Journal of Chromatography, 488 (1989) 283–293
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO 4558

ISOTOPE DILUTION GAS CHROMATOGRAPHY–MASS SPECTROMETRY FOR THE STUDY OF EICOSANOID METABOLISM IN HUMAN BLOOD PLATELETS

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SUMMARY

Stable isotope dilution gas chromatography–mass spectrometry provides one of the most important techniques for the quantitative measurement of eicosanoids. This technique was applied to the quantitation of hydroxyeicosatetraenoic acids, hydroxyheptadecatrienoic acid, thromboxane B_2 and prostaglandin $F_{2\alpha}$ formed during platelet aggregation after stimulation of gel-filtered platelets with thrombin (0.25 U/ml) or collagen (2 μg/ml). Similar amounts of hydroxyheptadecatrienoic acid and thromboxane B_2 were found after platelet activation. The ratio of formation of 12-hydroxyeicosatetraenoic acid to thromboxane B_2 varied from donor to donor. Only small amounts of prostaglandin $F_{2\alpha}$ (up to 200 pg per $2.0 \cdot 10^8$ platelets) and basic values of 15-hydroxyeicosatetraenoic acid (up to 100 pg per $2.0 \cdot 10^8$ platelets) were measured using gas chromatography with negative ion chemical ionization mass spectrometry. In addition, different stable isotope dilutions were prepared and are discussed in detail.

INTRODUCTION

In the past two decades the enzyme-catalysed metabolism of methylene-interrupted polyunsaturated fatty acids (PUFAs), particularly arachidonic acid (AA), has attracted increasing interest. Research has mainly been focused on the tissue-specific nature of PUFA synthesis, different metabolic pathways and the large

0378-4347/89/$03.50

number of biologically active compounds formed. Owing to the extremely low concentrations of eicosanoids in biological systems, the sensitivity and specificity of mass spectrometric techniques are of particular importance.

The term "eicosanoids" was introduced by Corey et al. in 1980 [1] and encompasses the large number of biologically active compounds formed from a C_{20} unsaturated fatty acid skeleton [2,3]. AA (5,8,11,14-all-*cis*-eicosatetraenoic acid), an essential fatty acid and the main representative amongst PUFAs, plays a key role as the precursor of a variety of eicosanoids [4]. Enzyme-catalysed metabolism of AA is generated by two main pathways. First, cyclooxygenase-catalysed fatty acid oxidation includes the formation of prostaglandins (PGs) and thromboxanes (TXs) [5]. The second pathway, catalysed by lipoxygenases [6], encompasses the formation of different non-cyclic hydroxyeicosatetraenoic (HETE) acids after reduction of the unstable hydroperoxy-eicosatetraenoic acids (HPETEs), and the formation of leukotrienes (LTs) [7] and lipoxins [8].

Various techniques, such as high-performance liquid chromatography [9], bioassay and radioimmunoassay [10], and gas chromatography (GC) with electron-capture detection [11] have been used to quantify eicosanoids in different cells, tissues and biological fluids. However, to attain high accuracy and precision, the necessity of isotope dilution mass spectrometry (MS) is generally emphasized [12]. GC coupled with MS using stable isotope dilution is undoubtedly the most sophisticated technique available for the detection and quantitative measurement of eicosanoids [13,14]. We applied this technique to measure the formation of AA metabolites after stimulation of gel-filtered platelets (GFPs) with thrombin or collagen.

EXPERIMENTAL

Materials

Arachidonic acid, Sil-A-200, soybean lipoxidase (E.C. 1.13.11.12) type IV, pseudocholinesterase (E.C. 3.1.1.8) type IV-S and type XI, TXB_2, $[^2H_4]PGF_{2\alpha}$ and thrombin (from bovine plasma grade I), sodium borohydride and indomethacin were purchased from Sigma (Munich, F.R.G.). N.N-Bis-(trimethylsilyl)trifluoroacetamide (BSTFA) and pyridine were from Pierce (Rockford, IL, U.S.A.), pentafluorobenzyl bromide (PFBBr) was from ICT (Vienna, Austria). [^{18}O] water and platinum dioxide were from Ventron (Karlsruhe, F.R.G.). Silicar CC4 was from Mallinckrodt (St. Louis, MO, U.S.A.) *tert.*-butyldimethylsilyl (tBDMS) chloride was from Fluka (Buchs, Switzerland), Sepharose 2B and Sephadex LH 20 were from Pharmacia (Uppsala, Sweden), human serum albumin (HSA) was from Behring (Marburg/Lahn, F.R.G.) and collagen reagent was from Hormon Chemie (Munich, F.R.G.). All other reagents and solvents of analytical grade were purchased from Merck (Darmstadt, F.R.G.)

Methods

Platelet aggregation studies. Blood was obtained from healthy volunteers who had taken no medication for at least 4 weeks. GFPs were prepared after column chromatography of platelet-rich plasma on Sepharose 2B, which was equilibrated

with freshly prepared calcium-free Tyrode (pH 7.3) containing HSA (0.2%, w/v) [15,16]. The platelet count was adjusted to $2.0 \cdot 10^8$ platelets/ml. Aggregations (500-μl samples of GFPs) were triggered with collagen (2 μg/ml) or thrombin (0.25 U/ml) as described previously [15], and stopped after different times with ice-cold ethanol and acidified to pH 3.2. After addition of ^{2}H- and ^{18}O-labelled internal standards (20 ng per 50 μl methanol), eicosanoids were extracted with diethyl ether and separated by silicic acid column chromatography as described previously [17,18].

Preparation of standards. 5-HETE was synthesized according to Corey et al. [19]. 12-HETE and hydroxyheptadecatrienoic acid (HHT) were synthesized from AA by incubating platelet suspensions in the presence or absence of indomethacin [20]. 15-HETE was prepared by incubation of AA with soybean lipoxidase (E.C. 1.13.11.12) type IV and type XI and subsequent reduction of 15-HPETE with sodium borohydride [21,22]. ^{18}O-Labelling of HETEs, HHT, TXB_2 and 2,3-dinor TXB_2 was accomplished according to procedures described elsewhere [18,23,24].

Derivatization. Methyl esters were prepared by addition of 2 ml of a fresh solution of diazomethane in diethyl ether–methanol (9:1, v/v) and allowed to react for 30 min at room temperature [24]. Hydrogenation of HETEs and HHT was carried out in methanolic solution with platinum dioxide as a catalyst and bubbling with hydrogen for 2 min at room temperature [24]. Pentafluorobenzyl (PFB) esters were obtained by treatment with 10 μl of diisopropylethylamine and 50 μl of a PFBBr solution in acetonitrile (7%, w/v) for 10 min at room temperature [25,26]. Trimethylsilyl (TMS) ethers were prepared by the use of 60 μl of BSTFA–pyridine (2:1, v/v) for 20 min at 60°C [23,24]. tBDMS ethers of hydrogenated and esterified HETEs and HHT were formed by treatment with 50 μl of a solution of tBDMS chloride in dimethylformamide (2 *M*) and 50 μl of a solution of imidazole in dimethylformamide (5 *M*) for 3 h at 70°C [27–29]. Methyloximation was performed by addition of 60 μl of a solution of methoxime hydrochloride in pyridine (2%, w/v) and overnight reaction [23].

Gas chromatography–mass spectrometry. A Finnigan GC 9610 coupled to a Finnigan 4500 mass spectrometer and an INCOS data system was used. It was equipped with a DB-5 fused-silica capillary column (30 m × 0.25 mm I.D., from J&W Scientific, Rancho Cordova, CA, U.S.A.) The splitless grob injector was kept at 260°C. For determination of HETEs and HHT, the column was kept at 100°C for 1 min, then programmed to 200°C at 25°C/min, then to 320°C at 5°C/min. For PGs and TXB_2, the column was kept at 170°C for 1 min, then programmed to 320°C at 40°C/min. The GC column was directly connected to the ion source of the mass spectrometer. Helium was used as the carrier gas. Negative ion chemical ionization (NICI) was carried out with methane as a moderating gas, an electron energy of 120 eV, and an emission current of 0.1 A. Electron impact (EI) spectra were recorded with an electron energy of 70 eV and an emission current of 0.2 A.

RESULTS AND DISCUSSION

The work-up procedure for AA metabolites on Silicar CC4 after extraction from biological media with diethyl ether provides savings of both time and solvent

in the separation and purification of eicosanoids. The percentage recovery of radioactivity after silicic acid column chromatography, measured by scintillation counting was ca. 85% for [^{14}C]15-HETE and more than 90% for [^{3}H]6-keto-$PGF_{1\alpha}$, implying similar results for other eicosanoids as verified by GC–MS [18,24]. Prior to extraction, biological samples should be treated with equal amounts of ethanol or acid to precipitate proteins and prevent back-exchange of ^{18}O-labelled standards by esterases. Detection limits for the eicosanoids determined are given in Table I.

Fig. 1 shows the partial EI mass spectrum of the hydrogenated methyl ester TMS ether derivative of 5-HETE and [$^{18}O_2$]5-HETE. This confirms that no oxygen exchange reaction occurs at the hydroxyl moiety. The isotopic compositions of different hydrogenated HETE methyl ester TMS ether derivatives after the first esterification–hydrolysis cycle in [^{18}O] water are given in Table II. The high yield of ^{18}O label for 5-HETE is apparent but cannot be explained by formation of the delta lactone [23].

For analysis of HETEs and HHT, tBDMS ethers were prepared rather than TMS ethers owing to the high resistance to hydrolysis and the lower instrumental background and incidence of interfering peaks during quantification according to the higher quantitation mass of the derivatives [18,23,24]. Under NICI conditions, HETE isomers provide the same fragment ion at [M−181] for silylated PFB ester derivatives. In order to obtain sufficient GC resolution for quantitation of these positional isomeric compounds, sophisticated GC temperature programmes were used. Furthermore, as HETEs exhibit different hydrogenation behaviour during derivatization [18], addition of a stable-isotope-labelled analogue of each isomer is essential (Fig. 2). Profiling of AA metabolites then provides high accuracy, sensitivity and specificity.

In the present study we were interested in cyclooxygenase and lipoxygenase metabolite formation after stimulation of GPFs with only physiological trigger substances. Results of previous experiments indicated that lipoxygenase and cyclooxygenase, different enzymes of the AA cascade, will work in parallel in blood platelets after stimulation [15].

TABLE I

DETECTION LIMITS OF HETEs, HHT, $PGF_{2\alpha}$ AND TXB_2 FOR GC-NICI-MS ANALYSIS

Values are based on a signal-to-noise ratio of 4:1 ($n=5$).

Compound	Detection limit (pg)
5-HETE	44.5
12-HETE	41.3
15-HETE	16.7
HHT	14.3
$PGF_{2\alpha}$	9.5
TXB_2	19.4

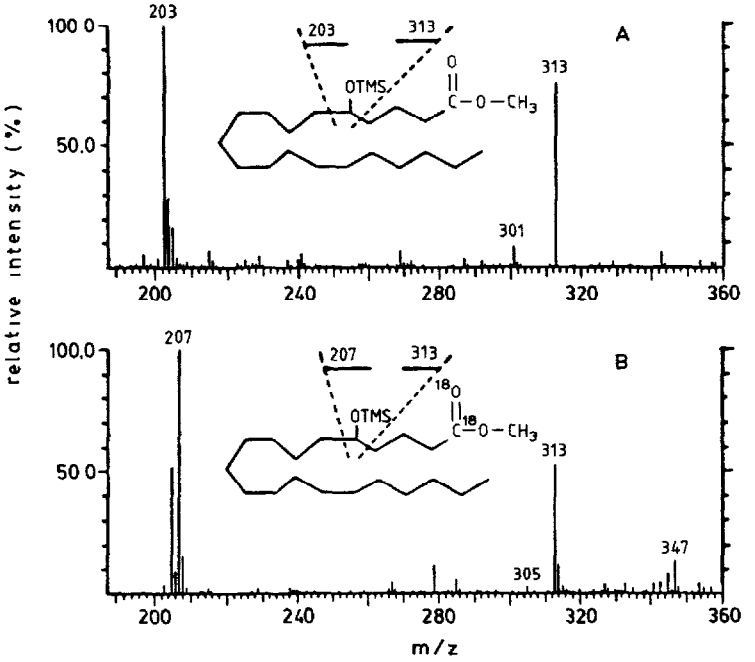

Fig. 1. Partial EI mass spectra of (A) 5-HETE and (B) base-catalysed ^{18}O-labelled [$^{18}O_2$]5-HETE as methyl ester TMS ether after catalytic hydrogenation.

TABLE II

ISOTOPIC COMPOSITION OF HYDROGENATED HETE METHYL ESTER TMS ETHERS AFTER ESTERIFICATION-HYDROLYSIS CYCLE IN [^{18}O]WATER

The atom percentage was calculated from the α-fragment ions relative to the TMS group containing the carboxylic acid moiety under EI conditions. Correction for natural abundance was made

Compound	Atom percentage		
	$^{16}O_2$	$^{16}O^{18}O$	$^{18}O_2$
5-HETE	9.0	91.0	0.0
8-HETE	92.6	7.4	0.0
11-HETE	94.7	5.3	0.0
12-HETE	73.2	26.8	0.0
15-HETE	100.0	0.0	0.0

Thrombin-induced platelet aggregation (0.25 U/ml GFP suspension) showed a very short lag-time (Fig. 3). Formation of 12-HETE, HHT and TXB_2 began subsequently after activation, reaching half-maximum production after 1.5 min. Plateau values for TXB_2 and HHT were found ca. 3 min after stimulation. The final amounts of TXB_2 and HHT were ca. $12.5 \pm 2.4 \cdot 10^{-2}$ nmoles/$2 \cdot 10^8$ GFPs and up to $22 \pm 3.1 \cdot 10^{-2}$ nmoles/$2 \cdot 10^8$ GFPs for 12-HETE for one platelet donor.

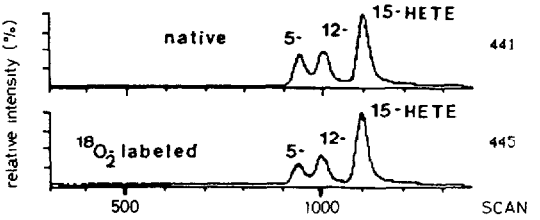

Fig. 2. Selected-ion monitoring mass chromatogram of a mixture of 15 ng each of 5-HETE, 12-HETE, 15-HETE and their $^{18}O_2$-labelled analogues. Eicosanoids were analysed by GC-NICI-MS as tBDMS ether PFB ester derivatives after catalytic hydrogenation. The ion chromatograms at *m/z* 441 and *m/z* 445 correspond to HETEs and [$^{18}O_2$]HETEs.

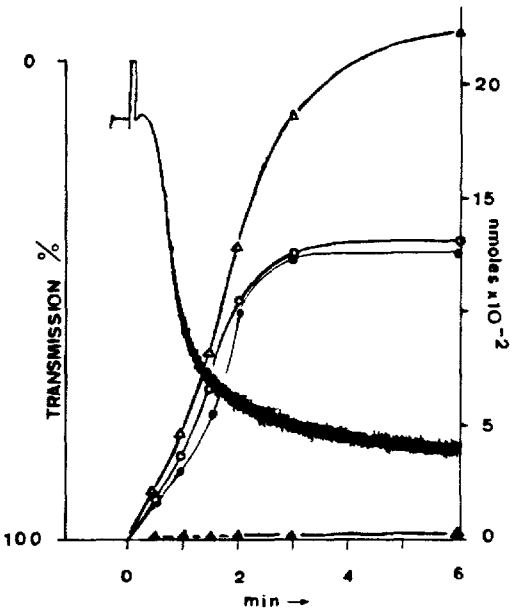

Fig. 3. Time-course of AA metabolites produced during thrombin-stimulated aggregation (0.25 U/ml GFP.). Aggregation was stopped at 0.5, 1, 1.5, 2, 3 and 6 min. After addition of internal standards, [$^{18}O_2$]5-HETE, [$^{18}O_2$]12-HETE, [$^{18}O_2$]15-HETE, [$^{18}O_2$]HHT, [$^2H_4$]$PGF_{2\alpha}$ and [$^{18}O_2$]TXB_2, separation and derivatization of eicosanoids were achieved as described in the Experimental section. Measurement was performed by GC-NICI-MS: HETEs and HHT were measured as PFB ester tBDMS ether derivatives after catalytic hydrogenation (*m/z* 441, natural occurring HETEs; *m/z* 399, natural occurring HHT; *m/z* 445 and *m/z* 403, the labelled analogues). TXB_2 and $PGF_{2\alpha}$ were analysed as TMS ether PFB ester methoxime derivatives (*m/z* 614, TXB_2; *m/z* 569, $PGF_{2\alpha}$; *m/z* 618 and *m/z* 573, the labelled analogues). Values are given as means of three determinations of one platelet donor. (●) = TXB_2; (○) = HHT; (△) = 12-HETE; (▲) = sum of 5-HETE, 15-HETE and $PGF_{2\alpha}$.

Only trace amounts were measured for 5-HETE and 15-HETE (up to 100 pg per $2 \cdot 10^8$ GFPs) and $PGF_{2\alpha}$ (up to 200 pg per $2 \cdot 10^8$ GFPs).

Collagen-triggered platelet aggregation (final concentration 2 μg/ml platelet suspension) showed a much longer lag-time. Fig. 4 represents the time-course of AA metabolites of another platelet donor. Also, collagen-induced stimulation confirms the similar increase of TXB_2 and HHT observed following thrombin

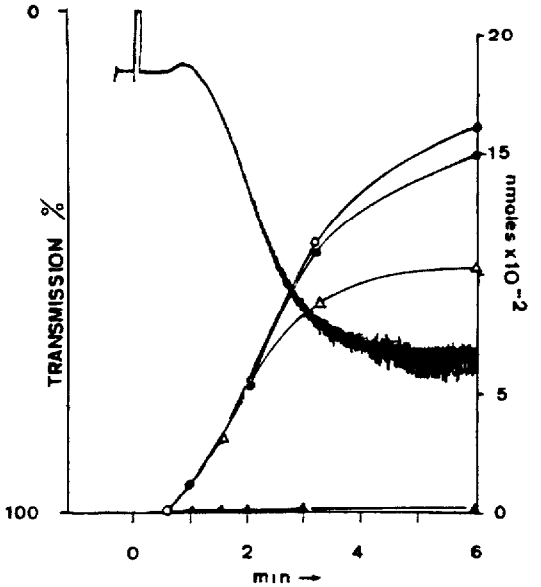

Fig. 4. Time-course of AA metabolites produced during collagen-stimulated aggregation (2 μg/ml GFP). Aggregation was stopped at 0.5, 1, 1.5, 2, 3 and 6 min. For work-up procedures see Fig. 3. Values are given as means of three determinations of one platelet donor. Symbols as in Fig. 3.

stimulation. The final amounts for both metabolites are ca. $15.4 \pm 1.8 \cdot 10^{-2}$ nmoles per $2 \cdot 10^8$ GFPs. Formation of 12-HETE increased up to 3 min, reaching almost a plateau value. The formation of TXB_2 and HHT did not reach a plateau value, but constantly increased up to 6 min. Also collagen-induced aggregation showed similar amounts of 5-HETE, 15-HETE, and $PGF_{2\alpha}$. However, it is worth noting that formation of AA metabolites during collagen-triggered aggregation does not start during the first 30 s, in contrast to thrombin stimulation. This suggests no liberation of AA from the phospholipid storage during the lag-time.

Both experiments confirm a molar ratio for TXB_2 and HHT of ca. 1:1. Also the very similar time-courses of the two compounds are apparent. 15-HETE is a platelet metabolite, which was originally detected by Wong et al. in 1985 [30]. We confirmed the basic levels of 15-HETE in human blood platelets using GC–NICI-MS with a detection limit in the low picogram range. In some experiments, up to 100 pg of 5-HETE were found after stimulation of blood platelets. This, however, could be due to leukocyte contamination during platelet preparation.

Preparation of stable isotopes is a well-reported feature for different AA metabolites [15,18,23,24,31]. They are readily prepared, and can be stored in stock solutions for an extended period without significant loss of label. Stable-isotope-labelled analogues of non-toxic compounds are harmless to mammals and can therefore be used for in vivo and in vitro experiments without special care. It is worth noting that substances labelled with stable isotopes have practically identical physical and chemical properties to the unlabelled naturally occurring compounds. All losses during extraction, separation and derivatization are assumed

to be identical for the naturally occurring and the labelled species. Known amounts of isotope-labelled compounds should be added to biological samples at the beginning of sample processing. Quantitation by GC–MS is finally achieved by comparing the peak areas of labelled and unlabelled compounds.

For the selection of the labelled compounds as internal standards, the following aspects should be considered: first, a high degree of isotope incorporation and total absence of the unlabelled compound are desirable; second, the mass difference between the labelled and the native compound should be sufficient to minimize interference from naturally occurring isotopes; third, precautions must be taken to prevent loss of label by back-exchange during 'work-up'.

The preparation of stable-isotope-labelled target eicosanoids can be achieved by deuterium-labelled arachidonate precursors or by preparation of ^{18}O-labelled compounds.

Preparation of deuterated eicosanoids

Eicosatetraynoic acid, a tetraacetylenic compound, is a precursor for the preparation of [$^{2}He_{8}$]AA [32,33]. Biochemical synthesis in turn from the deuterium-labelled arachidonate can be used to generate the desired eicosanoids. However, double-bond isomerization and reduced isotopic purity of the deuterated precursor restrict the use of deuterated compounds. Although Taber and coworkers [34,35] have reported a selective synthesis of octadeuterated 5-HETE for GC–MS quantitation, one report [36] concerning the limited application of deuterium-labelled methoximes as internal standards has appeared. However, it must be pointed out that deuterium labelling provides relatively high stability in biological media but instability to hydrogenation, as described for HETEs [18]. Although the high kinetic isotope effect of deuterium causes minimal differences in retention times from the naturally occurring metabolites [15], deuterated compounds are of proven use in PG analysis [37,38].

^{18}O-Labelling

One common structural feature of all AA metabolites is the carboxylic moiety. Labelled analogues can thus be prepared by exchange of the two carboxylic oxygen atoms with ^{18}O. These exchange reaction can be accomplished by acid catalysis, repetitive base-catalysed ester hydrolysis in [^{18}O]water, and enzymatically via esterases. Acid-catalysed exchange is preferable for AA, in contrast to PGs and HETEs [39].

However, repetitive base-catalysed ^{18}O-labelling hydrolysis [39–42] is widely used for other lipoxygenase and cyclooxygenase products, such as LTB_4 [4] and TXs [23,24]. Leis et al [23] reported that, in contrast to HETEs, the oxygen inbuilt for TXB_2 and 2,3-dinor TXB_2 is not limited to the carboxyl moiety (Fig. 5). Labelling is extended to a hydroxyl group and the oxygen of the hemiacetal. However, this four-fold labelling cannot be maintained, as back-exchange will occur during methyloximation on mechanistic grounds when derivatized together with PGs [23].

The third method of ^{18}O incorporation, the esterase-catalysed methyl ester hydrolysis of eicosanoids, was reported by Pickett and Murphy for labelling PG

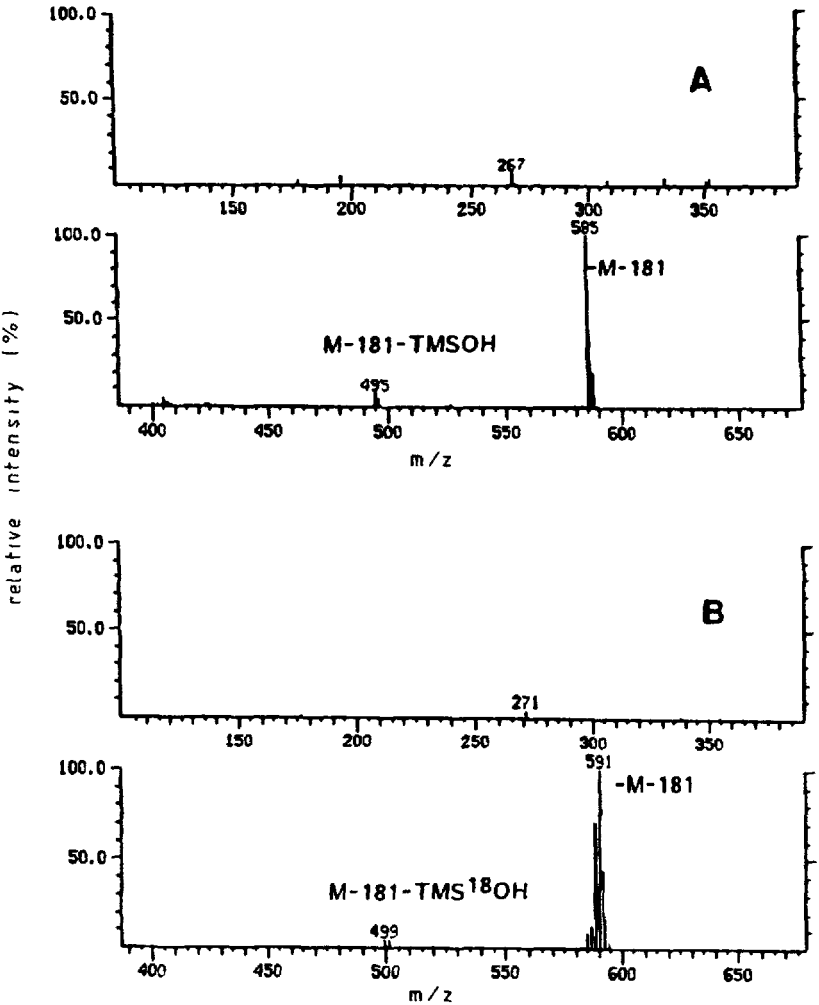

Fig. 5. Partial NICI mass spectra of TXB_2 PFB ester TMS ether (A) before and (B) after four cycles of base-catalysed hydrolysis in [^{18}O]water. The fragment ions at *m/z* 585 and *m/z* 591 correspond to the carboxylate anions formed by the loss of the PFB-tropylium cation. The signals at *m/z* 495 and *m/z* 499 correspond to a neutral loss of TMSOH and TMS^{18}OH.

$F_{2\alpha}$ with porcine esterase [43]. However, the rapidity of back-exchange reactions (the loss of ^{18}O) after incubation in physiological media demands attention [44]. Therefore, in turn, plasma esterases (such as different types of pseudocholinesterases) and liver esterases were applied to label lipoxygenase and cyclooxygenase products. PGD_2 and $PGF_{2\alpha}$ are substrates, but not as good as LTB4 or HETEs [44]. Leis and coworkers [18,23,24] reported that labelling of 5-HETE is more efficiently performed by base-catalysis, in contrast to 12-HETE, 15-HETE (Fig. 6) and 12-HHT (a cyclooxygenase breakdown product), resulting in more than 90% of the double-labelled species.

Thus ^{18}O-labelling provides high stability to hydrogenation but instability to

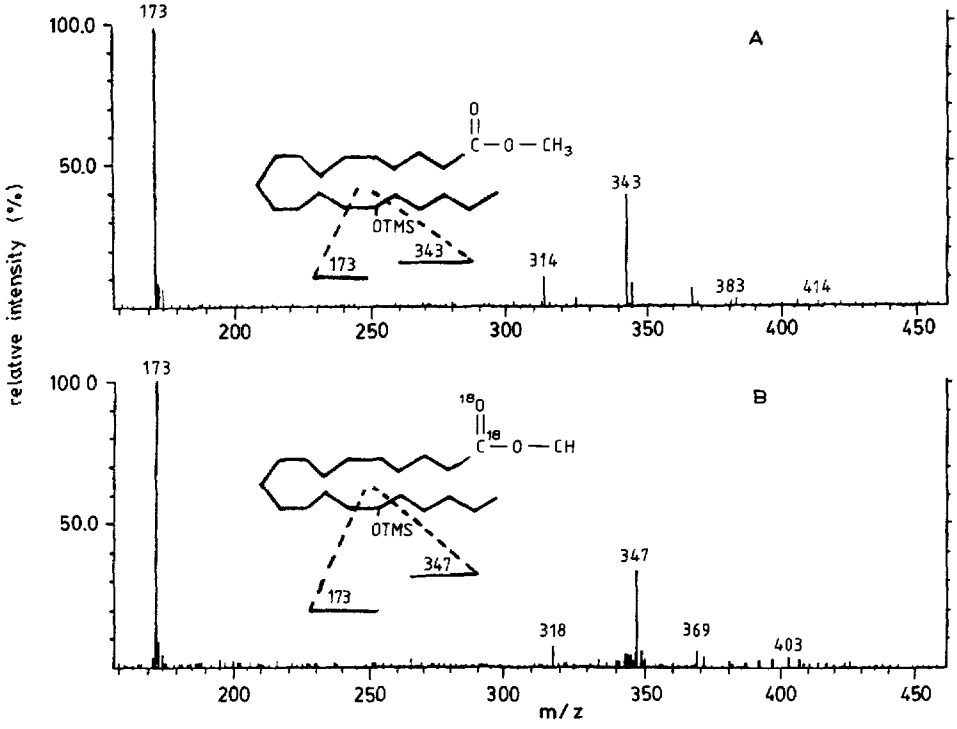

Fig. 6. Partial EI mass spectra of the hydrogenated methyl ester TMS ether derivative of (A) 15-HETE and (B) [$^{18}O_2$]15-HETE after esterase-catalysed ^{18}O-labelling.

various esterases present in biological media. In contrast to ^{2}H, the minor kinetic isotope effect of ^{18}O results in no difference in the retention times of the labelled and unlabelled species.

ACKNOWLEDGEMENTS

This work was supported by grants from the Fonds zur Förderung der Wissenschaftlichen Forschung (No 6528, S46, 5617 and 6882). We thank Dr. J. Pike (Upjohn Company, Kalamazoo, MI, U.S.A.) for providing 2,3-dinor-TX B_2, and Dr. P.M. Woollard (London, U.K.) for providing 8-HETE and 11-HETE.

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