Journal of Chromatography, 562 (1991) 237–247
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5564

Gas chromatographic–mass spectrometric characterisation of some novel hydroxyeicosatetraenoic acids formed on incubation of arachidonic acid with microsomes from induced rat livers

R. A. CLARE*

Department of Bioanalytical Sciences, Wellcome Research Laboratories, Beckenham (U.K.)

S. HUANG

Department of Biochemistry, University of Surrey, Guildford (U.K.)

M. V. DOIG

Department of Bioanalytical Sciences, Wellcome Research Laboratories, Beckenham (U.K.)

and

G. G. GIBSON

Department of Biochemistry, University of Surrey, Guildford (U.K.)

ABSTRACT

The biotransformation of arachidonic acid by rat liver microsomes from both control animals and animals pretreated with known inducers of cytochrome P-450 isoenzymes has been studied using a combination of reversed- and normal-phase high-performance liquid chromatography and combined gas chromatography–electron-impact mass spectrometry. The metabolite profiles observed were found to be dependent upon the inducing agent. Five metabolites were identified, namely 16-, 17-, 18-, 19- and 20-hydroxylated arachidonic acids. Of these the 16- and 17-isomers have not been reported as products of arachidonic acid metabolism by any biological system and the 18-isomer has not been reported as a product of liver metabolism.

INTRODUCTION

Much work has been done on the metabolism of arachidonic acid (AA) by the well established enzymatic pathways involving cyclooxygenase and lipoxygenase enzymes. However, in recent years, the metabolism by cytochrome P-450-dependent enzyme pathways has also become important following the recognition of the physiological significance of some of the products [1,2]. AA has been shown to be a very good substrate for a number of constitutive and xenobiotic-inducible cytochrome P-450 isoenzymes [3–7]. Three pathways of cytochrome P-450-catalysed AA metabolism have been recognised, namely olefin epoxidation

0378-4347/91/$03.50

producing epoxyeicosatrienoic acids (EETs), allylic hydroxylation producing hydroxyeicosatetraenoic acids (HETEs), analogous to those formed by the lipoxygenase pathway, and oxidation of the unactivated carbon at the ω-1 and ω positions yielding 19- and 20-hydroxyarachidonic acids, respectively.

One of the most striking features of cytochrome P-450-dependent AA metabolism is that different cytochrome P-450 isozymes display differing substrate regiospecificities and that pretreatment of animals with different xenobiotic inducers can dramatically alter AA metabolite profiles. This in turn can lead to significant changes in the physiological functions mediated by these metabolites.

The work was aimed at investigating the possibility that the known toxicities of polychlorinated biphenyl (PCB) isomers could be rationalised in terms of the AA metabolite profiles obtained from the induced liver microsomes. A number of HETEs were produced by these liver microsomes, two of which do not appear to have been previously identified as a product of AA metabolism in biological systems.

EXPERIMENTAL

Chemicals

3,4,5,3′4′-Pentachlorobiphenyl (PenCB), 99.0% pure, was obtained from Greyhound (Birkenhead, U.K.) [1-^{14}C]Arachidonic acid was obtained from Amersham International (Aylesbury, U.K.). NADPH, arachidonic acid and 3-methylcholanthrene (3-MC) were obtained from Sigma (Poole, U.K.). 1,2-Epoxy-3,3,3-trichloropropane was purchased from Aldrich (Gillingham, U.K.). Bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane (BSTFA–TMCS) was obtained from Pierce and Warriner (Chester, U.K.). Ethereal diazomethane was prepared from Diazald®, obtained from Aldrich, as recommended by the manufacturers. Clofibric acid was a gift from ICI Pharmaceuticals (Macclesfield, U.K.). All other reagents were analytical grade.

Animal pretreatment and microsome preparation

Male Wistar albino rats (University of Surrey Breeders), 90–100 g, were used. PenCB (5 mg/kg) or 3-MC (80 mg/kg) in corn oil were given as a single intraperitoneal dose five days before the animals were killed. Control rats were treated with an equal volume of corn oil vehicle. Clofibrate (250 mg/kg) in 0.9% NaCl was given by gastric intubation once daily for three days, the last dose being given 24 h before the animals were sacrificed. Liver microsomes were isolated from 20% (w/v) liver homogenates as described previously [8].

Microsomal pellets were resuspended in 100 m*M* Tris–HCl buffer pH 7.5 containing 20% (v/v) glycerol and stored at −70°C without significant loss of hydroxylase activity.

Incubation of arachidonic acid and extraction of metabolic products

Incubations were carried out essentially as described by Capdevila *et al.* [9] but with the following modifications. The incubation medium, 50 m*M* Tris–HCl pH 7.5, contained 150 m*M* KCl, 10 m*M* $MgCl_2$, 8 m*M* sodium isocitrate, 0.25 I.U./ml isocitrate dehydrogenase and 1 m*M* 1,2-epoxy-3,3,3-trichloropropane (an epoxide hydrase inhibitor). After preincubating 1 ml of a microsomal suspension in the above medium for 2 to 3 min at 35°C, [1-^{14}C]AA (2.5 μCi/μmol) was added to a final concentration of 0.1 m*M*. After 1 min the reaction was initiated by the addition of NADPH to give a final concentration of 1 m*M*. The reaction was allowed to proceed for 10 min and was then terminated by the addition of 50 μl of 0.1 *M* HCl. Incubation mixtures contained 1 mg/ml microsomal protein.

The reaction mixture was extracted three times with 2 ml of ethyl acetate containing 0.01% (w/v) of the antioxidant butylated hydroxytoluene (BHT) (3 min vortex-mixing followed by 5 min centrifugation at 300 *g*). The combined extracts were dried over anhydrous Na_2SO_4, filtered and evaporated to dryness under a stream of nitrogen. The residues were reconstitued in 100 μl of ethanol containing 0.01% (w/v) BHT for reversed-phase high-performance liquid chromatography (HPLC). Overall recovery of radioactivity was >90%.

High-performance liquid chromatography

Instrumentation. A Waters Model 510 chromatograph was coupled to a Berthold LB503 HPLC radioactivity detector which was interfaced to a Commodore PET (series 4000) computer.

Reversed-phase separations. These were carried out on a 250 mm × 4.6 mm I.D. column packed with 5-μm Ultrasphere ODS (Beckman). Gradient elution was used with the solvent programmed from 100% A (acetonitrile–water–acetic acid, 300:700:1) to 100% B (acetonitrile–acetic acid, 1000:1) over 50 min, at a flow-rate of 1.0 ml/min. Fractions collected for further analysis were taken to dryness under nitrogen at room temperature and reconstituted in hexane.

Normal-phase separations. These were performed on a 300 mm × 3.9 mm I.D. Spherisorb silica (5 μm) column (HPLC Technology, Macclesfield, U.K.) with a flow-rate of 3.0 ml/min, using isopropanol–hexane–acetic acid solvent systems. One of the following two solvent systems were used: (I) a linear gradient from 10:989:1 to 30:969:1 over 30 min; (II) 5:944:1 isocratically for 3.0 min and then a linear gradient to 15:984:1 over 30 min.

Derivatization

Fractions collected after normal-phase chromatography were taken to dryness under nitrogen and the residues taken up into 70 μl of methanol. Ethereal diazomethane (700 μl) was added and the solutions kept at room temperature for 10 min before removing solvent and excess reagent under nitrogen. The residue was dissolved in 70 μl of BSTFA–TMCS and the mixture was heated at 70°C for 60 min.

Hydrolysis, hydrogenation and resilylation

An aliquot of derivatised sample (30 μl) was added to 300 μl of water in a 1-ml vial and the mixture was shaken, using a vortex-mixer, for 7 min. It was then extracted twice with diethyl ether (500 and 300 μl) and the combined ethereal extracts were taken to dryness under nitrogen. The residue was dissolved in 250 μl of methanol and the solution purged with nitrogen prior to the addition of a small amount of Adam's catalyst (hydrated PtO_2). Hydrogen was bubbled through the suspension for 4 min. It was then purged again with nitrogen, the methanol decanted and the catalyst washed with a further 200 μl of methanol. The combined solutions were taken to dryness under nitrogen, 45 μl of BSTFA–TMCS were added and the resulting solution was heated at 70°C for 30 min.

Gas chromatography–mass spectrometry (GC–MS)

A Hewlett Packard 5890 gas chromatograph was directly coupled to a Finnigan MAT TSQ70 triple quadrupole mass spectrometer. Chromatography was carried out on a 30 m × 0.25 mm I.D. DB5 (WCOT) fused-silica capillary column (J & W Scientific), film thickness 0.25 μm, using splitless injection, with helium carrier gas at a head pressure of 0.103 MPa.

Injections (1–2 μl) were made with a column oven temperature of 150°C. This was maintained for 1 min, then programmed up to 240°C at 25°C/min and held for 22 min. The injection port and GC–MS interface were maintained at 250°C.

Electron-impact (EI) spectra were obtained with an ionization energy of 70 eV and a source temperature of 150°C.

A mixture of C_{16}–C_{24} fatty acid methyl esters was also injected and analysed by the GC—MS. Retention times (t_R) were measured from the time at which the column oven reached 240°C (4.6 min). Log t_R was plotted against the C value and a line that was linear between C_{20} and C_{24} was obtained. C values for the unknowns were calculated from this graph.

RESULTS

The reversed-phase chromatographic profiles obtained from the extracts of incubation media are shown in Fig. 1. It was found that after xenobiotic pretreatment there was an increase in the levels of material eluting between 31 and 34.5 min, accompanied by a varying degree of inhibition of the formation of other metabolites when compared with liver incubations. This was particularly marked in the case of clofibrate pretreatment.

Fig. 1B shows that without added NADPH, no products were formed by PenCB-induced microsomes. This, together with the fact that the formation of the major metabolite peaks was inhibited by up to 80% in the presence of carbon monoxide (data not shown), strongly indicates that their formation is cytochrome P-450-dependent.

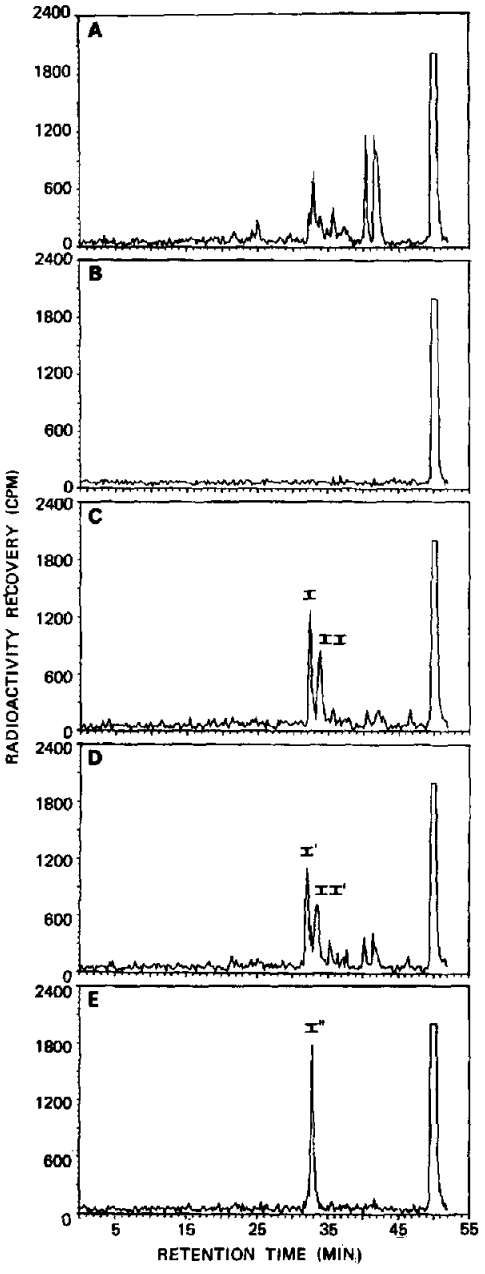

Fig. 1. Radiochromatograms obtained on reversed-phase HPLC of the extracts from liver microsomes from rats pretreated with (A) corn oil vehicle, (B) PenCB in the absence of NADPH, (C) PenCB, (D) 3-MC and (E) clofibrate.

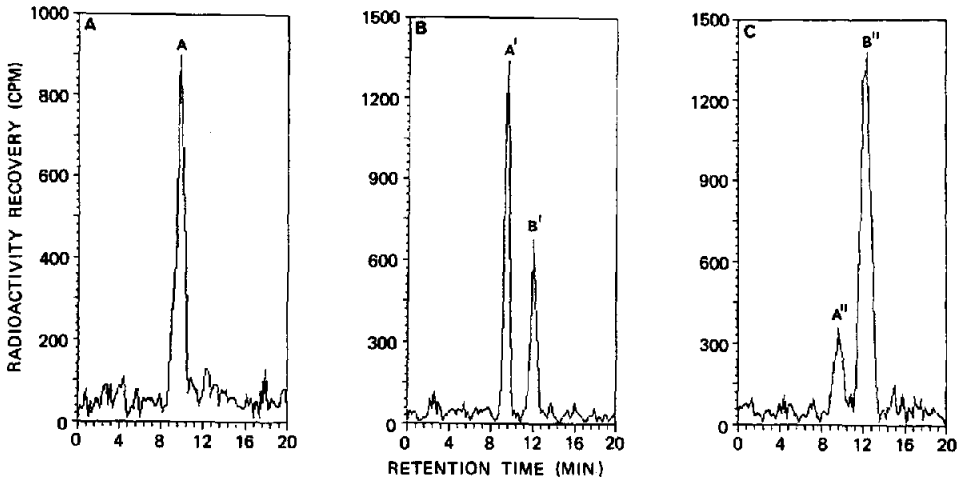

Fig. 2. Radiochromatograms obtained on normal-phase HPLC using solvent system I of fractions corresponding to peak I in the reversed-phase chromatograms from rats pretreated with (A) PenCB, (B) 3-MC and (C) clofibrate.

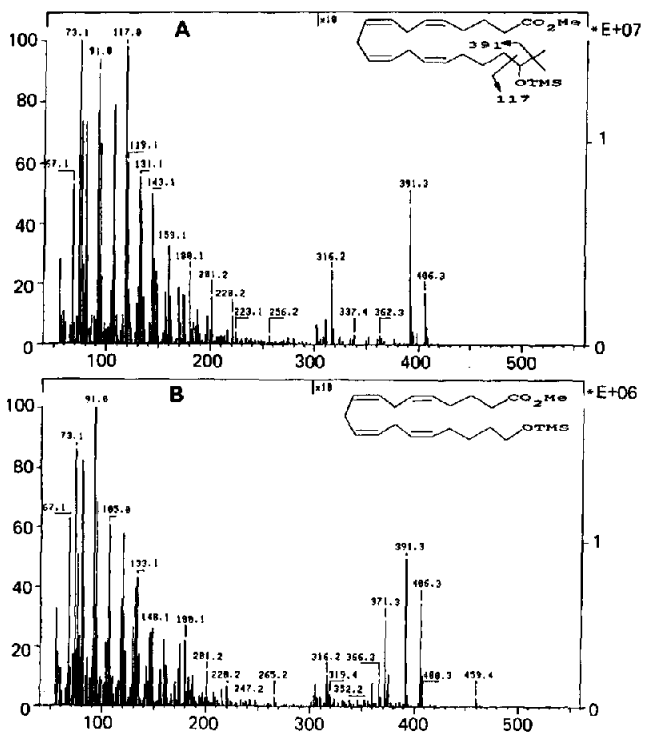

Fig. 3. Mass spectra obtained for (A) peak A from PenCB-treated microsomes (19-HETE) and (B) peak B″ from clofibrate-treated microsomes (20-HETE).

Normal-phase chromatography and GC–MS

In order to further purify the material in the fractions collected after reversed-phase chromatography they were rechromatographed using the described normal-phase systems. The more polar material was separated from peaks I, I′ and I″ using solvent system I and the less polar material from peaks II and II′ using solvent system II.

The fractions containing peaks I, I′ and I″ from PenCB-, 3-MC- and clofibrate-pretreated liver microsomal preparations when rechromatographed gave the traces shown in Fig. 2. Two components (A and B) were observed with the relative levels of each being strongly dependent on the inducing agent. PenCB pretreatment produced virtually 100% A, 3-MC approximately 60% A and clofibrate greater than 80% B.

We have previously shown that the major product formed by microsomes after clofibrate pretreatment (peak B″ in Fig. 2) is 20-hydroxyarachidonic acid [10]. The spectrum obtained in the present study (Fig. 3B) and the C-value of 22.8 are in good agreement with our previous work.

When the fraction containing peak A from the PenCB-induced microsome incubations was derivatized and subjected to GC–MS only one component, with a C value of 22.1, showing the molecular and M − 15 ions expected for a HETE at *m/z* 406 and 391 was found. This is shown in Fig. 3A. The C value and the spectrum are in good agreement with those obtained by Oliw *et al.* [11] for 19-hydroxyarachidonic acid. The intense ion at *m/z* 117 is formed by an α-cleavage to the O-trimethylsilyl (OTMS) group.

Normal-phase chromatography of fractions II and II′ from PenCB- and 3-

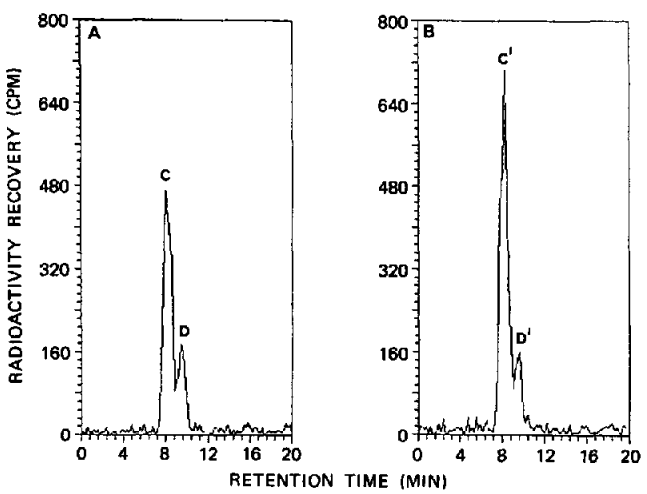

Fig. 4. Radiochromatograms obtained on normal-phase HPLC using solvent system II of fractions corresponding to peak II in the reversed-phase chromatograms from rats pretreated with (A) PenCB and (B) 3-MC.

MC-induced microsome incubations revealed two components labelled C and D. These chromatograms are shown in Fig. 4.

GC of derivatized samples of C and C′ showed the presence of two HETEs with C values of 21.7 and 22.0 which after hydrogenation gave two hydroxyeicosanoic acids (HEs) with C values of 22.4 and 22.7. The spectra obtained from the earlier running component before and after hydrogenation are shown in Figs. 5B

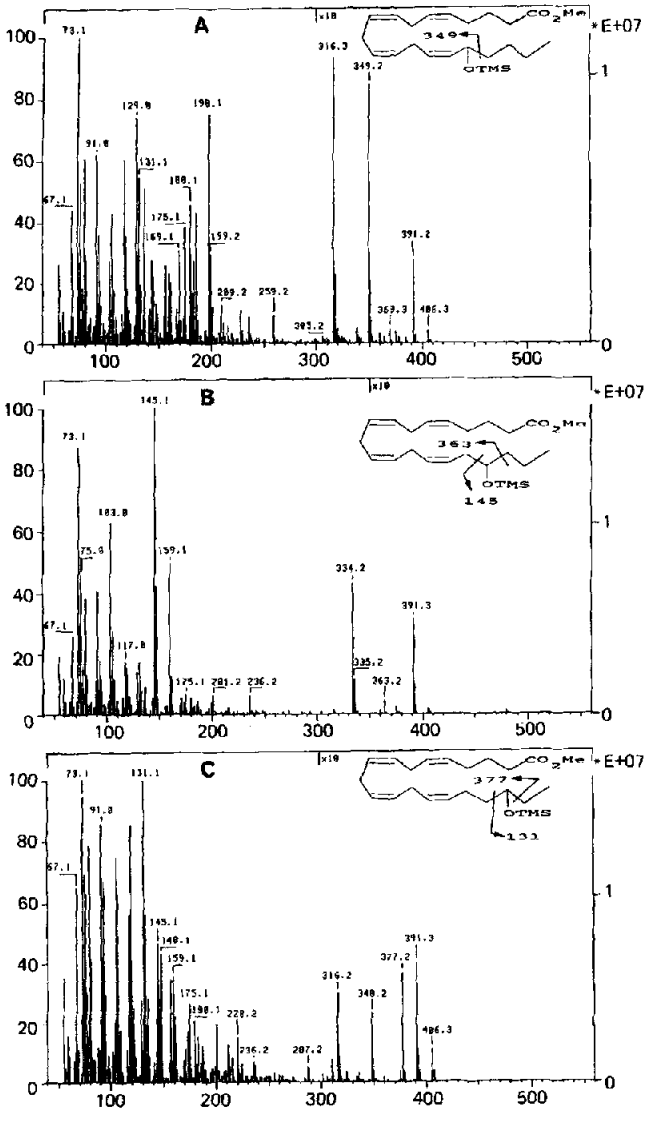

Fig. 5. (A) Mass spectra obtained for peak D (16-HETE) and (B,C) spectra obtained from peak C (17-HETE and 18-HETE, respectively).

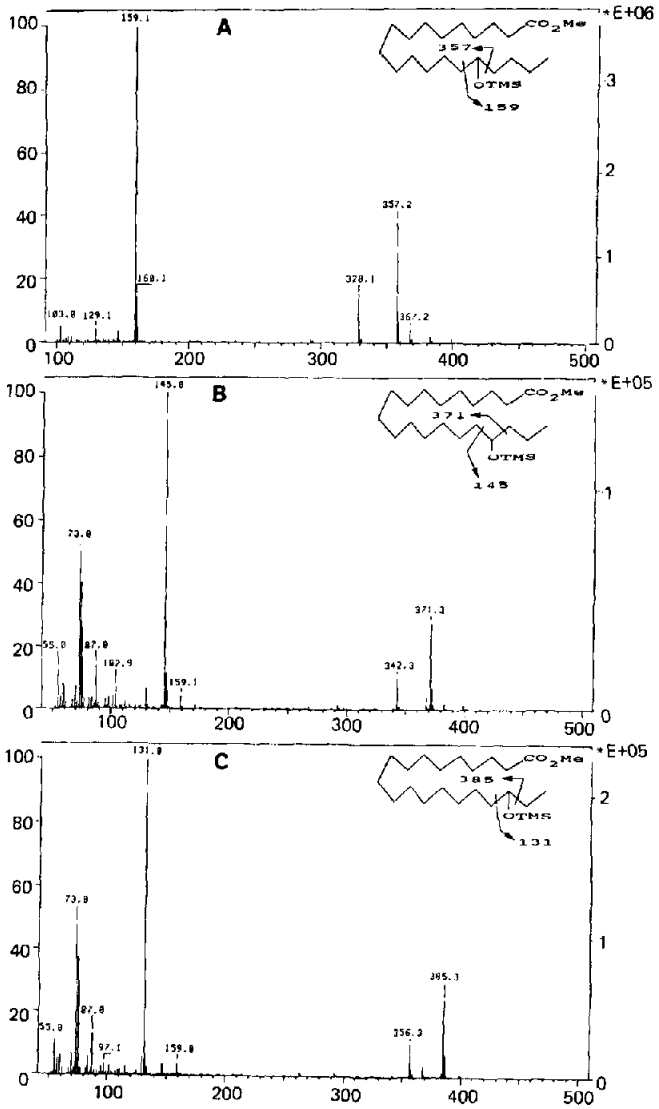

Fig. 6. Mass spectra of the components shown in Fig. 5 after catalytic hydrogenation.

and 6B, respectively. These are consistent with hydroxylation being on carbon 17, with the HETE derivative spectrum showing a strong ion at *m/z* 145 due to one of the α-cleavages to the OTMS group and a weak signal at *m/z* 363 for the other α-cleavage. After hydrogenation the ion at *m/z* 145 is still present but the other α-cleavage ion is shifted 8 a.m.u. to *m/z* 371 indicating that all double bonds lie between carbons 1 and 17.

Spectra obtained for the later-eluting component before and after hydrog-

enation (Figs. 5C and 6C) are consistent with the hydroxyl being on carbon 18 with α-cleavage ions for the HETE derivative at *m/z* 131 and 377 and those for the HE derivative being at *m/z* 131 and 385.

Examination of fraction D after derivatization revealed only one HETE with a C value of 21.3, which after reduction gave a HE which chromatographed with a C value of 22.2. The spectrum of the HE derivative (Fig. 6A) indicates that the hydroxyl group is on carbon 16 with the α-cleavage ions at *m/z* 159 and 357. In the spectrum of the HETE derivative (Fig. 5A) the α-cleavage ion at *m/z* 349 is apparent but no prominent ion at *m/z* 159 is observed. This is not unexpected since unsaturation at the α-carbon (C-15) is likely to inhibit this cleavage.

DISCUSSION

In the present study we have identified five monohydroxylated eicosatetraenoic acids formed when induced liver microsomes were incubated with AA in the presence of NADPH and molecular oxygen. Of these 16- and 17-HETEs have not previously been identified in biological systems and 18-HETE has not been identified as a product of liver microsomal metabolism. During the course of this work the formation of 18(*R*)-HETE by cynomolgus monkey seminal vesicle microsomes was reported by Oliw [12] and the C-values and spectra he found for the methyl ester trimethylsilyl ether derivatives of this compound and its hydrogenation product are in good agreement with those reported here.

The fact that the production of these HETEs depends on the presence of NADPH, that it is inhibited by carbon monoxide and that it is induced by compounds well known to be cytochrome P-450 inducers strongly suggests that it is mediated by a cytochrome P-450-dependent pathway.

The possible biological significance of these compounds in the mediation of the toxic effects of these inducing agents is currently under investigation.

ACKNOWLEDGEMENTS

This research was supported in part by a British Council postgraduate studentship (S.H.) and project grants from the M.R.C. (G.G.G.) and Wellcome Trust (G.G.G.).

REFERENCES

1 F. A. Fitzpatrick and R. C. Murphy, *Pharmacol. Rev.*, 40 (1989) 229.
2 W. L. Smith, *Biochem. J.*, 259 (1989) 315.
3 S. K. Bains, S. M. Gardiner, K. Mannweiler, D. Gillett and G. G. Gibson, *Biochem. Pharmacol.*, 34 (1985) 3221.
4 G. G. Gibson and S. K. Bains, *Biochem. Soc. Trans.*, 13 (1985) 850.
5 J. Capdevila, N. Chacos, J. Werringloer, R. A. Prough and R. W. Estabrook, *Proc. Natl. Acad. Sci. U.S.A.*, 78 (1982) 5363.

6 J. Capdevila, L. J. Marnett, N. Chacos, R. A. Prough and R. W. Estabrook *Proc. Natl. Acad. Sci. U.S.A.*, 79 (1982) 767.
7 M. L. Schwartzman, N. G. Abraham, M. A. Carroll, R. D. Levere and J. C. McGiff, *Biochem. J.*, 238 (1986) 283.
8 R. K. Sharma, B. G. Lake, J. Foster and G. G. Gibson, *Biochem. Pharmacol.*, 37 (1988) 1193.
9 J. Capdevila, Y. R. Kim, C. Martin-Wixtrom, J. R. Falck, S. Manna and R. W. Estabrook, *Arch. Biochem. Biophys.*, 243 (1985) 8.
10 R. K. Sharma, M. V. Doig, D. F. V. Lewis and G. G. Gibson, *Biochem. Pharmacol.*, 38 (1989) 3621.
11 E. H. Oliw, J. A. Lawson, A. R. Brash and J. A. Oates, *J. Biol. Chem.*, 256 (1982) 9924.
12 E. H. Oliw, *J. Biol. Chem.*, 264 (1989) 17 845.