

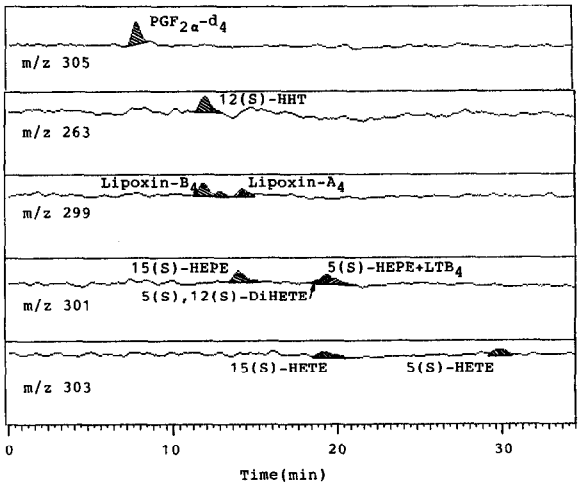

Fig. 4. SIM chromatogram of the acetyl derivatives of HPUFAs at *ca.* 0.5 pmol of HPUFAs prior to derivatization. HPLC and TSP conditions as described in Experimental.

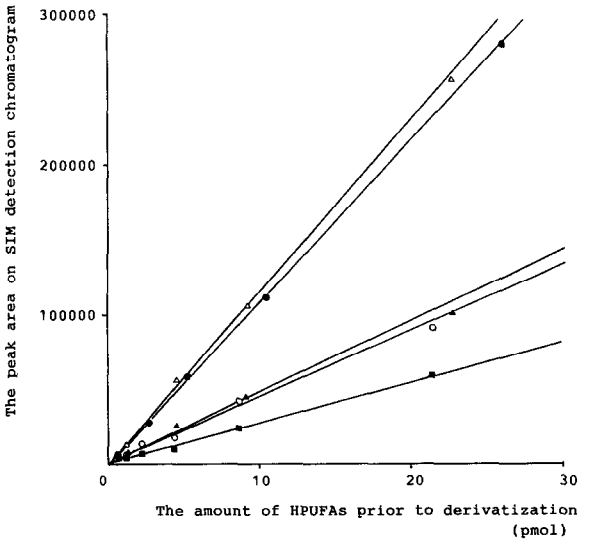

Fig. 5. Relationship between the peak area on the SIM chromatogram and the amount of HPUFAs prior to derivatization. HPLC and TSP conditions as described in Experimental. The peak area of each acetyl-HPUFA was corrected by the peak area of $PGF_{2\alpha}$-d_4, as the I.S. (△) 15(*S*)-HEPE; (●) 12(*S*)-HHT; (▲) 15(*S*)-HETE; (○) lipoxin B_4; (■) 5(*S*),6(*S*)-DiHETE.

[M + H − 60]	[M + Na − 120]	[M + NH_4 − 120]	[M + H − 120]	[M + Na − 180]	[M + NH_4 − 180]	[M + H − 180]
100						
100						
100						
100						
100						
100						
100						
100						
100						
100						
–	–	2	100			
–	–	3	100			
5	–	11	100			
–	–	2	100			
–	–	1	24	–	7	100
–	–	–	19	–	5	100

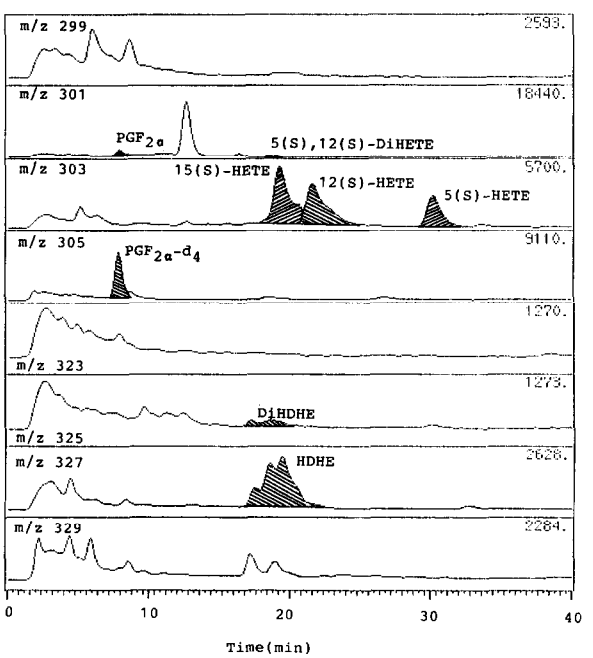

Fig. 6. SIM chromatograms of the acetyl derivative of extracts from incubation mixture of 20:4(n − 6) additional rat brain homogenate with indomethacin. HPLC and TSP conditions as described in Experimental. The number in the upper right-hand corner of each chromatogram is the ion count.

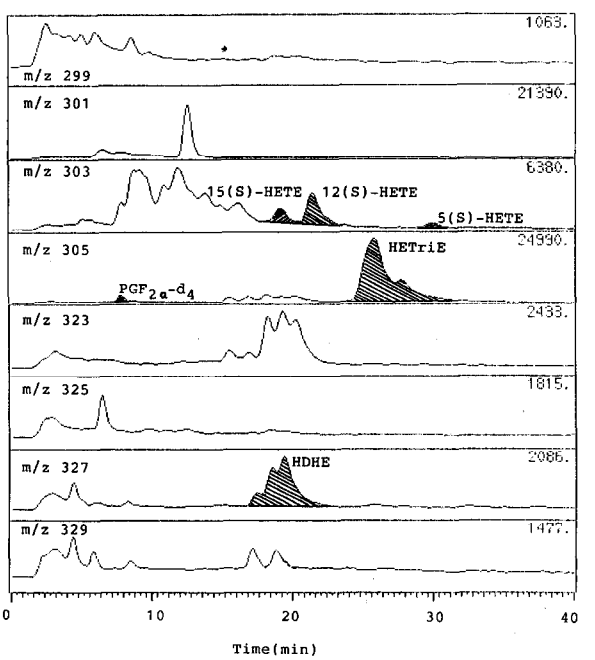

Fig. 7. SIM chromatograms of the acetyl derivative of extracts from incubation mixture of 20:3(n − 6) additional rat brain homogenate with indomethacin. HPLC and TSP conditions as described in Experimental. The number in the upper right-hand corner of each chromatogram is the ion count.

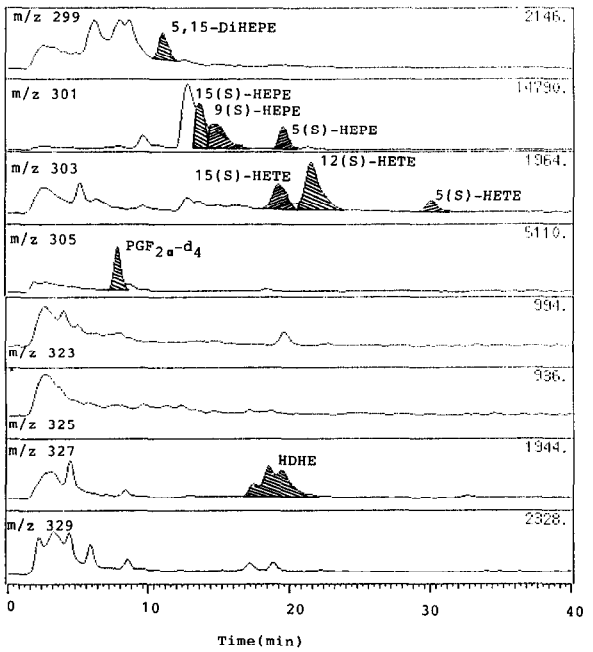

Fig. 8. SIM chromatograms of the acetyl derivative of extracts from incubation mixture of 20:5(n − 3) additional rat brain homogenate with indomethacin. HPLC and TSP conditions as described in Experimental. The number in the upper right-hand corner of each chromatogram is the ion count.

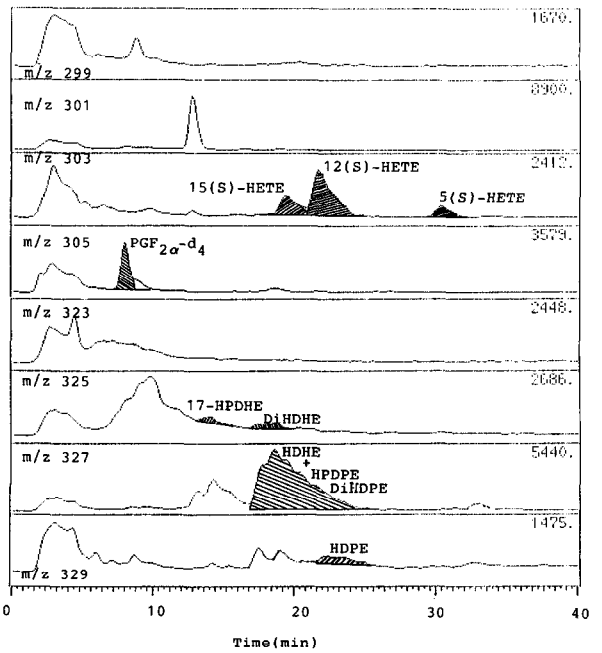

Fig. 9. SIM chromatograms of the acetyl derivative of extracts from incubation mixture of 22:6(n − 3) additional rat brain homogenate with indomethacin. HPLC and TSP conditions as described in Experimental. The number in the upper right-hand corner of each chromatogram is the ion count.

isomer, based on the bond structure of the hydroxyl groups.

On the chromatograms from the 20:4(n − 6) additional homogenate without indomethacin, peaks due to the derivatives of $PGF_{2\alpha}$, PGA_2 (E_2), PGD_2, PGA_1, 6-keto-PGE_1, 11-dehydro-TXB_2 and 15-keto-$PGF_{2\alpha}$ could be seen (Fig. 10).

TABLE II

RELATIVE ABUNDANCES OF IONS IN THE TSP SPECTRA OF HPUFAS OR THE ACETYL DERIVATIVES

Equal amounts of each HPUFA prior to derivatization or the acetyl derivatives applied to HPLC–TSP-MS and assayed under the conditions described in Experimental.

HPUFA	Base ion counts of HPUFA acetyl derivatives [M + H − n(60)] (%)	Each main ion counts of HPUFAs pror to derivatization[a] (%)			
		[M + Na]	[M + H − H_2O]	[M + H − 2(H_2O)]	[M + H − 3(H_2O)]
12(S)-HHT	100	3	15		
15(S)-HETE	100	7	21		
15(S)-HEPE	100	6	24		
5(S),6(S)-DiHETE	100	3	7	6	
LTB_4	100	3	10	15	
Lipoxin-A_4	100	2	3	6	1
Lipoxin-B_4	100	3	8	2	5

[a] These percentages are expressed as a proportion of the base ion counts of the HPUFA acetyl derivatives.

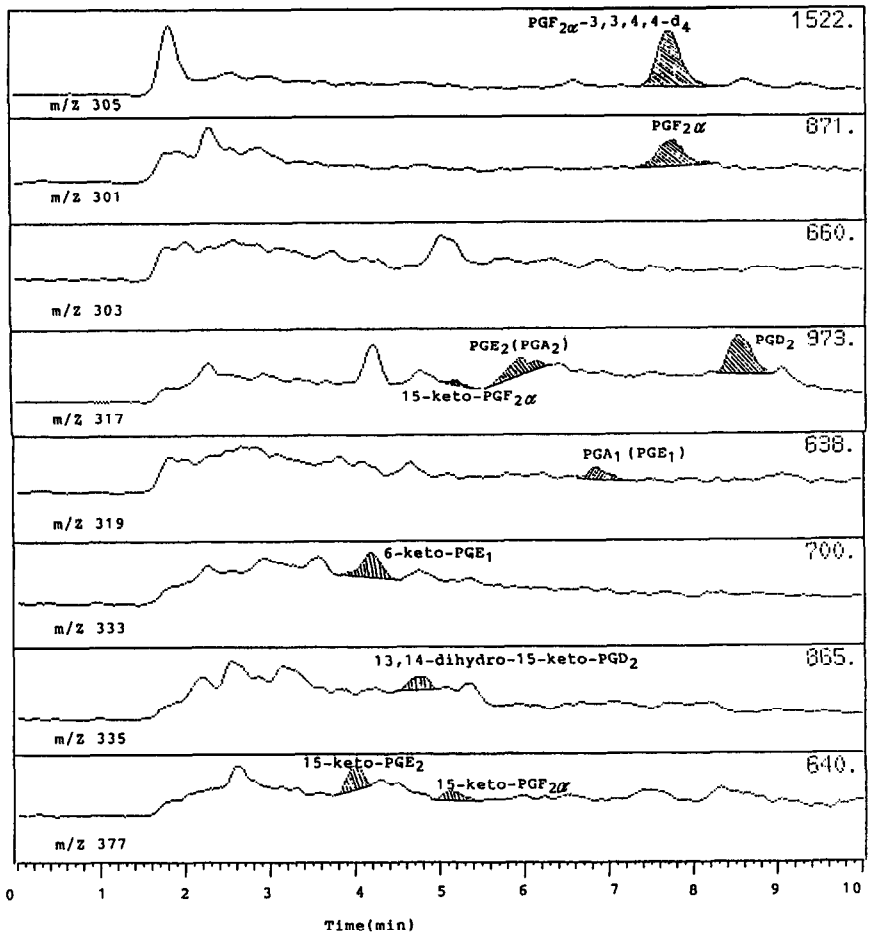

Fig. 10. SIM chromatograms of the acetyl derivative of extracts from incubation mixture of 20:4(n − 6) additional rat brain homogenate without indomethacin. HPLC and TSP conditions as described in Experimental. The number in the upper right-hand corner of each chromatogram is the ion count.

HPLC–TSP-MS techniques have been used previously for the determination of HPUFAs [16–18], but the sensitivity was not adequate because different fragment ions appeared following elimination of hydroxy groups, especially from di- or trihydroxy PUFAs (Table II).

However, in our method, the hydroxy groups of HPUFAs are acetylated, and thus the base ions ($[M + H - n(60)]^+$) in the mass spectra are strong without high fragmentation (Table I). Consequently, the sensitivity to HPUFAs is much improved without resort to the negative ionization mode or filament mode. The derivatives are characterized by a limited range of polarities, and thus HPLC retention times of the HPUFAs derivatives are only 35 min when a single mobile phase is used and detection is by TSP-MS. Also, *ca.* twenty kinds of HPUFA can be detected simultaneously by SIM of these base ions. In our method, the composition of the mobile phase on the TSP interface is fixed because a single mobile phase is used, thus lessening the variations in sensitivity.

This method is capable of simultaneously analysing all HPUFAs, including PG-related substances, by HPLC–TSP-MS analysis, and thus should be useful for studying the biosynthesis or metabolism of HPUFAs.

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