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Note

Analysis of dihydroxyeicosatetraneoic acids by gas chromatography—mass spectrometry

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The lipoxygenase metabolites of arachidonic acid are of especial interest to research into inflammation. The dihydroxy acid (diHETE), 5(*S*),12(*R*)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraeneoic acid (LTB_4), is one of the most potent endogenous chemoattractants for polymorphonuclear leukocytes (PMN) known [1] and has been found in increased concentrations in psoriatic skin [2]. The major monohydroxy acid found in skin is 12-hydroxyeicosatetraenoic acid (12-HETE). Although less potent than LTB_4 it is chemokinetic for PMN in vitro [3] and pro-inflammatory in vivo [4]. Raised levels are present in inflamed [5] and psoriatic skin [6]. A recent report has described the ω-oxidation by PMN of 12-HETE to 12,20-diHETE [7], and in view of the high concentrations of 12-HETE present in diseased skin, we have developed an analytical method for this metabolite.

EXPERIMENTAL

Materials

12-HETE and [^{3}H]12-HETE were obtained by photooxidation of arachidonic acid using methods already published [8]. LTB_4, 5(*S*),12(*S*)-diHETE and 12,20-diHETE were generous gifts from Dr. J. Rokach (Merck Frosst Labs., Montreal, Canada). [5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid was from Amersham International (Amersham, U.K.). High-performance liquid chromatography (HPLC) columns were from HPLC Technology, (Macclesfield,

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U.K.) and BDH (Poole, U.K.). HPLC solvents were from Fisons (Loughborough, U.K.). N-Methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) and *tert.*-butyldimethylsilylimidazole (tBDMSI), bromine, rhodium on alumina and other solvents and reagents were obtained from Aldrich (Gillingham, U.K.), *tert.*-butyldimethylsilyl chloride (tBDMSCl) and *tert.*-butyldiphenylsilyl chloride were obtained from Fluorochem (Glossop, U.K.). Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Pierce and Warriner (Chester, U.K.). The micro autosampler vials were supplied by Pye-Unicam (Cambridge, U.K.). *tert.*-Butylmethoxylphenylsilyl bromide (tBMPSBr) was prepared from *tert.*-butyldiphenylsilyl chloride by reaction with methanol followed by oxidation with bromine [9].

Hydrogenation of diHETEs

This was carried out essentially as described by Murphy [10]. The reaction was performed in autosampler vials which permitted the removal by centrifugation of the catalyst and limited the total volume to 30–50 μl.

Preparation of tBDMS ether-esters

The tBDMS ether-esters were prepared by treatment of the hydrogenated product with 50 μl of a solution of MTBSTFA–tBDMSI–tBDMSCl–acetonitrile (50:5:0.5:100) for 12 h at room temperature, or for 1 h at 45°C. The excess reagents and solvent were removed under vacuum and the residue dissolved in decane containing 5% MTBSTFA to guard the product against attack by moisture.

Preparation of tBMPS ethers

The diHETE was esterified with diazomethane (5 min at room temperature with 50 μl of a solution of diazomethane in diethyl ether–methanol, 95:5, v/v). After removal of diazomethane and solvent under nitrogen the methyl ester was treated with 50 μl of a 0.1 *M* solution of tBMPSBr in dichloromethane and 10 μl triethylamine for 1 h at room temperature. The excess reagents and solvent were removed in a stream of nitrogen and the product redissolved in BSTFA.

Gas chromatographic–mass spectrometric analysis

Gas chromatography–mass spectrometry (GC–MS) was performed on a VG Analytical (Manchester, U.K.), Model 305 mass spectrometer with an associated Model 2025 data system. The mass spectrometer was operated under electron-impact conditions at 40 eV ionisation energy. GC columns were mounted in a Varian Assoc. (Walton-on-Thames, U.K.), Model 3400 gas chromatograph fitted with a Varian on-column injector with independent temperature control. For scanned mass spectra the samples were injected onto and separated by a BP5 (SE52 equivalent) wide-bore capillary column (12 m × 0.53 mm I.D., SGE U.K., Milton Keynes, U.K.), maintained at 265°C and interfaced to the mass spectrometer by a jet separator. For selected-ion recording (SIR) analysis a 25 m × 0.33 mm I.D. SE-30 column, prepared in our laboratories, was connected directly to the mass spectrometer source. This column was temperature-programmed from 175°C to 300°C.

Oxidation of 12-HETE by PMN

Human PMN were prepared from peripheral blood by dextran sedimentation and resuspended in Dulbecco's phosphate-buffered saline. The leukocytes were mixed in the ratio 1:10 with platelets prepared from the platelet fraction, to give a final suspension of 10^8 viable PMN per ml. The mixed cell suspension, 3 ml, was incubated for 10 min at 37°C with 13 μM [^{3}H]arachidonic acid (specific activity 0.62 Ci/mmol). A PMN suspension without added platelets, 0.5 ml, was incubated for 10 min at 37°C with 16 μM [^{3}H]12-HETE (specific activity 0.3 Ci/mmol).

Extraction and HPLC purification of 12,20-diHETE

The incubation supernatants were acidified to pH 3.5 and extracted with ethyl acetate. The resulting lipid was redissolved in 0.75 ml of 1-chlorobutane and partitioned against an equal volume of phosphate buffer, pH 8.5. The arachidonic acid and HETEs were selectively extracted into the chlorobutane while the diHETEs remained in the aqueous phase and were recovered from it with ethyl acetate [6]. Part of the diHETE fraction was methylated with diazomethane. The diHETEs were analysed by HPLC on a 250 mm × 4.6 mm Nucleosil 50 5-μm silica column connected to on-line radioactivity and UV detectors. Solvent systems used were hexane–propan-2-ol–acetic acid (94:6:0.01) (solvent A) for the methyl esters, and hexane—propan-2-ol—methanol—acetic acid (88:7:5:0.1) (solvent B) for the free acids. In all cases elution was isocratic at 1 ml/min.

RESULTS AND DISCUSSION

Derivatisation procedures adopted for the GC—MS analysis of diHETEs in the literature include the use of the methyl ester trimethylsilyl (TMS) ethers [11], the pentafluorobenzyl ester TMS ethers (with negative-ion MS) [12] and the tBDMS derivatives of both the alcohol and carboxylic acid moieties [10]. The hydrogenation of the four double bonds in the diHETEs, advocated in ref. 10, leads to a derivative with two major advantages over the unsaturated molecule; the GC behaviour is greatly improved both in thermal stability and peak shape and the overall sensitivity of a GC—MS assay, monitoring the $[M - 57]^+$ ion of the tri-tBDMS derivative is greatly increased over that obtainable with other derivatives. Using the procedure described in Experimental we have derivatised a series of dilutions of LTB_4 and have detected, by monitoring this ion, amounts below 1 pg injected on-column with a signal-to-noise ratio of greater than 5:1. An identical procedure, when employed for the derivatisation of the synthetic 12,20-diHETE, led to the tri-tBDMS derivative, the mass spectrum of which is shown in Fig. 1. The equivalent fatty acid methyl ester chain length of this product was 33.5 (compared to 31.7 for the derivative of LTB_4). As expected for a tBDMS derivative, the $[M - 57]^+$ ion dominates this spectrum and, of the other ions seen, the two most structurally informative are those at *m/z* 387 and *m/z* 443 which correspond to α-fission adjacent to the derivatised hydroxyl group on C-12. There are no ions in the spectrum diagnostic for the 20-OH group, and it is not possible to distinguish between,

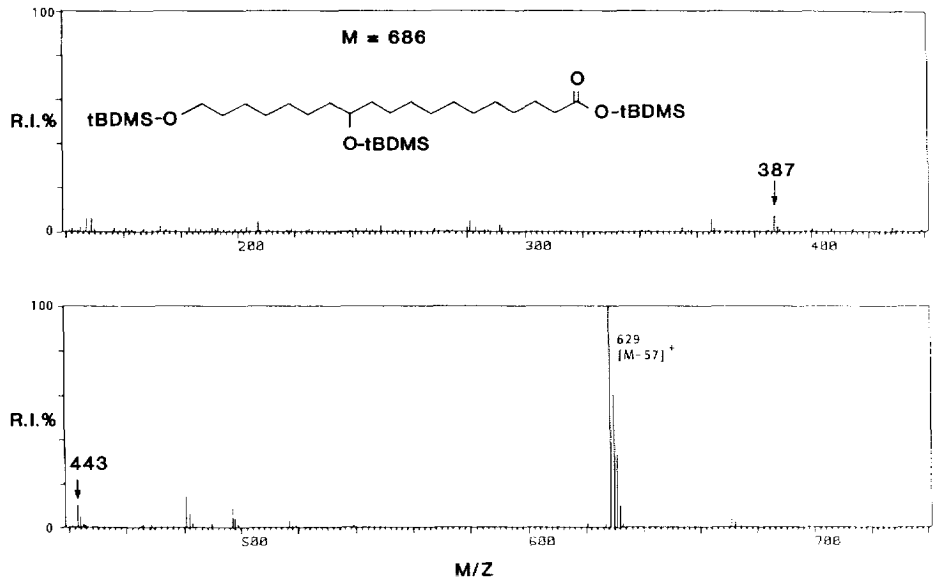

Fig. 1. 40-eV Mass spectrum of 12,20-diHETE after reduction and derivatisation as the tri-tBDMS ether-ester.

for instance, the C-19 and the C-20 hydroxylated metabolites, from the mass spectrum alone.

In their original report Wong et al. [7] described the oxidation of 12,20-diHETE with chromic acid—acetic acid to produce the 12-keto dicarboxylic acid from the mass spectrum of which they were able to obtain an unambiguous structure. We have used an alternative procedure which gives better sensitivity at low concentrations. Guindon et al. [9] reported the development of a *tert.*-butyl-substituted silylation reagent, tBMPSBr which, when reacted in dichloromethane solvent with triethylamine as catalyst, produced the tBMPS ether of primary alcohols, but left secondary and tertiary hydroxyl groups unsubstituted. Hydrogenated 12,20-diHETE was first esterified with diazomethane and then treated with the Guindon reagent. The product was then dissolved in BSTFA and this produced the expected methyl ester, TMS ether, tBMPS ether, the mass spectrum of which is shown in Fig. 2. This spectrum showed a prominent $[M - 57]^+$ ion at *m/z* 565, a smaller one at $[M - 31]^+$ and a base peak, following α-fission next to the 12-OH group, at *m/z* 301. The equivalent chain length of this derivative was 33.3. 5,12-diHETE when treated under the same conditions produced only the methyl ester, di-TMS ether, as would be expected for a product with no primary alcohol groups.

We have tested the method on the product of PMN-mediated oxidation of 12-HETE. The mixed cell suspension incubated with [^{3}H]arachidonic acid and an incubation of PMNs alone, with [^{3}H]12-HETE, each gave a labelled product with UV absorption at 235 nm which, after methylation, eluted at 23 min on HPLC using solvent A. Under the same conditions authentic LTB_4 methyl ester eluted at 28.8 min. The relative elution times for the labelled product and for LTB_4 are the same as those reported by Wong et al. [7]. When

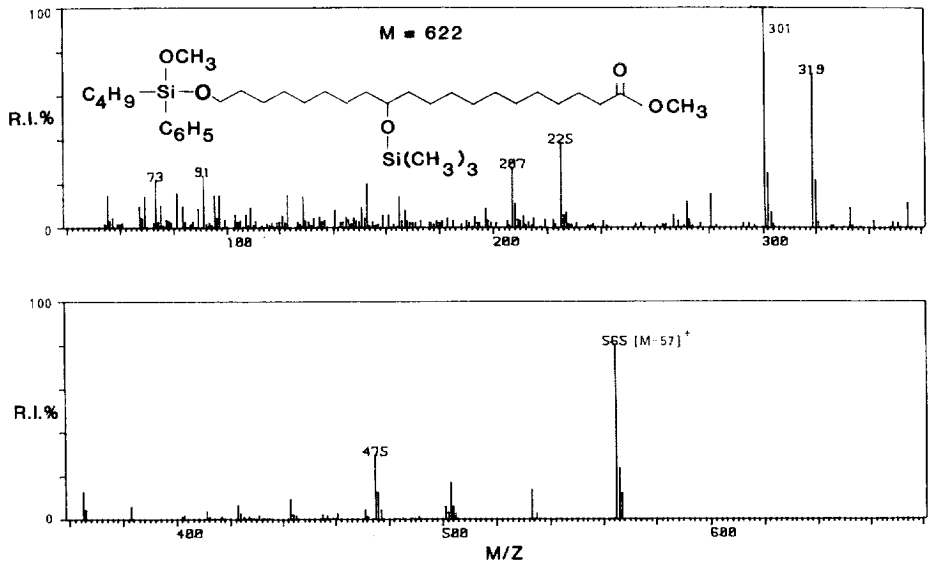

Fig. 2. 40-eV Mass spectrum of 12,20-diHETE after reduction and derivatisation as the methyl ester, TMS ether, tBMPS ether.

chromatographed as the free acid, with solvent B, a radioactive and UV-absorbing product from both the mixed cell and the PMN alone incubations chromatographed at 13.6 min, a time identical to that for authentic 12,20-diHETE and different to those for LTB_4 (16.4 min) and 5(*S*),12(*S*)-diHETE (15.4 min).

Part of this product, as the free acid, was reduced and derivatised as described in Experimental to the tri-tBDMS derivative and analysed by GC–MS, monitoring the response at *m/z* 629 and also at *m/z* 387 and 423. A further sample was also reduced and derivatised as the methyl ester, TMS ether, tBMPS ether and analysed by monitoring at *m/z* 565. The chromatographic tracings (Figs. 3 and 4) show clear evidence for the elution of material at the correct retention times.

This paper describes a procedure for the rapid and sensitive detection of diHETEs in biological fluids. A rigorous quantitative assay would require the addition of an isotopically substituted internal standard and the di-[^{18}O]-carboxylic acid as described by Strife and Murphy [13] is the obvious candidate.

All of the reactions can be carried out on low picogram quantities of material without excessive losses because the number of transfers of solutions from one vial to another has been minimised. The reduction procedure does not appear to produce more than 5% of hydrogenolysis products and the overall recoveries for LTB_4 through the reduction and tri-tBDMS derivatisation procedures are over 75%. The limitation of the process lies in the size and involatility of the tBDMS products and the consequent length of the retention times for these derivatives on GC. In spite of the high GC column oven temperatures employed, no decomposition has been observed in the peak shapes eluting from the GC–MS system and the upper temperature limit of 330°C of most fused-silica SE-30 GC columns makes this technique appear

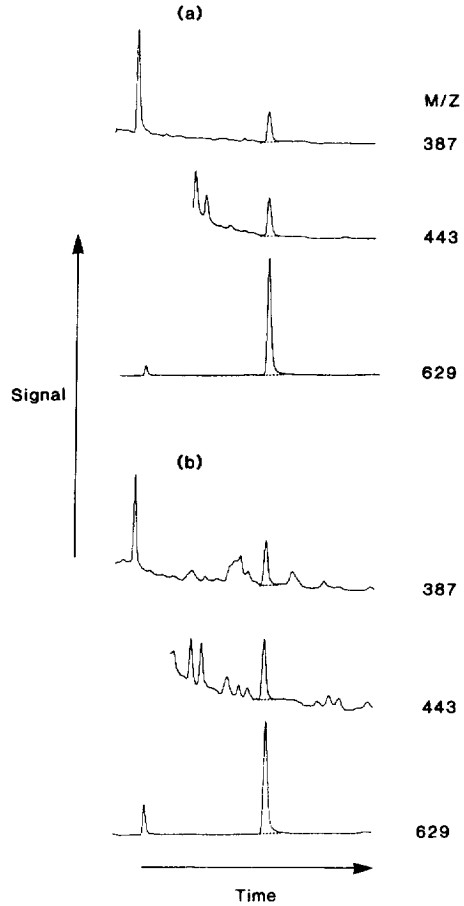

Fig. 3. Selected-ion recording traces of (a) synthetic and (b) biologically produced 12,20-diHETE after reduction and tBDMS derivatisation. Monitored at *m/z* 629, 443 and 387.

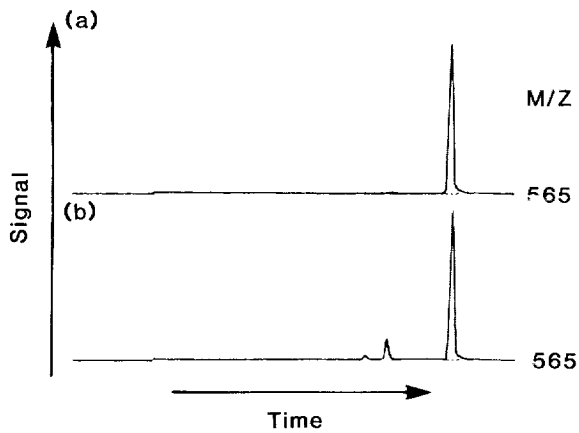

Fig. 4. Selected-ion recording traces of (a) synthetic and (b) biologically produced 12,20-diHETE after reduction and methyl ester, TMS and TBMPS derivatisation. Monitored at *m/z* 565.

applicable to similar derivatives of the hydroxylated metabolites of unsaturated fatty acids up to C_{26} in chain length.

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