ELSEVIER

Journal of Chromatography B, 652 (1994) 123–136

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

High-performance liquid chromatography–thermospray mass spectrometry of epoxy polyunsaturated fatty acids and epoxyhydroxy polyunsaturated fatty acids from an incubation mixture of rat tissue homogenate

Mototeru Yamane[a,*], Akihisa Abe[a], Sayoko Yamane[b]

[a]*Department of Biochemistry, Tokyo Medical College, 6-1-1, Shinjuku, Shinjuku-ku, Tokyo, Japan*
[b]*Department of Laboratory Medicine, Ohmori Hospital, Toho University School of Medicine, 6-11-1 Ohmori-nishi, Ohta-ku, Tokyo, Japan*

(First received September 14th, 1993; revised manuscript received November 9th, 1993)

Abstract

A method for the analysis of epoxy polyunsaturated fatty acids (EpPUFAs) and epoxyhydroxy polyunsaturated fatty acids (EpHPUFAs) in rat tissue homogenate, with 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, has been developed. Extraction with dichloromethane at pH 4–5 and concentration in the presence of pyridine were performed. Spectral analysis of chromatograms obtained with high-performance liquid chromatography–thermospray mass spectrometry showed the presence of EpPUFAs, EpHPUFAs and dihydroxy metabolites (DiHPUFAs) of EpPUFAs corresponding to each precursor fatty acid. On a selected-ion monitoring chromatogram, many EpPUFAs, EpHPUFAs and DiHPUFAs in an extract from an incubation mixture of each precursor fatty acid in aged rat tissue homogenate were detected simultaneously within 70 min. EpPUFAs and DiHPUFAs derived from 20:3 ($n-6$) or 20:5 ($n-3$) were detected in significant amounts. From these results, a highly active cytochrome P450 system or non-enzymic oxidative reactions in aged rat tissue homogenate were suggested.

1. Introduction

Hydroxylation of polyunsaturated fatty acids has been observed in rat brain, with 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 substrate [1–5]. Lipoxygenase enzymes, cytochrome P450-dependent mono-oxygenase enzymes [6], and peroxidative processes [7,8] can all produce hydroxy derivatives of these fatty acids. Enantiomer separation of these monohydroxy polyunsaturated fatty acids (HPUFAs) using a chiral-phase column is available for investigation of the processes described above [3]. However, although this method is capable of proving the occurrence of lipoxygenase processes, direct proof of cytochrome P450 processes is not obtained.

* Corresponding author.

0378-4347/94/$07.00 © 1994 Elsevier Science B.V. All rights reserved
SSDI 0378-4347(93)E0394-6

Cytochrome P450 epoxygenase-dependent metabolism of these fatty acids as substrates may result in the formation of several isomeric epoxy polyunsaturated fatty acids (EpPUFAs). Because these EpPUFAs and the dihydroxy polyunsaturated fatty acids (DiHPUFAs) of hydrolysate of the EpPUFAs cannot contain conjugated double bonds, highly selective detection of these compounds by high-performance liquid chromatography (HPLC) with UV detection is difficult. Although gas chromatography–mass spectrometry (GC–MS) of separated EpPUFAs or DiHPUFAs by an HPLC–radioactive detector system is the most reliable method at present [9–16], the simultaneous determination of several EpPUFAs and DiHPUFAs has not been reported.

On the other hand, hydroperoxy polyunsaturated fatty acids (HPPUFAs), produced by lipoxygenase or autooxidation processes, are enzymically or non-enzymically converted into epoxyhydroxy poly-unsaturated fatty acids (EpHPUFAs) corresponding to each HPPUFA [17–24]. Because these EpHPUFAs and trihydroxy polyunsaturated fatty acids (TriHPUFAs) of hydrolysate of the EpHPUFAs cannot contain conjugated double bonds, highly selective detection of these compounds by HPLC–UV is also difficult. Although GC–MS of separated EpHPUFAs or TriHPUFAs by an HPLC-radioactive detector system is the most reliable method at present [17–24], the simultaneous determination of several EpHPUFAs and TriHPUFAs has not been reported.

This paper describes a method for the simultaneous detection of EpPUFAs, DiHPUFAs and EpHPUFAs corresponding to each precursor fatty acid in rat tissue homogenate by HPLC–thermospray (TSP)-MS.

2. Experimental

2.1. Standards and reagents

Cascade Biochem (Reading, UK) supplied the following EpPUFA standards, racemic 5,6-epoxyeicosatrienoic acid (5,6-EpETriE), 8,9-EpETriE, 11,12-EpETriE, and 14,15-EpETriE; DiHPUFA standards, racemic 5,6-dihydroxyeicosatrienoic acid (5,6-DiHETriE), racemic 5,6-DiHETriE-1,5-lactone (5,6-DiHETrE-δ-lactone), 8,9-DiHETriE, 11,12-DiHETriE, and 14,15-DiHETriE; EpHPUFA standards, 14(*S*), 15(*S*)-epoxy-13(*R*,*S*)-hydroxy-eicosatrienoic acid [14(*S*), 15(*S*)-Ep-13(*R*,*S*)-HETriE], 13(*S*), 14(*S*)-epoxy-15(*S*)-hydroxy-eicosatrienoic acid [13(*S*), 14(*S*)-Ep-15(*S*)-HETriE], 11(*S*), 12(*S*)-epoxy-8(*R*,*S*)-hydroxy-eicosatrienoic acid methyl ester [11(*S*), 12(*S*)-Ep-8(*R*,*S*)-HETriE methyl ester; Hepoxilin-A_3 methyl ester], and 11(*S*), 12(*S*)-epoxy-10(*R*,*S*)-hydroxyeicosatrienoic acid [11(*S*), 12(*S*)-Ep-10(*R*,*S*)-HETriE; Hepoxilin-B_3]; HPUFA standards, 5(*S*)-hydroxyeicosatetraenoic acid [5(*S*)-HETE], 8(*S*)-HETE, 9(*S*)-HETE, 11(*S*)-HETE, 12(*S*)-HETE, 15(*S*)-HETE, 5(*S*)-hydroxyeicosapentaenoic acid-[5(*S*)-HEPE], 8(*S*)-HEPE, 9(*S*)-HEPE, 11(*S*)-HEPE, 12(*S*)-HEPE, and 15(*S*)-HEPE; HPPUFA standards, 5(*S*)-hydroxyperoxyeicosatetraenoic acid [5(*S*)-HPETE], 12(*S*)-HPETE, 15(*S*)-HPETE and 12(*S*)-[5,6,8,9,11,12,14,15-$^2H_8$]-HETE [12(*S*)-HETE-d_8].

Hepoxilin-A_3 methyl ester was hydrolysed with 1.2 *M* sodium hydroxide in 50% ethanol, acidified to pH 4 with 2 *M* hydrochloride, and applied to a Sep-Pak C_{18} cartridge (Waters, Milford, MA, USA) equilibrated with water. The cartridge was washed with 6 ml of water, and the Hepoxilin-A_3 free acid in the cartridge was eluted with 5 ml of acetonitrile. (±)-5-Hydroxyeicosatrienoic acid [(±)-5-HETriE] and (±)-15-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 ($n-3$)] and *cis*-4,7,10,13,16,19-docosahexaenoic acid [22:6 ($n-3$)] were obtained from Sigma (St. Louis, MO, USA).

Epoxides of 20:3 ($n-6$), 20:5 ($n-3$) and 22:6 ($n-3$) were synthesized by slowly adding one equivalent of *m*-chloroperoxybenzoic acid in dichloromethane to 20:3 ($n-6$), 20:5 ($n-3$) or 22:6 ($n-3$) dichloromethane solution over a period of 3 h at room temperature, as described previously [11,25,26]. The reaction mixture was

supplemented with 0.05 ml of pyridine and concentrated to a pyridine solution under reduced pressure. The residual pyridine solution was suspended in 4 ml of water and applied to a Sep-Pak C_{18} cartridge equilibrated with water. The cartridge was washed with 6 ml of water to remove pyridine. EpPUFA fractions in the cartridge were eluted with 6 ml of acetonitrile, and the eluent was analysed by HPLC–TSP-MS. The fraction corresponding to each epoxide in this analysis was collected and converted into each DiHPUFA by treatment with a solution of 15% formic acid–acetonitrile (1:1, v/v) for 1 h at room temperature. Each DiHPUFA from 20:3 ($n-6$), 20:5 ($n-3$) or 22:6 ($n-3$) was analysed by HPLC–TSP-MS.

14-Hydroperoxydocosahexaenoic acid (14-HPDHE) and 17-HPDHE were enzymically prepared with human platelets or soybean lipoxygenase from 22:6 ($n-3$), as described previously [5]. 4-HPDHE and 20-HPDHE were prepared from an autooxidative product of 22:6 ($n-3$), as described previously [8]. Each of these HPDHE isomers was purified using the HPLC–UV system and a Sep-Pak C_{18} cartridge, as described previously [5]. Each HPDHE or HPETE isomer was converted into the corresponding epoxyhydroxy docosapentaenoic acid (EpHDPE) or epoxyhydroxy eicosatrienoic acid (EpHETriE) by treatment with hematine, as described previously [20,22,24]. These EpHPUFAs were partially purified on a Sep-Pak C_{18} cartridge and analysed by HPLC–TSP-MS.

The other solvents and reagents were of analytical-reagent or chromatographic grade.

2.2. Extraction from rat tissue homogenate

An aged male Wistar rat (600 g) was killed by decapitation, and its brain was immediately excised at low temperature. Also 1 ml of 2,4,6-trinitrobenzene-sulphonic acid (TNB) 50% ethanol solution (120 mg/ml) was injected into the large intestine via the anus of another aged male Wistar rat (600 g), which was killed by decapitation 4 days after the injection, and a part of the intestine of a TNB colitis was cut down. The tissue (1.5 g of brain or 2.0 g of large intestine) was cut into pieces of *ca.* 2 × 2 mm and washed twice by decantation with 5 ml of 0.85% sodium chloride. The pieces were suspended in 25 ml (brain) or 33 ml (intestine) of 50 m*M* Tris–HCl buffer (pH 7.5) and Polytron (Kinematica, Switzerland) homogenized.

Aliquots of 20:3 ($n-6$), 20:4 ($n-6$), 20:5 ($n-3$) or 22:6 ($n-3$), containing *ca.* 1300 nmol in ethanol, were evaporated until dry in incubation tubes under reduced pressure. Rat brain or intestine homogenate (5 ml each) was added, and the mixture was homogenized in a vortex-mixer to disperse the substrate. Each mixture was incubated at 37°C for 30 min in a shaker operated at 120 rpm. The incubation mixture was acidified to *ca.* pH 4 with 15% formic acid and extracted twice with dichloromethane. The dichloromethane layer was supplemented with pyridine (5 μl/ml dichloromethane) and filtered with No. 5A filter paper (Toyo Roshi, Tokyo, Japan). The filtrate was concentrated to a pyridine solution under reduced pressure. The residual pyridine solution was suspended in 4 ml of water and applied to a Sep-Pak C_{18} cartridge equilibrated with water. The cartridge was washed with 6 ml of water to remove pyridine. The fraction including EpPUFAs, EpHPUFAs, HPUFAs and DiHPUFAs in the cartridge were eluted with 6 ml of acetonitrile, and the eluent was evaporated to dryness under reduced pressure. The residue was dissolved in 100 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. HPLC separation was carried out using a Nucleosil 100 $5C_{18}$ column (5 μm particle size, 150 mm × 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 (4:1:5, 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, the optimal vaporizer control,

vaporizer tip, vapour, block and tip heater temperatures were maintained at 155, 295, 330, 345 and 345°C, respectively, under electron-beam-on (40 μA) or -off conditions.

3. Results and discussion

DiHPUFA, EpPUFA and EpHPUFA standards each showed a characteristic MS pattern (Fig. 1). The common base ion was (MH − H_2O), and the MS patterns of EpPUFAs and EpHPUFAs are characterized by high ion intensities of the molecular ion (MH) and the quasi-molecular ion (MNH_4, MNa).

HPLC–TSP-MS analysis of each reaction mixture from 20:3 ($n-6$) and *m*-chloroperoxybenzoic acid is shown in Fig. 2. From the mass spectrum of each main peak on a total ion chromatogram (TIC) and the behaviour on reversed-phase chromatography of each oxidation product corresponding to each precursor fatty acid, peaks A, B, C, D, E and F in Fig. 2 were assigned as 11,12-epoxy-14,15-epoxyeicosamonoenoic acid (11,12-Ep-14,15-Ep-EME), 8,9-Ep-14,15-EpEME, 8,9-Ep-11,12-EpEME,14,15-epoxyeicosadienoic acid (14,15-EpEDE), 11,12-EpEDE and 8,9-EpEDE, respectively. 14,15-EpEDE (peak D) and 11,12-EpEDE (peak E) yielded decomposition ions at *m/z* 223 and 183, respectively, as illustrated in Fig.3. 11,12-Ep-14,15-EpEME (peak A) yielded decomposition ions at *m/z* 157, 181, 183, 199, 237, 239 and 255, as illustrated in Fig. 3. 8,9-Ep-14,15-EpEME (peak B) yielded decomposition ions at *m/z* 159, 181, 237, 239 and 255, as illustrated in Fig. 3. 8,9-Ep-11,12-EpEME (peak C) yielded decomposition ions at *m/z* 159, 179, 181, 197, 199 and 215, as illustrated in Fig. 3. Similarly, each regioisomer of the diepoxides and monoepoxides

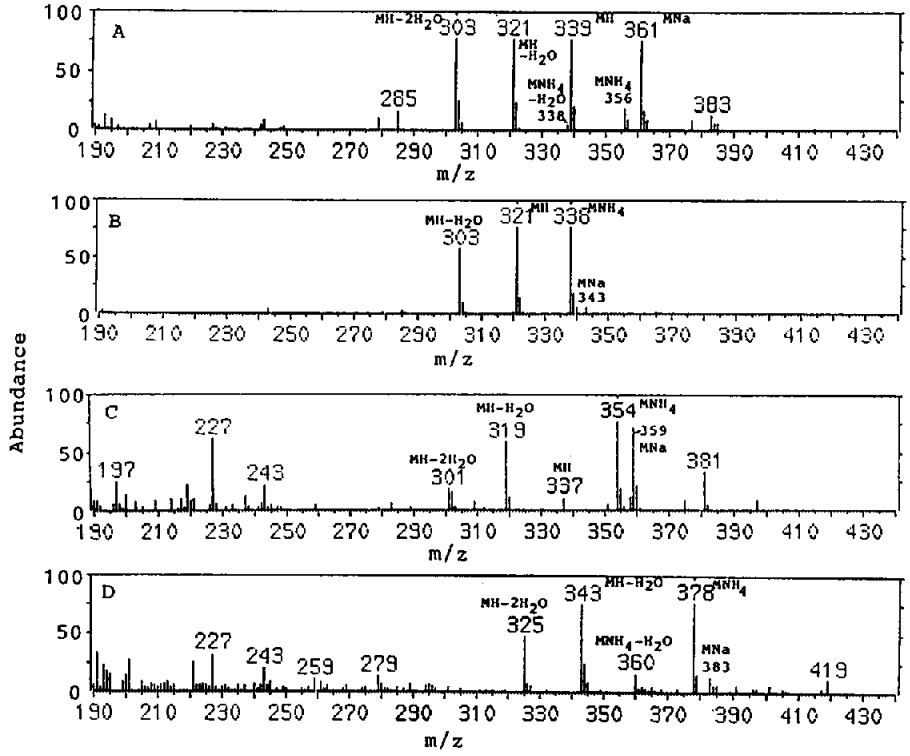

Fig. 1. HPLC–TSP-MS spectra obtained from DiHETriE, EpETriE and EpHETriE standards. HPLC and TSP conditions as described in Experimental, with the filament off. (A) 11,12-DiHETriE; (B) 5,6-EpETriE; (C) Hepoxilin-B_3; (D) EpHDPE derived from 17-HPDHE.

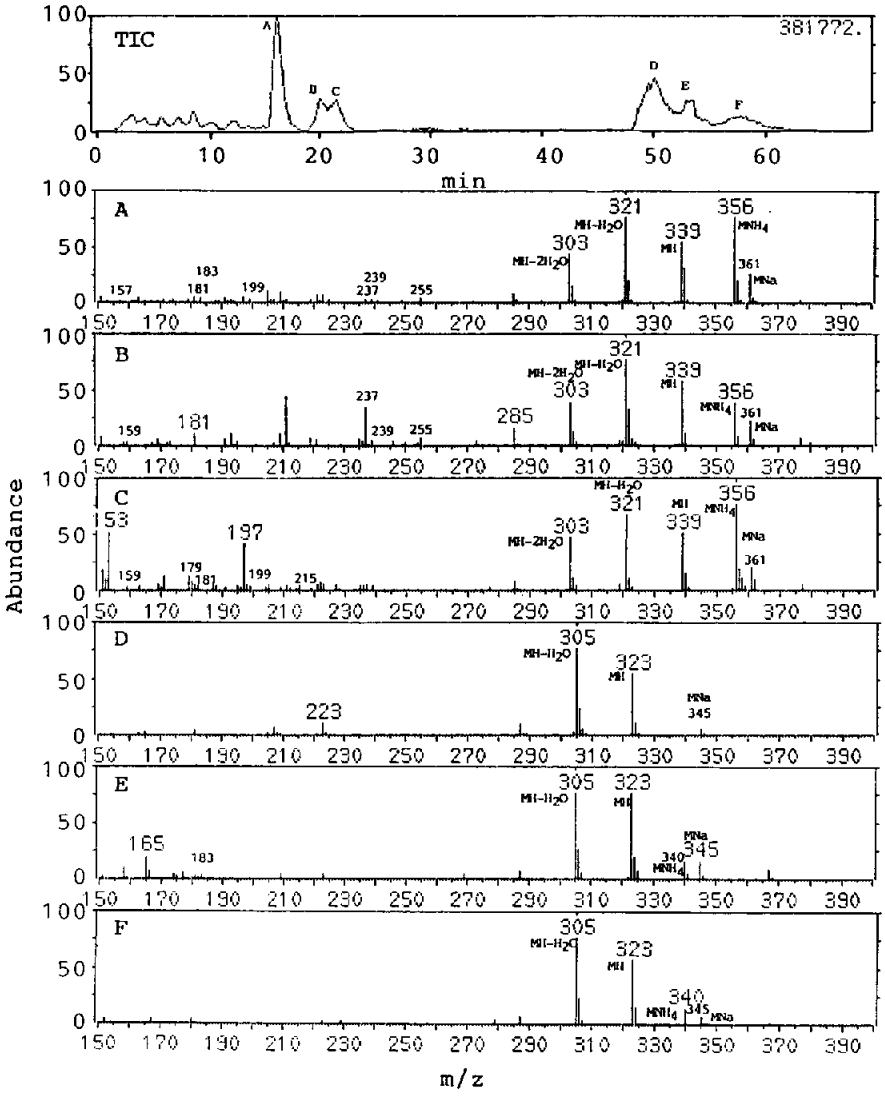

Fig. 2. TIC profile and corresponding mass spectra obtained from the reaction mixture of 20:3 ($n-6$) and *m*-chloroperoxybenzoic acid. HPLC and TSP conditions as described in Experimental, with the filament off.

derived from 20:5 ($n-3$) or 22:6 ($n-3$) was identified by MS spectral pattern (data not shown) accompanying decomposition ions in HPLC–TSP-MS analysis of each reaction mixture from 20:5 ($n-3$) or 22:6 ($n-3$) and *m*-chloroperoxybenzoic acid. For example, except for the base ion and the molecular ion, 14,15-Ep-17,18-EpETriE of diepoxyeicosatrienoic acid (DiEpETriE) yielded decomposition ions at m/z 219, 221, 237, 275, 277 and 293; 17,18-epoxyeicosatetraenoic acid (17,18-EpETE) yielded ions at m/z 259 and 261; 14,15-EpETE yielded ions at m/z 219 and 221; 11,12-EpETE yielded ions at m/z 179 and 181; and 8,9-EpETE yielded ions at m/z 161, 163 and 179. As well as the base ion and the molecular ion, 16,17-Ep-19,20-EpDTE of diepoxydocosatetraenoic acid (DiEpDTE) yielded decomposition ions at m/z 247, 263, 301, 303 and 319; 19,20-epoxydocosapentaenoic acid (19,20-EpDPE) yielded ions at m/z

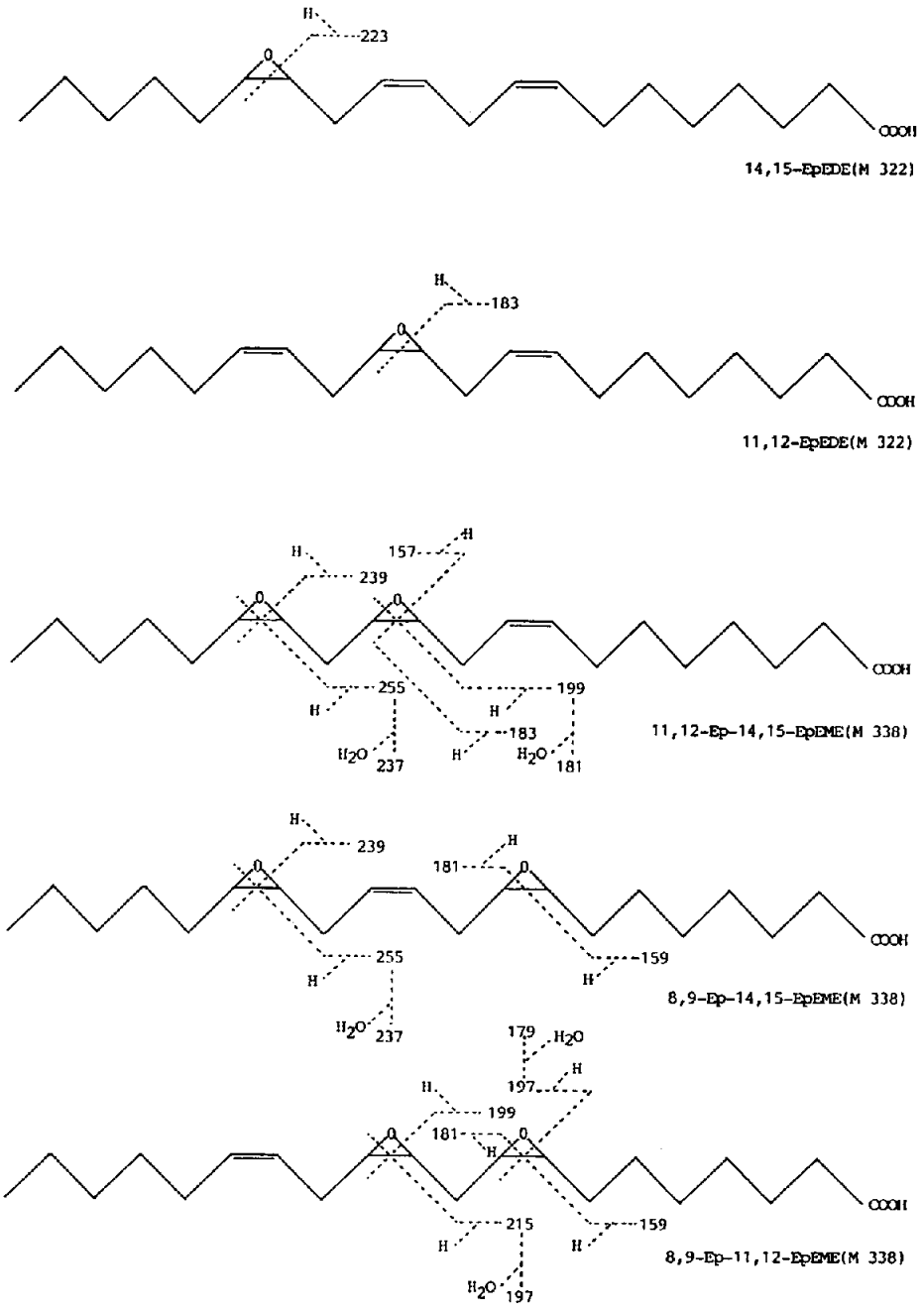

Fig. 3. MS fragmentation patterns.

285 and 287; 16,17-EpDPE yielded ions at m/z 245 and 247; 13,14-EpDPE yielded ions at m/z 205 and 207; 10,11-EpDPE yielded ions at m/z 161, 165 and 167.

Ion chromatogram profiles of m/z 305 and 356, and mass spectra of each peak obtained from an incubation mixture of 20:3 ($n-6$) additional rat TNB colitis homogenate, are shown in

Fig. 4. By comparison with the chromatographic behaviour and mass spectra of peaks A–F in Fig. 2 or authentic materials, including the acidic hydrolysates of each synthetic EpEDE, peaks A, B and C in Fig. 4 were assigned as 14,15-dihydroxyeicosadienoic acid (14,15-DiHEDE), 11,12-DiHEDE and 8,9-DiHEDE, respectively; peaks D, E, F, G and H were assigned as individual regioisomers of HETriE; peaks I, J and K were assigned as 14,15-EpEDE, 11,12-EpEDE and 8,9-EpEDE, respectively; peak L was assigned as EpHEDE. Peak I (14,15-EpEDE) yielded a decomposition ion at m/z 223, as illustrated in Fig. 3.

Ion chromatogram profiles of m/z 301 and 352, and mass spectra of each peak obtained from an incubation mixture of 20:5 ($n-3$) additional rat TNB colitis homogenate are shown in Fig. 5. By comparison with the chromatographic behaviour and mass spectra as described above or authentic materials, including the acidic hydrolysates of each synthetic EpETE, peak A in Fig. 5 was assigned as a mixture of 14,15-dihydroxyeicosatetraenoic acid (14,15-DiHETE) and 11,12-DiHETE; peaks B, C, D, E and F were assigned as 18-HEPE, 15-HEPE, 12-HEPE, 9-HEPE and 5-HEPE, respectively; peaks H, I, J and K were assigned as 17,18-EpETE, 14,15-EpETE, 11,12-EpETE and 8,9-EpETE, respectively; peak L, with a base ion of m/z 279, was assigned as a byproduct resulting from the Sep-Pak C_{18} cartridge; peak M was assigned as epoxyhydroxyeicosatetraenoic acid (EpHETE); peak G can be regarded as 5,6-DiHETE-δ-lactone, but this was not confirmed. Peak H (17,18-EpETE) yielded a decomposition ion at m/z 261, as described above.

SIM chromatograms of authentic regioisomers of DiHETriE and EpETriE of *ca.* each 100 pmol are shown in Fig. 6-I. Peaks A, B, C and D on m/z 356, 339 or 321 were assigned as 14,15-DiHETriE, 11,12-DiHETriE, 8,9-DiHETriE and 5,6-DiHETriE, respectively; peak E, with a base ion of m/z 279, was assigned as a byproduct resulting from the Sep-Pak C_{18} cartridge; peaks F, G, H and I on m/z 338 or 321 were assigned as 14,15-EpETriE, 11,12-EpETriE, 8,9-EpETriE and 5,6-EpETriE, respectively. A SIM chromatogram of MH (m/z 339) or MH $-$ H_2O (m/z 321) was suitable for the detection of DiHETriE regioisomers. A SIM chromatogram of MH (m/z 321) was suitable for the detection of EpETriE regioisomers.

SIM chromatograms of authentic regioisomers of EpHETriE are shown in Fig. 6-II. Peak A was assigned as EpHETriE derived from 5-HPETE; peaks B and C were assigned as hepoxilin-A_3; peaks D and E were assigned as hepoxilin-B_3. SIM chromatograms of MNH_4 (m/z 354), MNa (m/z 359) or MH $-$ H_2O (m/z 319) were suitable for the detection of hepoxilin-A_3, hepoxilin-B_3 and EpHETriE derived from 5-HPETE.

SIM chromatograms of EpHDPE regioisomers prepared from a hematine treatment of each HPDHE isomer are shown in Fig. 6-III. Peak A was assigned as trihydroxydocosapentoenoic acids (TriHDPEs) derived from isomers of EpHDPE; peak B was assigned as EpHDPE derived from 20-HPDHE; peaks C and D were assigned as EpHDPE regioisomers derived from 17-HPDHE; peak E was assigned as EpHDPE derived from 4-HPDHE; peaks F and G were assigned as EpHDPE regioisomers derived from 14-HPDHE. Although the MH $-$ $2H_2O$ (m/z 325) ion was suitable for the detection of EpHDPE from 20-HPDHE, the MNH_4 (m/z 378), MNa (m/z 383) or MH $-$ H_2O (m/z 343) ions were suitable for the detection of EpHDPEs derived from 4-HPDHE, 14-HPDHE, and 17-HPDHE.

SIM chromatograms of an extract from an incubation mixture of 20:3 ($n-6$) additional rat brain homogenate are shown in Fig. 7. Peaks A and B on m/z 363 or 305 were assigned as 14,15-DiHEDE and 11,12-DiHEDE, respectively; peaks C, D, and E on m/z 361 were assigned as DiHETriE (C) and isomers (D and E) of epoxyhydroxy eicosadienoic acid (EpHEDE) or diepoxyeicosamonoenoic acid (DiEpEME), respectively; peaks F, G, and H on m/z 345 or 305 were assigned as isomers of HETriE; peaks I, J and K on m/z 345 were assigned as 14,15-EpEDE, 11,12-EpEDE and 8,9-EpEDE, respectively.

SIM chromatograms of an extract from an incubation mixture of 20:4 ($n-6$) additional rat brain homogenate are shown in Fig. 8. Peaks A, B, C and D on m/z 356 or 321 in Fig. 8 were

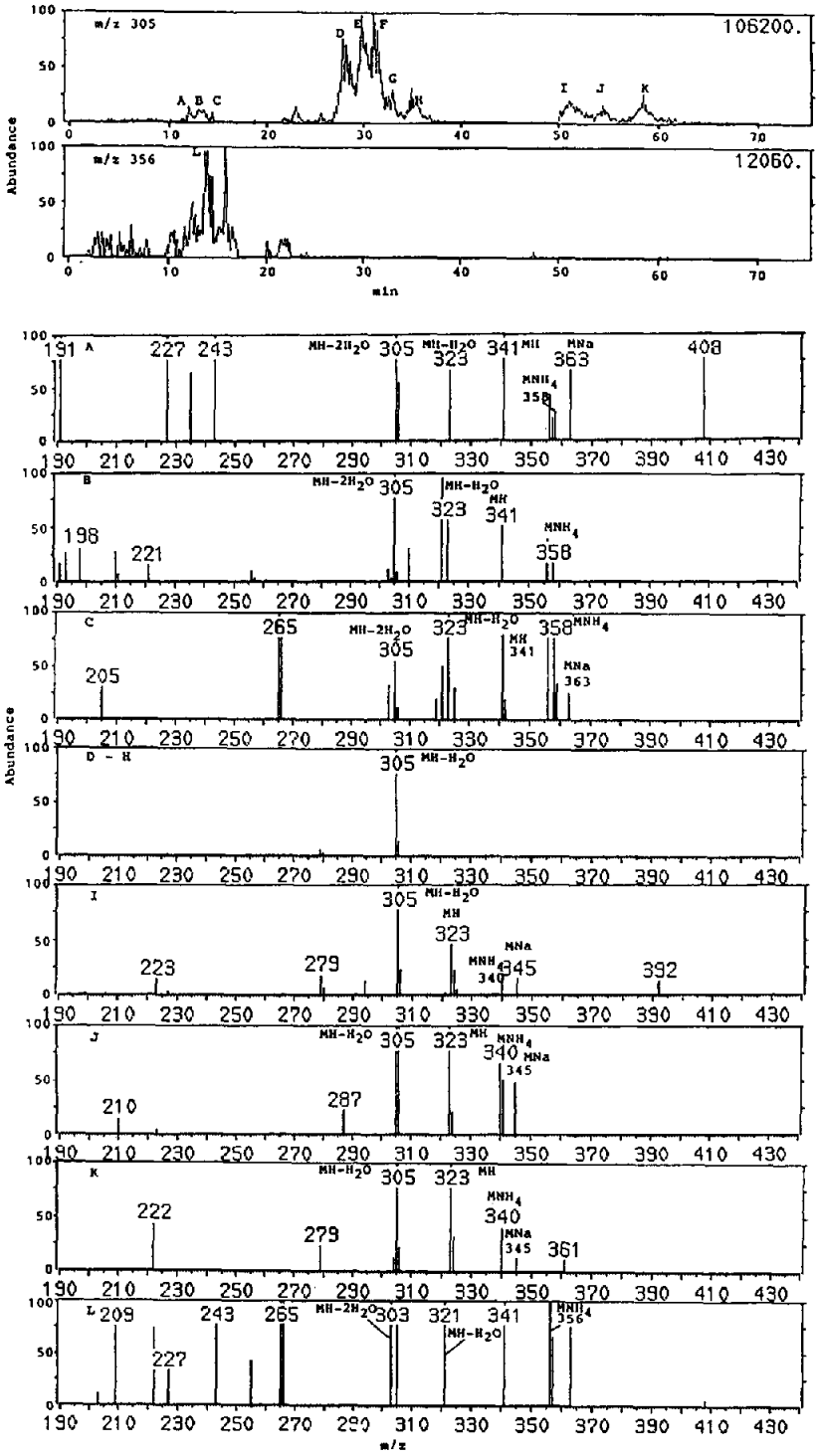

Fig. 4. Mass chromatogram profile on m/z 305 or 356 and corresponding mass spectra obtained from an incubation mixture of 20:3 ($n-6$) additional rat TNB colitis homogenate. HPLC and TSP conditions as described in Experimental, with the filament on (40 μA).

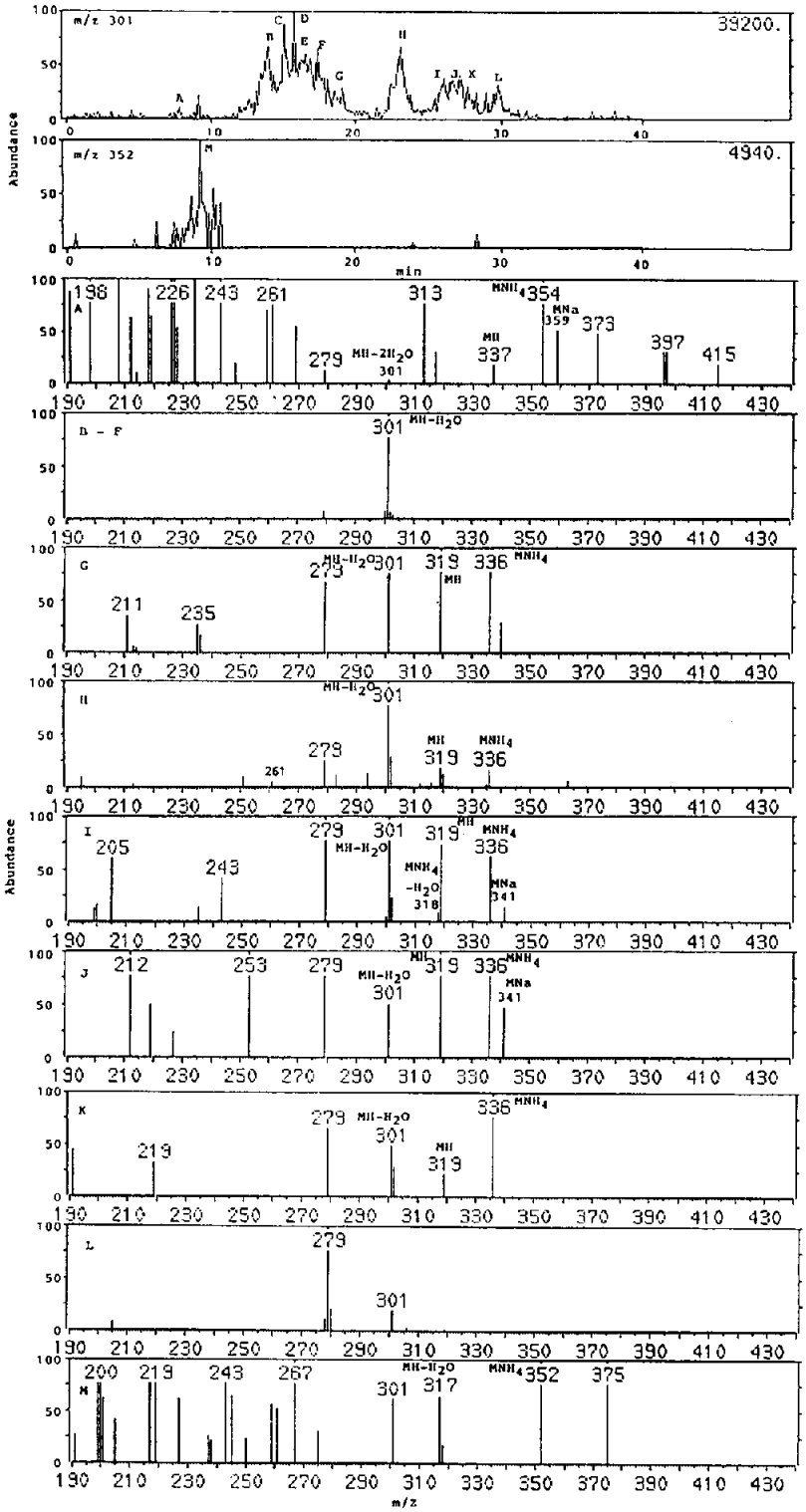

Fig. 5. Mass chromatogram profile on m/z 301 or 352 and corresponding mass spectra obtained from an incubation mixture of 20:5 ($n-3$) additional rat TNB colitis homogenate. HPLC and TSP conditions as described in Experimental, with the filament on (40 μA).

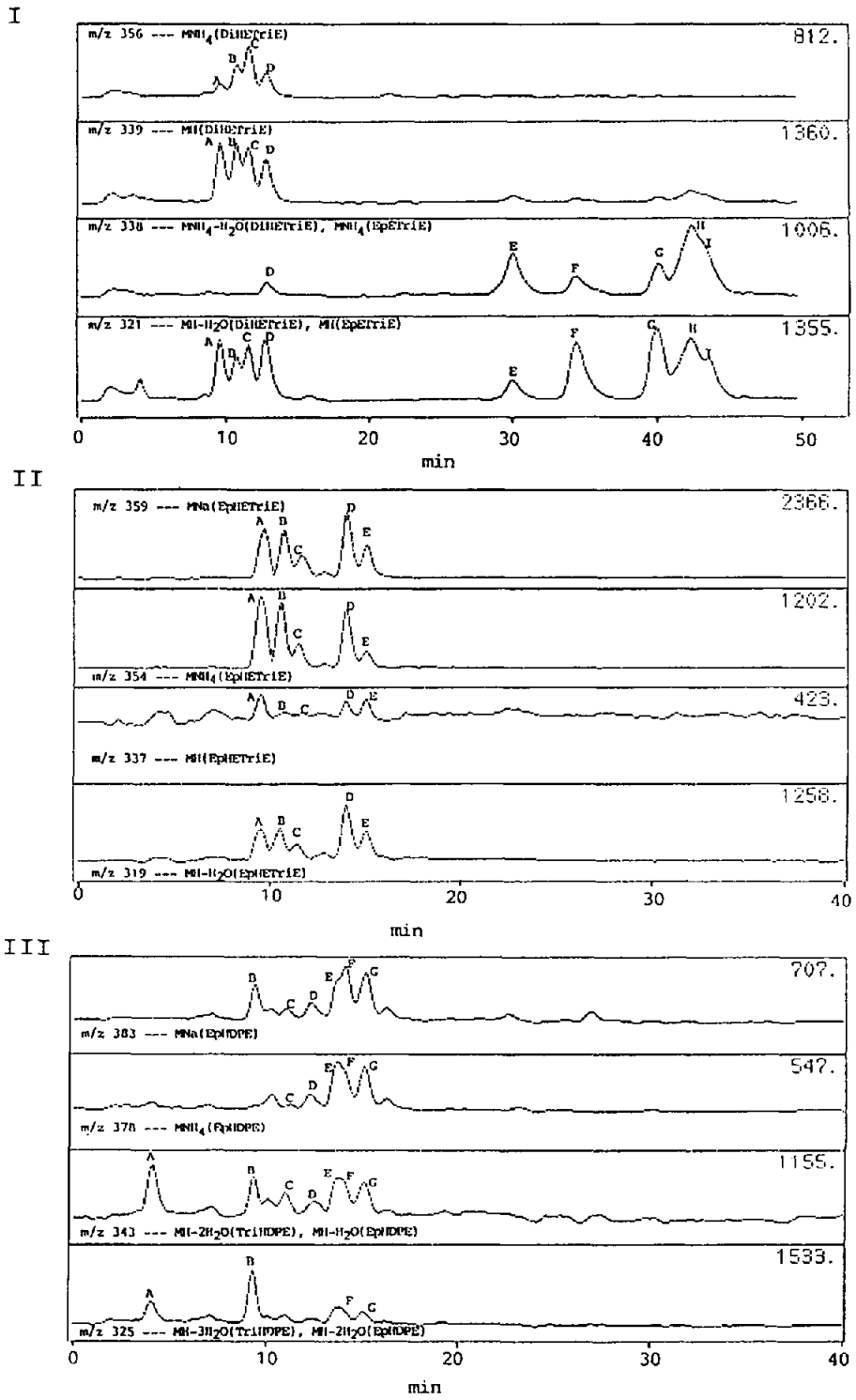

Fig. 6. SIM chromatograms of authentic regioisomers of DiHETriE, EpETriE, EpHETriE and EpHDPE. HPLC and TSP conditions as described in Experimental, with the filament off. (I) DiHETriEs and EpETriEs, *ca.* 100 pmol each; (II) EpHETriEs; (III) EpHDPEs prepared from hematine treatment of each HPDHE isomer. The number in the upper right-hand corner of each chromatogram is the ion count.

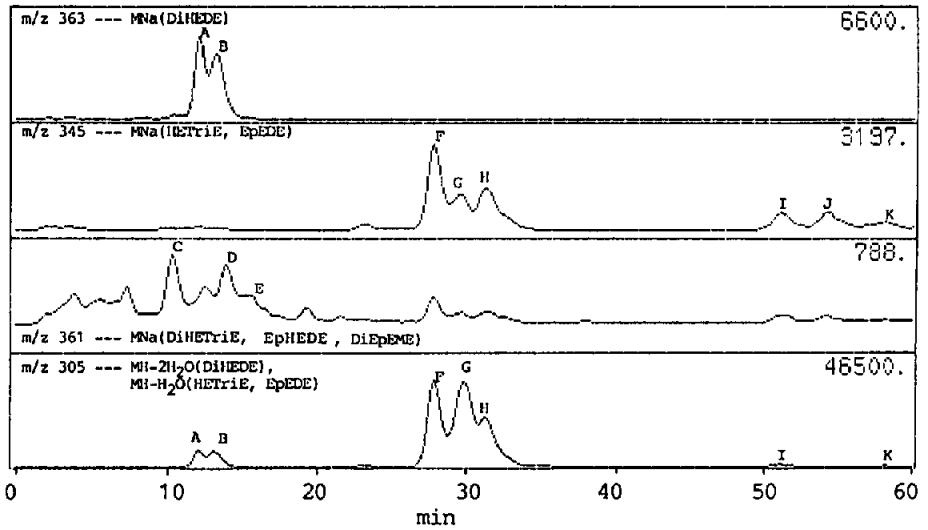

Fig. 7. SIM chromatograms of an extract from an incubation mixture of 20:3 ($n-6$) additional rat brain homogenate. HPLC and TSP conditions as described in Experimental, with the filament off. The number in the upper right-hand corner of each chromatogram is the ion count.

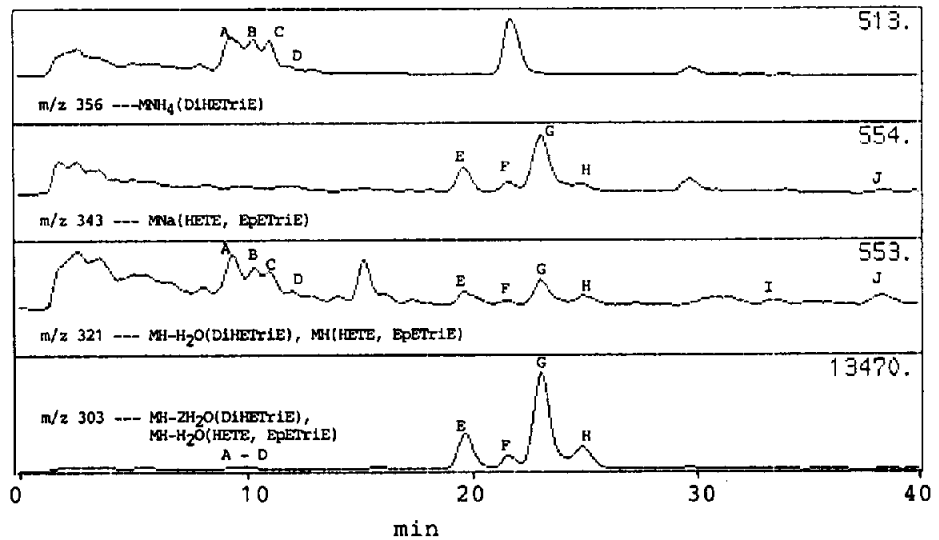

Fig. 8. SIM chromatograms of an extract from an incubation mixture of 20:4 ($n-6$) additional rat brain homogenate. HPLC and TSP conditions as described in Experimental, with the filament off. The number in the upper right-hand corner of each chromatogram is the ion count.

assigned as 14,15-DiHETriE, 11,12-DiHETriE, 8,9-DiHETriE and 5,6-DiHETriE, respectively; peaks E, F, G and H on m/z 343, 321 or 303 were assigned as 15-HETE, 11-HETE, 12-HETE and 5-HETE, respectively; peaks I and J on m/z 321 were assigned as 14,15-EpETriE and 11,12-EpETriE, respectively.

SIM chromatograms of an extract from an incubation mixture of 20:5 ($n-3$) additional rat brain homogenate are shown in Fig. 9. Peaks A

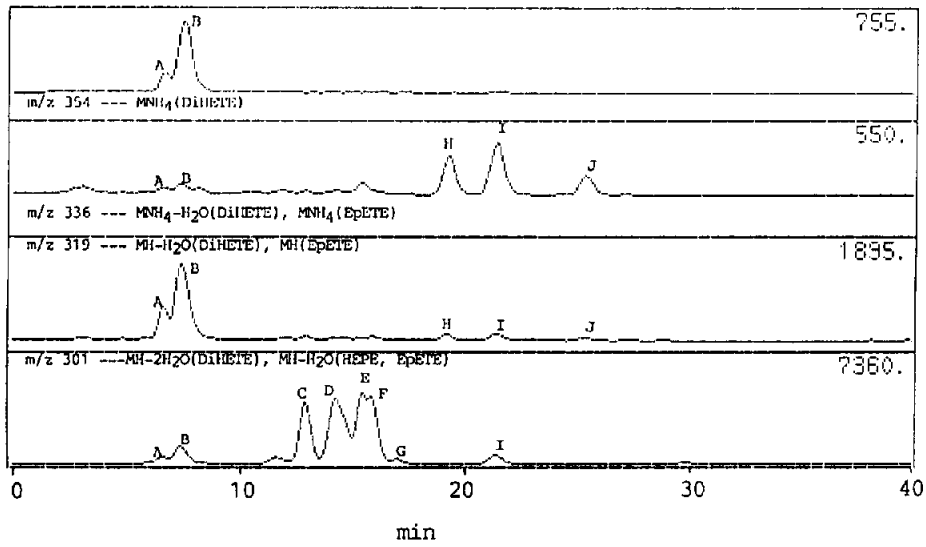

Fig. 9. SIM chromatograms of an extract from an incubation mixture of 20:5 ($n-3$) additional rat brain homogenate. HPLC and TSP conditions as described in Experimental, with the filament off. The number in the upper right-hand corner of each chromatogram is the ion count.

and B on m/z 354, 336, 319 or 301 were assigned as 17,18-DiHETE and a mixture of 14,15-DiHETE and 11,12-DiHETE, respectively; peaks C, D, E, F and G on m/z 301 were assigned as 18-HEPE, 15-HEPE, 12-HEPE, 9-HEPE and 5-HEPE, respectively; peaks I and J on m/z 336 or 319 were assigned as 17,18-EpETE and 11,12-EpETE, respectively; peak H can be regarded as 5,6-DiHETE-δ-lactone, but this was not confirmed.

SIM chromatograms of an extract from an incubation mixture of 22:6 ($n-3$) additional rat brain homogenate are shown in Fig. 10. Peaks A, B, C and D on m/z 380, 362 or 345 in Fig. 10 were assigned as isomers of dihydroxydocosapentaenoic acid (DiHDPE); peaks E, F, G, H, I and J on m/z 327 were assigned as 20-HDHE, 17-HDHE, 14-HDHE, 11-HDHE, 7-HDHE and 4-HDHE, respectively; peaks K and L on m/z 362, 345 or 327 were assigned as 19,20-EpDPE and 13,14-EpDPE, respectively.

The formation of EpETriE by cytochrome P450 epoxygenase and their metabolism *in vivo* is largely unknown. Epoxides of arachidonic acid, especially 5,6-EpETriE, are difficult to measure because they undergo chemical changes during isolation and sample workup [27]. In the same way, hydroxyepoxides of arachidonic acid are difficult to measure because they undergo chemical changes during isolation and sample workup [17–24]. In the present method, these EpPUFAs and EpHPUFAs were quickly extracted with dichloromethane at pH 4–5 and concentrated in the presence of pyridine in order to prevent chemical changes. On the SIM detection chromatogram of HPLC–TSP-MS, many EpPUFAs, EpHPUFAs and DiHPUFAs were detected simultaneously within 70 min without chemical derivatization or hydrolysis.

As shown in Figs. 4, 5, 7 and 9 and Table 1, EpPUFAs and DiHPUFAs derived from 20:3 ($n-6$) or 20:5 ($n-3$) were detected in quantity in aged rat brain or TNB colitis homogenate. From these results, the high activity of the cytochrome P450 system or non-enzymic oxidative reactions of these fatty acids as substrate in the aged rat tissue homogenate was suggested. The 20:4 ($n-6$) epoxides, EpETriEs, are found in microsomal preparations of rabbit liver [9] and kidney [28], and also in human urine [29]. There is increasing evidence that EpETriEs are released from vascular and inflammatory cells [26].

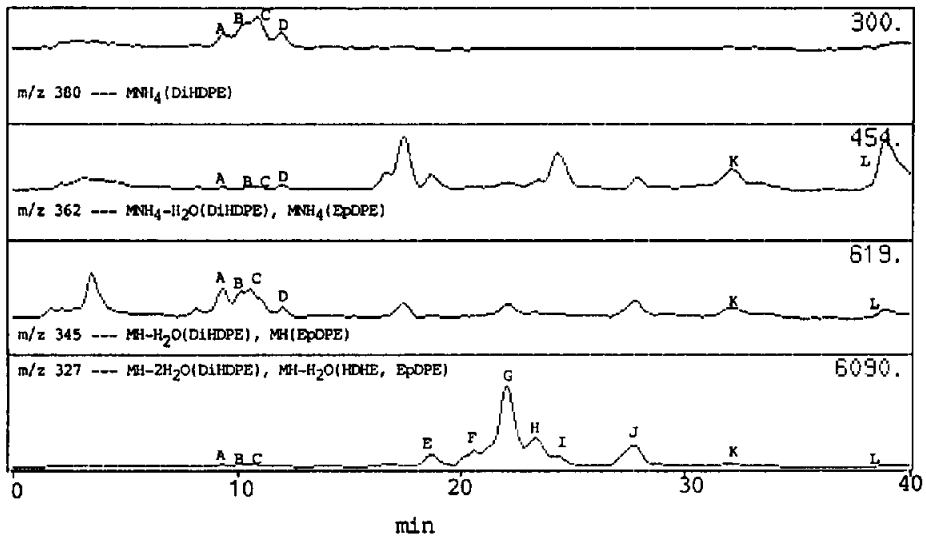

Fig. 10. SIM chromatograms of an extract from an incubation mixture of 22:6 ($n-3$) additional rat brain homogenate. HPLC and TSP conditions as described in Experimental, with the filament off. The number in the upper right-hand corner of each chromatogram is the ion count.

Table 1
Conversion of each precursor fatty acid by rat TNB colitis homogenate

Precursor fatty acid	Conversion of each precursor fatty acid (%)[a]				
	EpPUFAs	DiHPUFAs	HPUFAs	EpHPUFAs	Prostaglandins[b]
20:3 ($n-6$)	5.8	0.9	16.6	0.5	0.6
20:4 ($n-6$)	0.02	0.1	0.4	0.04	0.7
20:5 ($n-3$)	1.7	0.3	1.9	0.6	0.8
22:6 ($n-3$)	0.1	0.01	0.5	0.1	–

[a] Percentage conversion was calculated from the amount of each product measured with 12(*S*)-HETE-d_8 as the internal standard and the amount of precursor fatty acid.
[b] Prostaglandins were measured as previously described [36].

Several of these compounds have been reported to inhibit Na^+/K^+-ATPase [30], mobilize microsomal calcium [31], dilate microvessels [32], inhibit platelet aggregation [26], antagonize the vasoconstriction effects of thromboxane-A_2 in stenosed coronary arteries [12], and inhibit cyclooxygenase activity [26] and the release of somatostatin [33], leutinizing hormone [34], insulin [35] and glucagon [35].

The metabolism of 20:3 ($n-6$), 20:5 ($n-3$) or 22:6 ($n-3$) to EpPUFAs or EpHPUFAs raises the question of their biological effects.

4. References

[1] T. Miyamoto, J.A. Lindgren, T. Hokfelt and B. Samuelsson, *FEBSs Lett.*, 216 (1987) 123.
[2] J.A. Lindgren, T. Hokfelt, S.E. Dahlen, C. Patrono and B. Samuelsson, *Proc. Natl. Acad. Sci., USA*, 81 (1987) 6212.
[3] H.Y. Kim, S. Sawazaki and N. Salem, Jr., *Biochem. Biophys. Res. Commun.*, 174 (1991) 729.
[4] M. Yamane and A. Abe, *J. Chromatogr.*, 575 (1992) 7.
[5] M. Yamane, A. Abe, S. Yamane and F. Ishikawa, *J. Chromatogr.*, 579 (1992) 25.

[6] M. VanRollins, R.C. Baker, H.W. Sprecher and R.C. Murphy, *J. Biol. Chem.*, 259 (1984) 5776.
[7] M. VanRollins and R.C. Murphy, *J. Lipid Res.*, 25 (1984) 507.
[8] H.Y. Kim and N. Salem, Jr., *Prostaglandins*, 37 (1989) 105.
[9] E.H. Oliw, F.P. Guengerich and J.A. Oates, *J. Biol. Chem.*, 257 (1982) 3771.
[10] L.R. Ballou, B.K. Lam, P.Y.-K. Wong and W.Y. Cheung, *Proc. Natl. Acad. Sci., USA*, 84 (1987) 6990.
[11] M. Balazy and A.S. Nies, *Biomed. Environmental Mass Spectrom.*, 18 (1989) 328.
[12] M. Rosolowsky, J.R. Falck, J.T. Willerson and W.B. Campbell, *Circ. Res.*, 66 (1990) 608.
[13] M. VanRollins, *Lipids*, 25 (1990) 481.
[14] K. Bernstrom, K. Kayganich and R.C. Murphy, *Anal. Biochem.*, 198 (1991) 203.
[15] M. Balazy, *J. Biol. Chem.*, 266 (1991) 23561.
[16] F. Dray, B.V.-L. Normand, A. Deroussent, I. Briquet, M.-M. Gabellec, S. Nakamura, L.M. Wahl, A. Gouyette and Z.S. Salahuddin, *Biochim. Biophys. Acta.*, 1180 (1992) 83.
[17] R.L. Jones, P.J. Kerry, N.L. Poyser, I.C. Walker and N.H. Wilson, *Prostaglandins*, 16 (1978) 583.
[18] I.C. Walker, R.L. Jones and N.H. Wilson, *Prostaglandins*, 18 (1979) 173.
[19] C.R. Pace-Asciak, E. Granstrom and B. Samuelsson, *J. Biol. Chem.*, 258 (1983) 6835.
[20] C.R. Pace-Asciak, *J. Biol. Chem.*, 259 (1984) 8332.
[21] R.W. Bryant, T. Schewe, S.M. Rapoport and J.M. Bailey, *J. Biol. Chem.*, 260 (1985) 3548.
[22] C.R. Pace-Asciak, *Prostaglandins Leukotrienes Med.*, 22 (1986) 1.
[23] M. Hamberg, C.A. Herman and R.P. Herman, *Biochim. Biophys. Acta*, 877 (1986) 447.
[24] C.R. Pace-Asciak, *Biochem. Biophys. Res. Commun.*, 151 (1988) 493.
[25] S.-K. Chung and A.I. Scott, *Tetrahedron Lett.*, 35 (1974) 3023.
[26] F.A. Fitzpatrick, M.D. Ennis, M.E. Baze, M.A. Wynalda, J.E. McGee and W.F. Liggett, *J. Biol. Chem.*, 261 (1986) 15334.
[27] J. Capdevila, L.J. Marnett, N. Chacos, R.A. Prough and R.W. Estabrook, *Proc. Natl. Acad. Sci., USA*, 79 (1982) 767.
[28] J.R. Falck, V.J. Schueler, H.R. Jacobson, A.K. Sinddhanta, B. Pramanik and J. Capdevila, *J. Lipid Res.*, 28 (1987) 846.
[29] R. Toto, A. Siddhanta, S. Manna, B. Pramanik, J.R. Falk and J. Capdevila, *Biochim. Biophys. Acta*, 919 (1987) 132.
[30] M.L. Schwartzman, M.A. Caroll, N.R. Ferreri, E. Songu-Mize and J.C. McGiff, *Nature*, 314 (1985) 620.
[31] P. Kutsky, J.R. Falk, G. Weiss, S. Manna, N. Chacos and J. Capdevila, *Prostaglandins*, 26 (1983) 13.
[32] K.G. Proctor, J.R. Falck and J. Capdevila, *Circ. Res.*, 60 (1987) 50.
[33] J. Capdevila, N. Chacos, J.R. Falk, S. Manna, A. Negro-Vilar and S.R. Ojeda, *Endocrinology*, 113 (1983) 421.
[34] G.D. Snyder, J. Capdevila, N. Chacos, S. Manna and J.R. Falk, *Proc. Natl. Acad. Sci. USA*, 80 (1983) 3504.
[35] J.R. Falk, S. Manna, J. Moltz, N. Chacos and J. Capdevila, *Biochem. Biophys. Res. Commun.*, 114 (1983) 743.
[36] M. Yamane and A. Abe, *J. Chromatogr.*, 568 (1991) 11.