Journal of Chromatography, 306 (1984) 1—21
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1988

LIPOXYGENASE PRODUCTS

A NOVEL GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC ASSAY FOR MONOHYDROXY FATTY ACIDS

P.M. WOOLLARD* and A.I. MALLET

Institute of Dermatology, University of London, Homerton Grove, London E9 6BX (U.K.)

(First received August 1st, 1983; revised manuscript received October 21st, 1983)

SUMMARY

We present a novel mass spectrometric method for the profiling of monohydroxy fatty acids and illustrate its use for the analysis of lesional skin of patients with psoriasis.

By combination of vapour phase hydrogenation and gas chromatography—mass spectrometry with selected ion monitoring the position of the substituted hydroxyl group of each fatty acid was determined in their methyl ester trimethylsilyl ether derivatives. Reduction was instantaneous and quantitative allowing detection of less than 100 picogram of each compound. Biological extracts, derivatised as methyl ester *tert.*-butyldimethylsilyl ethers, were purified by reversed-phase high-performance liquid chromatography. Separation was dependent on carbon chain length and degree of unsaturation, but not on the position of the silyl ether group. Subsequent conversion of the *tert.*-butyldimethylsilyl ethers to trimethylsilyl ethers facilitated detection of each of the positional hydroxyl isomers as described. Distinction between double bond isomers was possible when they were separated on gas chromatography prior to reduction.

INTRODUCTION

Unsaturated fatty acids are metabolised by lipoxygenase enzymes to their respective hydroperoxide products. Subsequent enzymic or non-enzymic events lead either to the formation of further oxidative products or to their reduction to monohydroxylated fatty acids.

Both mammalian and plant enzymes exhibit varying degrees of specificity for a wide range of fatty acid substrates and the pattern of monohydroxylated metabolites produced tends to be characteristic of the tissue in which it is found. Whereas the major product of lipoxygenase action in human platelets is 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) [1], human eosinophils

0378-4347/84/$03.00

produce mainly 15-HETE [2], while human neutrophils produce 12-, 9-, 8- and 5-HETEs [3]. The availability of more than one substrate may also lead to the formation of a variety of metabolites. Rabbit polymorphonuclear leukocytes convert arachidonic acid primarily into 5-HETE, while 8-hydroxy-9,11,14-eicosatrienoic acid is the major product when dihomo-gamma-linolenic acid is utilised [4].

Changes in lipoxygenase activity have been demonstrated in certain human inflammatory diseases. In particular, patients with the common skin disorder, psoriasis, show significantly increased concentrations of certain monohydroxy fatty acids in their lesional as opposed to uninvolved skin [5, 6]. Since we have found that human skin produces several monohydroxylated products derived from at least three precursor fatty acids [6], we were interested in profiling metabolites from these and other possible precursors in patients with psoriasis and in unaffected individuals. The significance of these hydroxy fatty acids is unclear, but several metabolites derived from arachidonic acid have been shown to be chemotactic and chemokinetic for human neutrophils [7], the infiltration of which has been implicated in the pathogenesis of the disease [8]. As a large number of precursor fatty acids have been shown to be present in human skin [9] stimulation of lipoxygenases in normal or pathological conditions may result in the formation of a wide range of hydroxylated products.

For the screening of monohydroxy fatty acids in lesional psoriatic skin, published methods lack the required specificity: we therefore describe a novel gas chromatography—mass spectrometric (GC—MS) assay with improved selectivity and sensitivity capable of measuring a wide range of monohydroxy fatty acids.

EXPERIMENTAL

Materials

All unsaturated fatty acids and N,O-bis-trimethylsilyl trifluoroacetamide (BSTFA) were purchased from Sigma London (Poole, U.K.). 12-Hydroxystearic acid and ricinoleic acid were supplied by Applied Science Labs. (Chester, U.K.). All other hydroxy fatty acids were synthesised by a photo-oxidative method previously described [6] and outlined below.

[1-^{14}C] Arachidonic acid was supplied by Amersham International (Amersham, U.K.) and was used for the synthesis of radioactive hydroxy fatty acids [6]. The *tert.*-butyldimethylchlorosilane, imidazole, tetrabutylammonium fluoride, dimethylformamide, tetrahydrofuran and *tert.*-butylmethyl ether (Fluorochem) were purchased from Fluka (Glossop, U.K.). Col Treet™ (Regis) was supplied by Phase Separations (Clwyd, U.K.).

The catalysts, palladium(II) chloride and rhodium(III) chloride trihydrate were obtained from Aldrich (Gillingham, U.K.) and the platinum(IV) oxide was from Fluka. The support phases on which the catalysts were coated were Diatomite CQ (100—120 mesh) obtained from Pye Unicam (Cambridge, U.K.) and Chromosorb G HP (100—120 mesh) supplied by Phase Separations.

Methanol and *n*-heptane, which were redistilled from the Analar-grade reagents and the 2,4,4-trimethylpentane were obtained from BDH (Poole, U.K.). All other solvents (HPLC grade) and Fisofluor I scintillation fluid were from Fisons (Loughborough, U.K.).

Preparation of monohydroxy fatty acids

Monohydroxy fatty acids were prepared by photolysis of their respective unsaturated fatty acids using a modification of the method of Porter et al. [10] as described previously [6]. Briefly, each fatty acid in solution in methanol containing 0.01% methylene blue (w/v) was irradiated with three 500-W Phillips PF 318 P2/3 bulbs, the reaction being cooled (less than 10°C) using a circulating water jacket. The hydroperoxides so formed were reduced with sodium borohydride and the products were subjected to preliminary purification on Lipidex 5000^{TM} (100 ml bed volume; 20 cm column length) (Packard, Reading, U.K.) reversed-phase gel partition chromatography using methanol—water—1,2-dichloroethane—acetic acid (750:150:150:1), from which the monohydroxy fatty acid fraction was collected. Isolation of the positional isomers was achieved by using a semipreparative Spherisorb S5W straight-phase high-performance liquid chromatography (HPLC) column (25 cm × 8 mm) eluted with hexane—propan-2-ol—methanol—acetic acid (975:22:26:1). Where individual components co-eluted, separation was attained, where possible, by adjusting the solvent system. 14-HETE, which is not separated from 15-HETE using the above solvent system, was purified by using the same solvents in the proportion 1070:5:15:1. Those compounds containing a conjugated diene were detected by UV absorbance at 235 nm; 14-HETE was detected at 205 nm.

Extraction

Samples of psoriatic scale (25 mg) obtained by gentle abrasion of skin lesions were vortexed with a mixture of sodium acetate buffer (0.1 *M*, pH 3.5, 3 ml) and ethyl acetate (3 ml). After centrifugation the organic phase of each sample was removed and the process was repeated with an equal volume of ethyl acetate. The pooled organic phase was evaporated under nitrogen. Non-polar lipids were then removed by partition of the residue between methanol (3 ml) and *n*-heptane (4 ml), the heptane layer being discarded. The methanol was evaporated prior to derivatisation. (See Fig. 1, step 1.)

Methylation

Methyl esters were prepared by reacting the methanolic residues twice (Fig. 1, step 1) with methanolic—ethereal (1:9) diazomethane (100 μl). (See Fig. 1, step 2.)

tert.-Butyldimethylsilyl ether formation

tert.-Butyldimethylsilyl (tBDMS) ether derivatives were prepared using a solution of *tert.*-butyldimethylchlorosilane (1 *M*) and imidazole (0.5 *M*) in dimethylformamide (100 μl), a modification of the reagent described by Corey and Venkateswarlu [11]. The reaction was carried out at 40°C for 30 min when it was terminated by addition of water (100 μl). Fatty acid methyl esters and methyl ester, tBDMS ether derivatives were extracted twice into hexane (2 × 1 ml). The pooled hexane phase (2 ml) was then washed with an equal volume of water to remove traces of imidazole prior to evaporation of the solvent and HPLC of the derivatives. (See Fig. 1, step 2.)

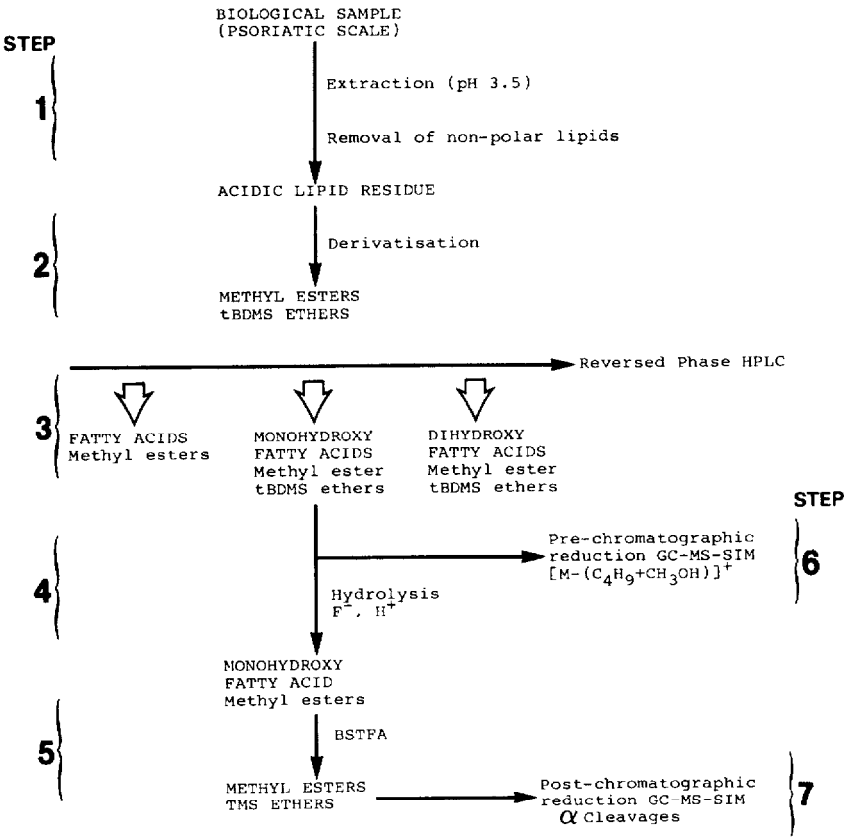

Fig. 1. Flow diagram of assay procedure.

Reversed-phase high-performance liquid chromatography

This was carried out on a Spherisorb analytical S5 ODS column eluted with methanol—water (925:75) at a flow-rate of 1 ml/min. Effluent was collected at the relevant times (see Results) and the solvent removed by vortex evaporation at 40° C. (See Fig. 1, step 3.)

Hydrolysis of tert.-butyldimethylsilyl derivatives

The methyl ester tBDMS ether derivatives purified by HPLC were treated with a solution of tetrabutylammonium fluoride (100 m*M*, 100 μl) at 40° C for 1 h; sodium acetate buffer (0.1 *M*, pH 3.5, 200 μl) was added and the hydrolysis products extracted twice into *tert.*-butylmethyl ether (2 × 500 μl). After evaporation of the pooled organic phase (1 ml) the products were rederivatised as the trimethylsilyl (TMS) ethers (Fig. 1, step 5.)

Alternatively a solution containing acetic acid—methanol—water (2:2:1, 100 μl) was used as the hydrolytic reagent. The reaction was carried out at 40° C overnight. The products were diluted with water (200 μl) and extracted as above. (See Fig. 1, step 4.)

Trimethylsilyl ether formation

Following hydrolysis of the tBDMS ether derivatives, TMS ether derivatives

were formed by addition of BSTFA (25 μl) to the hydroxy fatty acid methyl ester residues. (See Fig. 1, step 5.)

Thin-layer chromatography

Thin-layer chromatography (TLC) was carried out on neutral silica gel on glass-backed plates (200 × 50 × 0.25 mm) (Anachem, Luton, U.K.) developed to 150 mm using a solvent system consisting of 2,4,4-trimethylpentane—ethyl acetate (1:1). Radioactive samples applied as a spot (5 mm diameter) were removed in sections (5 mm) after development. Standard samples were visualised with iodine vapour.

Scintillation counting

Liquid scintillation counting was carried out using an LKB 1012 Liquid Scintillation Counter. Radioactive samples (^{14}C) were added to scintillant (3 ml) and the dpm values were obtained after correction for quenching using the automatic external standard channels ratio method and a quench calibration curve. Silica gel sections from TLC plates were added directly to the scintillation vials and counted as described.

Gas chromatography

Packed column chromatography. GC was performed on either a Pye Model 104 Series gas chromatograph with flame ionisation detection or a Pye Model 204 Series gas chromatograph interfaced via a jet separator to the mass spectrometer described below.

Glass columns (1.5 m × 2 mm I.D.) packed with 3% OV-1 on Gas-Chrom Q (100—120 mesh) were employed, except where hydrogen was used as the carrier gas (80 kPa head press), when a narrower glass column (1.5 m × 1.2 mm I.D.) packed with 3% OV-1 on Gas-Chrom Q (100—120 mesh) was used.

Capillary column chromatography. GC was carried out on a fused silica OV-17 coated capillary column (25 m × 0.3 mm) attached to an all-glass dropping needle injector (Chrompack, London, U.K.) using hydrogen as carrier gas (33—167 kPa head press). GC—MS was carried out using the gas chromatograph described above. Where flame ionisation detection was employed GC was carried out on a Packard 428 instrument also attached to a dropping needle injector. In this case helium was used as carrier gas (115 kPa head press).

Hydrogenation

Liquid phase hydrogenation. Hydroxy fatty acid methyl esters (100 μg each) were dissolved in methanol (50 μl). To this a suspension of platinum(IV) oxide (10 mg/ml, 200 μl) was added and after purging with nitrogen the compounds were reduced by bubbling with hydrogen for 2 min. The catalyst was then rapidly sedimented by centrifugation in an Eppendorf 5412 centrifuge at maximum speed for 2 min. The supernatant was removed and evaporated at 40° C prior to formation of either TMS or tBDMS ether derivatives.

Vapour phase hydrogenation. The catalysts were prepared as reported previously [12]. Palladium(II) chloride was dissolved in methanol with the addition of a few drops of conc. hydrochloric acid. A weighed quantity of the

support phase, either Gas-Chrom Q (100—120 mesh) or Chromosorb G HP (Phase Sep, 100—120 mesh) was then added to give the final degree of palladium coating required. Batches of catalyst were prepared with coatings between 1 and 10% by weight. The solvent was removed by rotary evaporation under reduced pressure at 70°C followed by vacuum desiccation. The catalyst was then packed into an empty column, placed in the gas chromatograph oven and activated with hydrogen gas at 250°C for 30—60 min, the effluent being combusted in the flame ionisation detector. The support phase was then deactivated by injection of BSTFA or Col Treet.

Catalytic reduction on the packed column

On-column hydrogenation did not necessitate any reconstruction of the GC apparatus. Reduction was achieved using hydrogen as the carrier gas and a plug of the catalyst was situated at a chosen site on the GC column.

(A) Pre-chromatographic reduction. A plug of catalyst (4 cm) contained by glass wool was placed at the top of the glass column contained in the injector port enabling the temperature of catalysis to be controlled.

(B) Post-chromatographic reduction. In this case the plug of catalyst (10 cm) was placed at the end of the GC column just prior to the interface with the mass spectrometer. (See Fig. 1, step 6.)

Capillary column reduction

On-column hydrogenation employed a catalytic chamber packed with the prepared catalyst which was deactivated with BSTFA before use. The catalytic chamber consisted of a length of glass-lined steel tubing (15 cm × 1.6 mm I.D.) with union fittings (1.6 mm) (SGE, Milton Keynes, U.K.) connecting chromatographic and interface capillary columns. A number of configurations were investigated for interfacing the catalytic chamber and the mass spectrometer two of which are described below:

Configuration 1: introduction of the effluent from the catalytic chamber directly into the source of the mass spectrometer;

Configuration 2: addition of helium make-up gas prior to the catalytic chamber and jet separator interface.

Addition of helium make-up gas at a wide range of flow-rates prior to the catalytic chamber had no detrimental effect on the efficiency of reduction and eliminated any dead space in the catalytic chamber, thus improving peak shape while also providing the neccessary flow through the jet separator. Use of configuration 2 (Fig. 2) enabled optimum conditions of chromatography, recovery and reduction to be achieved. (See Fig. 1, step 7.)

Mass spectrometry

MS was performed on a VG Analytical Model 305 single focusing magnetic sector mass spectrometer. Mass spectra were acquired at an ionising energy of 40 eV with a trap current of 500 μA operating at a source temperature of 200°C. Selected ion monitoring was carried out by switching the accelerating voltage focusing masses between *m/z* 207 and *m/z* 385. The source tuning was relatively unaffected over this range, however at the higher masses monitored the signal was attenuated due to the decrease in ion energy.

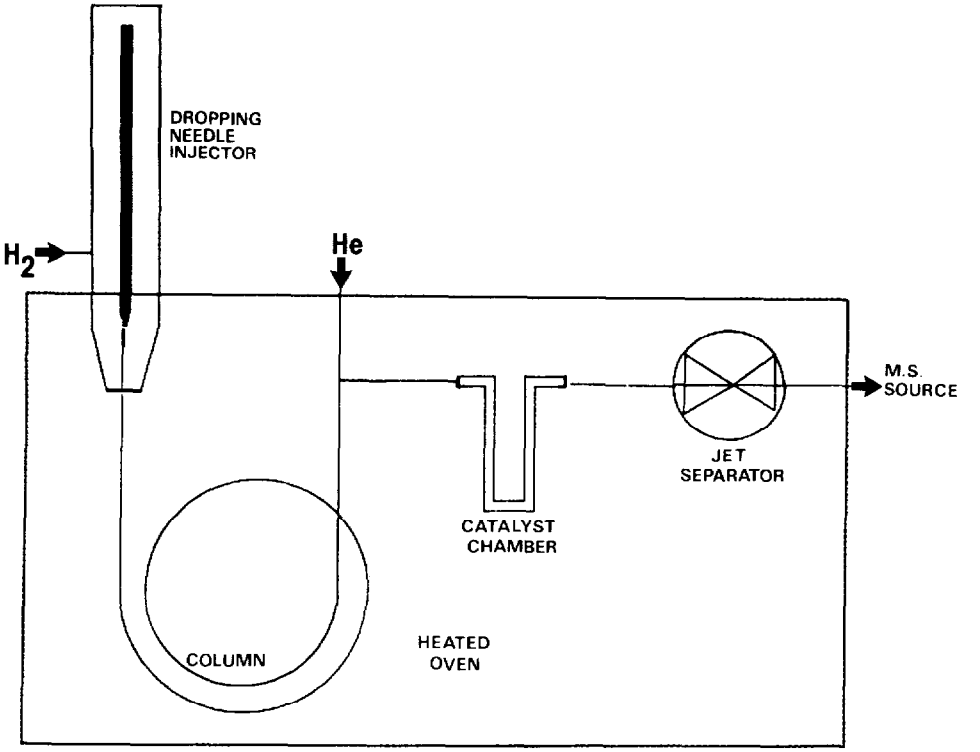

Fig. 2. Scheme of capillary column GC—MS incorporating post-chromatographic reduction (configuration 2).

RESULTS AND DISCUSSION

The electron impact mass spectra of the TMS and tBDMS ether derivatives of hydroxy fatty acids are stongly characterised by the presence or absence of unsaturation within the molecule [13]. Both derivatives of the unsaturated methyl esters predominantly yield a single intense ion in their mass spectra due to α-cleavage at the ether group, as illustrated by these derivatives of 12-HETE (Fig. 3a and b), however two ions could be expected to be produced by this fission. That only one of the α-cleavage fragments is usually present is a reflection of the greater resonance stability gained by formation of that ion in conjugating with the diene in the hydrocarbon chain.

On hydrogenation of the molecule however, the methyl ester TMS ether derivatives yield both α-cleavage fragment ions (Fig. 3c), widely used determinants for the position of the hydroxyl substitution in the parent molecule. In contrast, the methyl ester tBDMS ether derivatives of the saturated compounds exhibit characteristically intense high mass ions where the $[M-(C_4H_9 + CH_3OH)]^+$ ion is usually the base peak (Fig. 3d) [13]. We therefore decided that reduction of the methyl ester TMS ether derivatives would allow specific analysis of fatty acids substituted at any position on the carbon chain.

Such a procedure has previously been demonstrated [14], where reduction was carried out prior to GC—MS. However, when a number of unsaturated

fatty acid precursors are available, as occurs in human skin, it is important to eliminate the possibility that hydroxy fatty acids with differing degrees of unsaturation will be reduced to form identical compounds.

Sample preparation

Conversion of the polar lipid fraction derived from psoriatic scale as described above, into the methyl ester tBDMS ethers, transformed both fatty acids and their hydroxylated metabolites into non-polar derivatives which were stable to the reversed-phase HPLC system used.

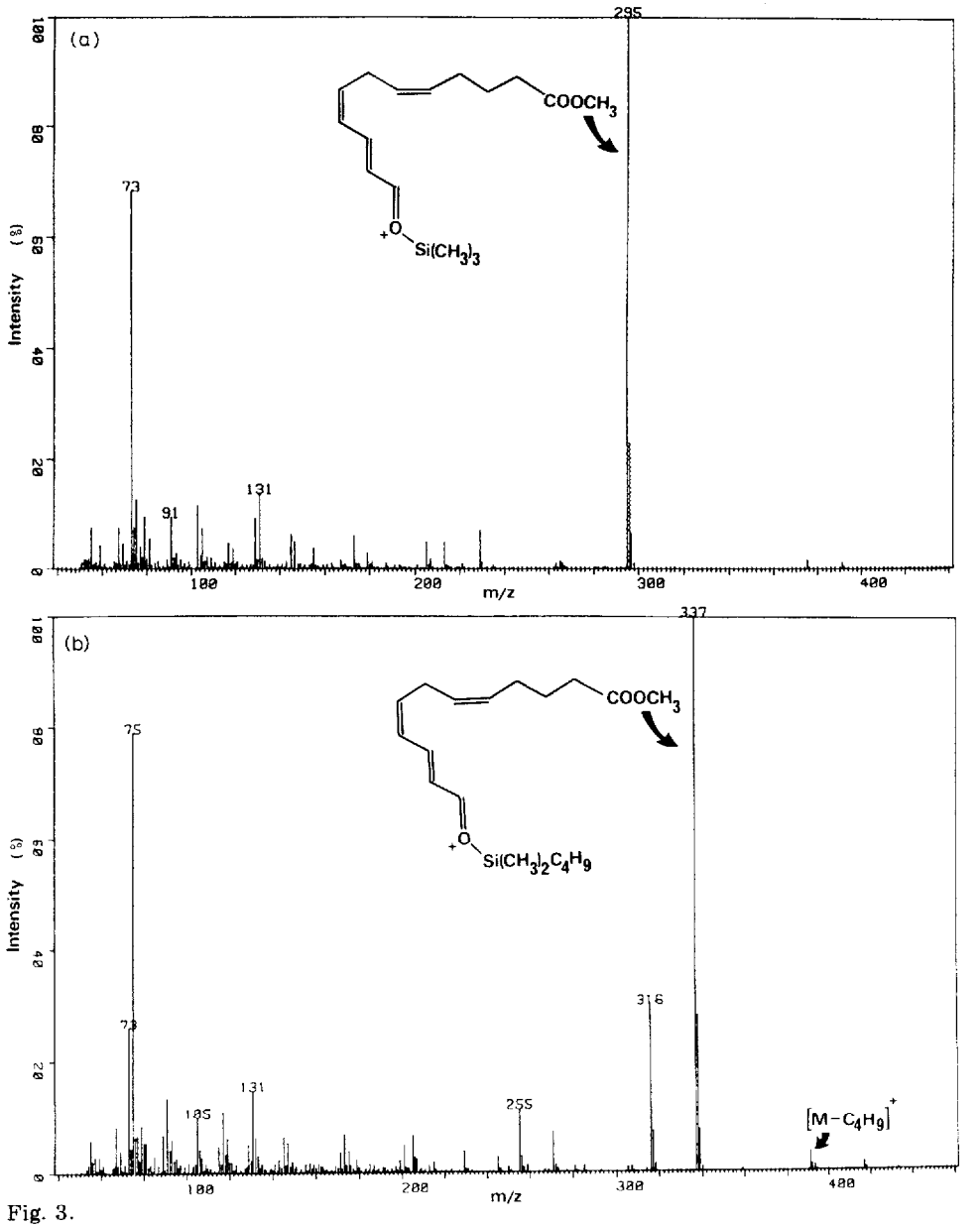

Fig. 3.

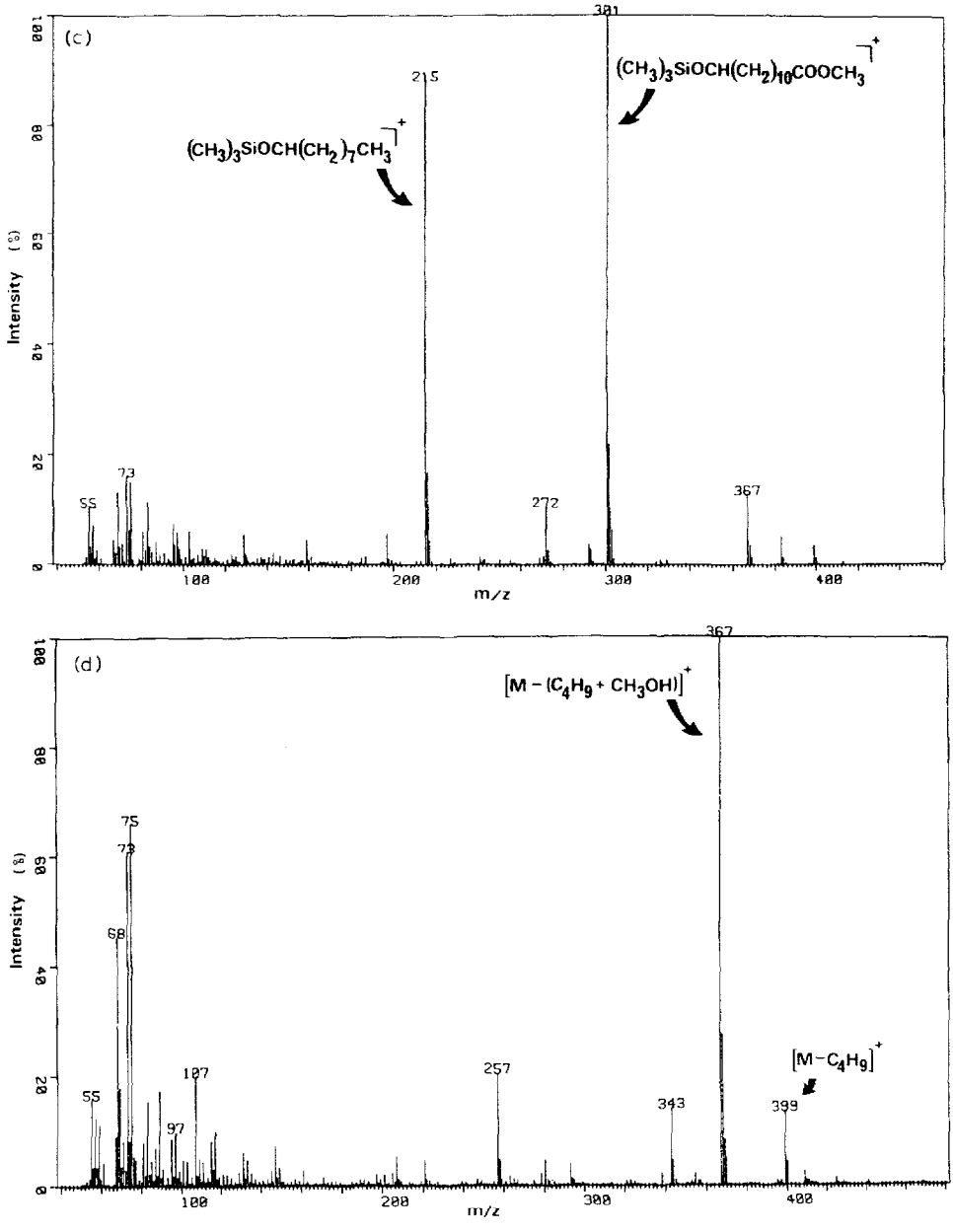

Fig. 3. Mass spectra of 12-HETE derivatives: (a) methyl ester TMS ether, (b) methyl ester tBDMS ether; and after hydrogenation of the double bonds, (c) methyl ester TMS ether, (d) methyl ester tBDMS ether.

Subjection of the derivatives obtained from 25 mg of scale to reversed-phase HPLC yielded UV absorbance profiles, one of which is shown in Fig. 4. The retention characteristics of the methyl ester tBDMS ether derivatives were influenced only by those factors affecting their polarity and were based therefore on three criteria: (1) the number of carbon atoms in the fatty acid

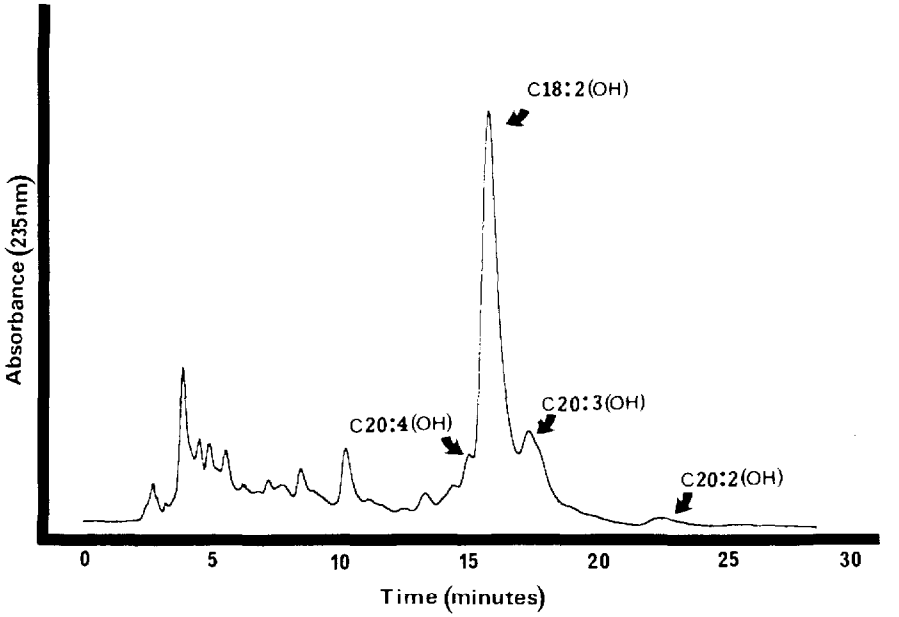

Fig. 4. HPLC profile of the methyl ester tBDMS ether derivatives of the organic extract from 25 mg of lesional psoriatic scale. UV absorbance was monitored at 235 nm. The labelled peaks indicate the identity of the mixture of derivatised hydroxy fatty acid positional isomers (OH) differing in carbon chain length (C_{20} and C_{18}) and number of double bonds (:2, :3, :4). HPLC was carried out on an analytical Spherisorb S5 ODS column using as the mobile phase methanol—water (925:75).

skeleton; (2) the number of double bonds in the fatty acid skeleton; (3) the number of substituted hydroxyl groups present.

The position of the substituted hydroxyl group had no significant effect on the retention time. This enabled collection of all the monohydroxylated metabolite derivatives from any particular fatty acid precursor in a single fraction. Separation based on the degree of unsaturation was especially important where the oxidation products of $C_{20:4}$, $C_{20:3}$ and $C_{20:2}$ fatty acids were present. In addition the separation of the double bond congeners was governed by an exponential relationship between the number of double bonds and the retention time (Fig. 5). From this relationship the retention time of any chosen monohydroxylated derivative could be predicted. The derivatives of the 8,11,14- and 11,14,17-eicosatrienoic acid metabolites, differing only in the position of their double bonds were partially separated by HPLC and were collected in the same fraction. It was also convenient to collect in one fraction the metabolite derivatives of arachidonic and linoleic acids.

Analysis of high-performance liquid chromatography fractions

GC—MS analysis of the effluent HPLC fractions could be carried out on the methyl ester tBDMS ether derivatives or after removal of the tBDMS group using tetrabutylammonium fluoride and re-derivatisation as TMS ethers. In either case the use of psoriatic scale led to the formation of mixed mass spectra as shown for the methyl ester tBDMS ether derivatives of 9- and 13-hydroxy-octadecadienoic acid (HODD) (Fig. 6). The C_{20} hydroxy fatty acid derivatives,

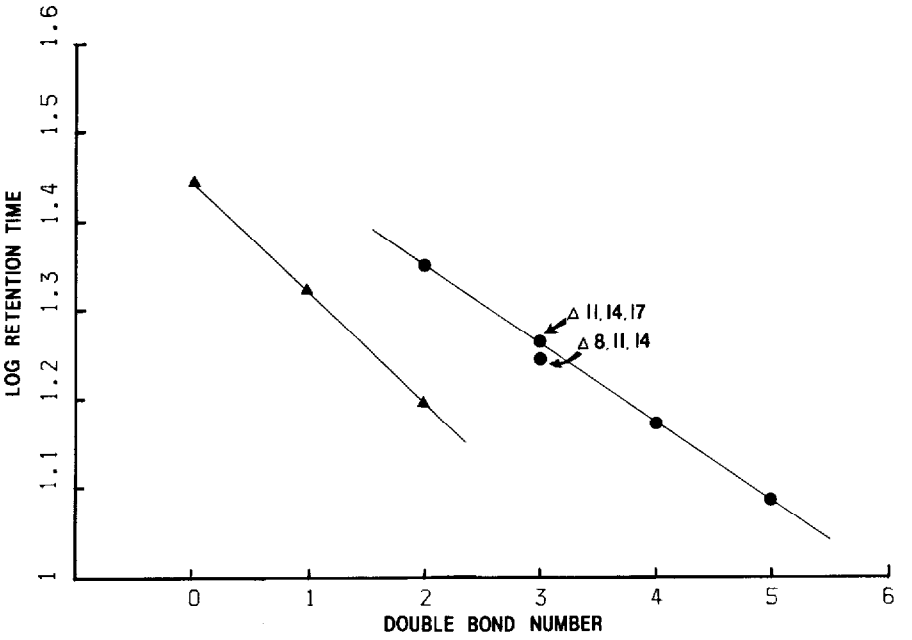

Fig. 5. Relationship between the log retention time (HPLC) and the number of double bonds in the carbon chain of the methyl ester tBDMS ether derivatives of C_{18} (▲) and C_{20} (●) hydroxy fatty acids. Derivatives containing conjugated double bonds were monitored for UV absorbance at 236 nm. Ricinoleic acid ($C_{18:1}$) and 12-hydroxy stearic acid ($C_{18:0}$) derivatives were detected by GC analysis of the HPLC fractions.

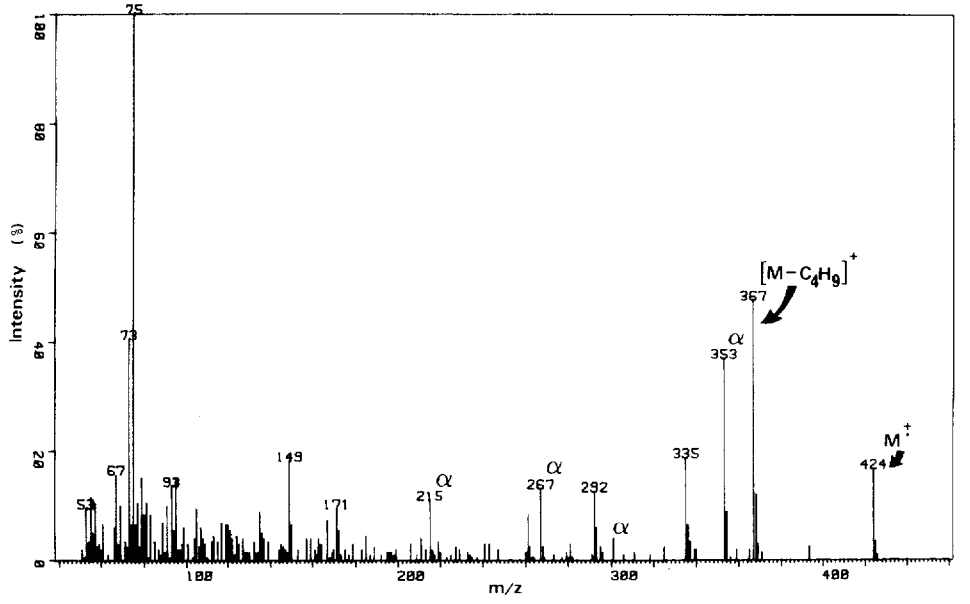

Fig. 6. Mixed mass spectrum of the $C_{18:2}$ (OH) fraction (Fig. 4) containing the methyl ester tBDMS ether derivatives of 9- and 13-hydroxy octadecadienoic acid (HODD). α-Cleavage ions are present at *m/z* 267 and *m/z* 301 (9-HODD) and *m/z* 215 and *m/z* 353 (13-HODD).

however, were generally present in a concentration too low for the acquisition of their full mass spectra.

An HPLC fraction containing the precursor fatty acids as their methyl esters could also be obtained, but its analysis will not be reported in this paper.

Hydrolysis of tert.-butyldimethylsilyl derivatives

The necessary conditions for hydrolysis of the tBDMS group were dependent on the presence of unsaturation in the hydrocarbon chain. Tetrabutylammonium fluoride in tetrahydrofuran (100 m*M*) achieved rapid quantitative removal of the tBDMS group in all of the $C_{20:4}$ ether derivatives except for 5-HETE where the reaction resulted in the formation of an unstable unidentified product. The ease of hydrolysis was ranked as follows: 8- and 12-HETE > 9- and 11-HETE > 14- and 15-HETE, with the reaction for all HETE compounds being complete within 45 min at 40°C. The removal of the tBDMS group of 12-hydroxystearic acid methyl ester was considerably less effective with this reagent, while increasing the concentration of tetrabutylammonium fluoride (1 *M*) caused concomitant removal of the methyl ester group. The use of acid hydrolysis at 40°C overnight with the reagent described was more successful for the saturated derivative with no apparent losses occurring, however for the $C_{20:4}$ ether derivatives hydrolysis was not quantitative. (See Fig. 1, step 4.)

Recovery of hydroxy eicosatetraenoic acids

Psoriatic scale samples (25 mg) spiked in duplicate with [1-^{14}C]12-HETE (23 ng, 58 mCi/mmol), [1-^{14}C]8-HETE (32 ng, 58 mCi/mmol), and [1-^{14}C]5-HETE (31 ng, 58 mCi/mmol) were processed through the assay system and analysed after HPLC (Fig. 1, step 3). One sample of each HETE derivative was subjected to scintillation counting, while the duplicate was hydrolysed with tetrabutylammonium fluoride (12-HETE and 8-HETE) or acetic acid reagents (5-HETE) (Fig. 1, step 4). These hydrolysates were then subjected to TLC and subsequent scintillation counting. Total percentage recovery of radioactivity for each compound was as follows: 12-HETE (49.1%, n = 2), 8-HETE (54.9%, n = 2), and 5-HETE (47.8%, n = 2).

Hydrolysis of 8-HETE and 12-HETE tBDMS derivatives was shown to be virtually quantitative by TLC, less than 0.5% of the silyl derivatives remaining. For the 5-HETE derivative only 58% of the recovered radioactivity was hydrolysed under acid conditions. In addition, hydrolysis was studied using GC—flame ionisation detection (FID). Amounts (approx. 2.5 μg each) of [1-^{14}C]HETE methyl ester tBDMS ether derivatives (40.6—87 μCi/mmol) were hydrolysed under both conditions described, before subjection to scintillation counting or reaction with BSTFA. Recovery of radioactivity was greater than 93% (average 97.8 ± 1.3%, n = 12, mean ± S.E.M.) for all HETEs when hydrolysed with tetrabutylammonium fluoride. GC—FID analysis after reaction of the hydrolytic products with BSTFA showed no tBDMS derivative remaining and indicated quantitative formation of the TMS ethers, except for that of the 5-HETE derivative. Under acid conditions however, hydrolysis of all the hydroxy fatty acid derivatives was shown to be incomplete.

On-column hydrogenation

For all compounds reduced, whether unsaturated fatty acid methyl esters or their hydroxylated TMS and tBDMS ether derivatives complete vapour phase reduction was possible during GC. No significant differences were detected between their mass spectra and those obtained by reduction prior to silylation and GC–MS. In addition vapour phase reduction could be carried out using both packed and capillary columns.

Introduction of the catalyst at the top of the column resulted in immediate reduction upon injection and hence chromatographic separation of the reduced components. No significant increase in retention times was detected whether reduction was carried out on-column or the previously reduced compounds were chromatographed. Thus reduction of the double bonds of the hydroxy fatty acid derivatives removed their effect on the retention characteristics.

By incorporation of the catalyst at the detector end of the column separation of the parent derivatives occurred before their reduction. This was advantageous in that all the chromatographic properties of the parent derivatives were conserved until MS itself. Hydrogenation of a mixture of compounds prior to GC may result in the reduction of more than one component to an identical compound or the formation of complex chromatograms. However, by using GC analysis with and without post-chromatographic reduction, both parent and reduced components can be easily matched from the chromatograms without complication.

The characteristics of vapour phase reduction

Using GC–MS it was possible to monitor simultaneously reduction efficiency, catalyst inactivation, hydrogenolysis, peak shape and absolute sensitivity. Several different configurations of catalyst position, chromatography column and MS interface were used (see Experimental). It was evident from these that the amount of hydrogen present in the catalytic chamber was always in large excess during chromatography. The catalyst, however, was susceptible to coating by chromatographic phase and subsequent inactivation at low palladium coverage (1%). With greater catalyst coverage (up to 10%) the chamber could be re-used many times without loss of catalytic efficiency, especially in combination with the capillary column.

Estimation of catalytic efficiency was carried out by monitoring the α-cleavage ion for 8-HETE methyl ester TMS ether at m/z values between m/z 265 (totally unreduced) and m/z 271 (totally reduced). Post-chromatographic reduction using a wide range of carrier flow-rates and catalyst concentrations resulted in almost total reduction (maximum signal at m/z 271) with a constant residual 3% signal remaining at m/z 269 (one double bond remaining intact).

Employment of palladium as catalyst kept hydrogenolysis of the derivatives to a minimum and mass spectra obtained after post-chromatographic reduction showed insignificant breakdown of the compounds. Similarly pre-chromatographic reduction led to the formation of single peaks without evidence of hydrocarbon products. In contrast, hydrogenation of the HETE methyl esters using PtO_2 in methanol gave 3–10% conversion to arachidic acid methyl ester. Where rhodium (5% coverage) was employed as the vapour phase catalyst, breakdown of the derivatives was considerable.

Gas chromatography—mass spectrometry with selected ion monitoring

Hydroxy fatty acids produced by photo-oxidation were subjected to HPLC as their methyl ester tBDMS ether derivatives. Subsequent GC—MS-selected ion monitoring (SIM) analysis of the $[M-(C_4H_9 + CH_3OH)]^+$ ion after pre-chromatographic reduction gave no distinction between the isomers, but yielded a trace which was the sum of the individual components (Fig. 7). For samples derived from psoriatic skin this information was similar to that obtained after HPLC, however greater sensitivity was obtained from the GC—MS—SIM analysis.

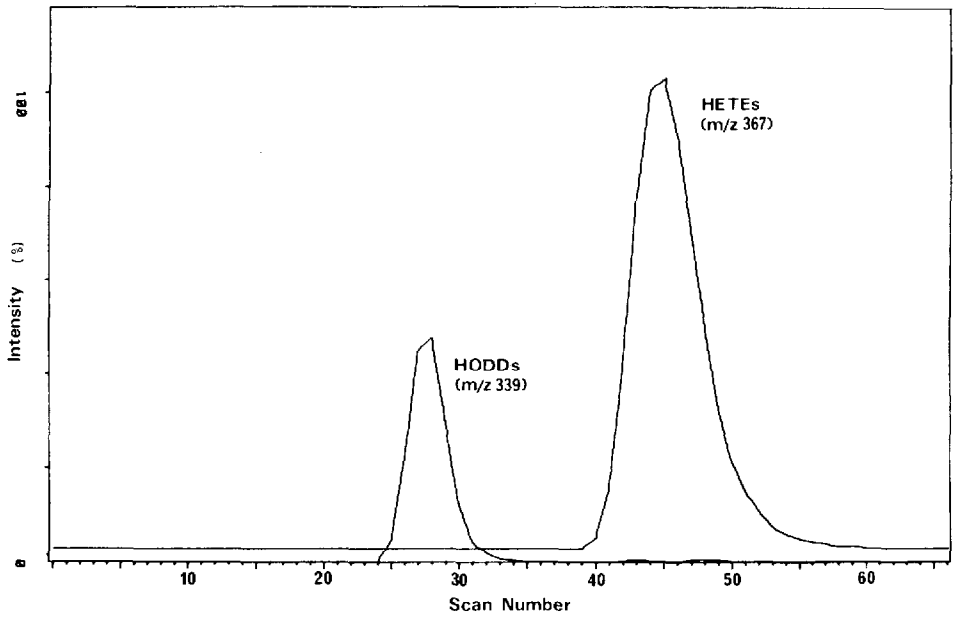

Fig. 7. Ion chromatogram of the $[M-(C_4H_9 + CH_3OH)]^+$ ion obtained for 20 ng of each component: 9- and 13-HODD (*m/z* 339) and 5-, 8-, 9-, 11-, 12-, 14- and 15-HETE (*m/z* 367) after pre-chromatographic reduction. GC was carried out on a packed column (1.5 m × 1.2 mm I.D.) containing 3% OV-1 on Gas-Chrom Q (100—120 mesh). Hydrogen was used as the carrier gas.

On the other hand, information on each of the components was obtained by GC—MS—SIM analysis with post-chromatographic reduction if the methyl ester TMS ether derivatives were used. Identification of the hydroxy fatty acids synthesised from a single precursor was possible simply by monitoring the *m/z* values corresponding to either α-cleavage fragment ion for each derivative (Table I). Scale samples from ten patients with psoriasis were then processed and analysed in the same manner. GC—MS—SIM analysis of the HPLC fraction containing the HODD and HETE compounds is illustrated in Fig. 8. Ion traces for up to ten derivatives could be obtained from a single injection and in almost every case noise levels were very low and chromatograms were free of interfering peaks.

Semi-quantitative data obtained from this analysis were similar to that obtained from an earlier study [6]. 13-HODD was present at highest concentrations (13.8 ± 5.3 ng/mg scale, $n = 10$; mean ± S.E.M.) with lower 9-HODD

TABLE I

EQUIVALENT CHAIN LENGTHS OF THE METHYL ESTER TMS ETHER DERIVATIVES OF HYDROXY FATTY ACIDS

Hydroxy fatty acid	Equivalent chain lengths		Ions monitored (*m/z*)
	OV-1 packed	OV-17 capillary	
12(OH) C20 Δ5,8,10,14	21.3	21.8	301, 215
14(OH) C20 Δ5,8,11,15	21.2	21.4	329, 187
15(OH) C20 Δ5,8,11,13	21.3	21.8	343, 173
11(OH) C20 Δ5,8,12,14	21.3	21.8	287, 229
9(OH) C20 Δ5,7,11,14	21.3	21.8	259, 257
8(OH) C20 Δ5,9,11,14	21.3	21.8	271, 245
5(OH) C20 Δ6,8,11,14	21.4	21.8	313, 203
12(OH) C20 Δ8,10,14	21.5	21.9	301, 215
14(OH) C20 Δ8,11,15	21.3	21.5	329, 187
15(OH) C20 Δ8,11,13	21.5	21.9	343, 173
11(OH) C20 Δ8,12,14	21.6	21.9	287, 229
8(OH) C20 Δ9,11,14	21.6	21.9	271, 245
12(OH) C20 Δ10,14,17	21.6	21.8	301, 215
15(OH) C20 Δ11,13,17	21.8	22.3	343, 173
14(OH) C20 Δ11,15,17	21.8	22.2	329, 187
17(OH) C20 Δ11,14,18	21.8	22.0	371, 145
18(OH) C20 Δ11,14,16	22.2	22.6	385, 131
11(OH) C20 Δ12,14,17	21.9	22.2	287, 229
12(OH) C20 Δ10,14	21.7	21.7	301, 215
14(OH) C20 Δ11,15	21.7	21.7	329, 187
15(OH) C20 Δ11,13	21.9	22.0	343, 173
11(OH) C20 Δ12,14	21.9	22.0	287, 229
12(OH) C18 Δ9,13	19.7	19.7	301, 187
13(OH) C18 Δ9,11	19.9	20.0	315, 173
9(OH) C18 Δ10,12	19.9	20.0	259, 229
12(OH) C18 Δ9	19.8	—	301, 187
12(OH) C18 —	20.1	—	301, 187

concentrations (5.5 ± 1.8 ng/mg scale, n = 10; mean ± S.E.M.). 12-HETE was the most abundant metabolite of arachidonic acid (0.73 ± 0.3 ng/mg scale, n = 10; mean ± S.E.M.) and the rank order of concentration was as follows: 12-HETE > 8-HETE > 15-HETE ≈ 9-HETE > 11-HETE.

Quantification

Stable isotope labelled compounds have frequently been used as internal standards in the quantification of the metabolites of arachidonic acid. Deuterium-labelled HETE analogues are conveniently synthesised by photo-oxidation of [5,6,8,9,11,12,14,15-octadeutero]-arachidonic acid [15];

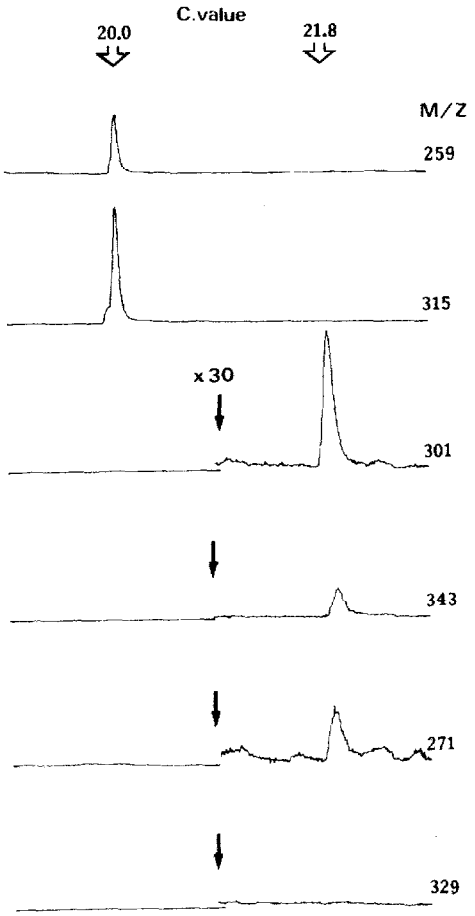

Fig. 8. GC—MS—SIM analysis of the methyl ester TMS ether derivatives of the HODD [$C_{18:2}$ (OH)] and HETE [$C_{20:4}$ (OH)] HPLC fraction (Fig. 4), obtained from 25 mg of psoriatic scale. Post-chromatographic reduction was carried out using a capillary column in configuration 1 (Experimental) with hydrogen as carrier gas. Equivalent chain lengths (C value) of peaks are indicated and ions monitored correspond to one α-cleavage fragment for each derivative: *m/z* 259, 9-HODD; *m/z* 315, 13-HODD; *m/z* 301, 12-HETE; *m/z* 343, 15-HETE; *m/z* 271, 8-HETE; *m/z* 329, 14-HETE (absent). Data were obtained from a single injection.

however where catalytic reduction is employed they are not suitable owing to exchange of the deuterium atoms during hydrogenation of the double bonds.

Given that the hydroxy fatty acid derivatives were purified by HPLC in a batchwise fashion it was desirable to choose a single internal standard for each fraction collected. For the HETE and HODD fraction, the choice of 14-HETE as a suitable internal standard was made after the scrutiny of scale samples from the ten patients studied. In none of these samples was 14-HETE detected and it is therefore unlikely to be produced in lesional skin (Fig. 8). Further the lack of a conjugated diene and thus the absence of the UV chromophore (λ_{max} 236) monitored during HPLC enabled all quantitative information from

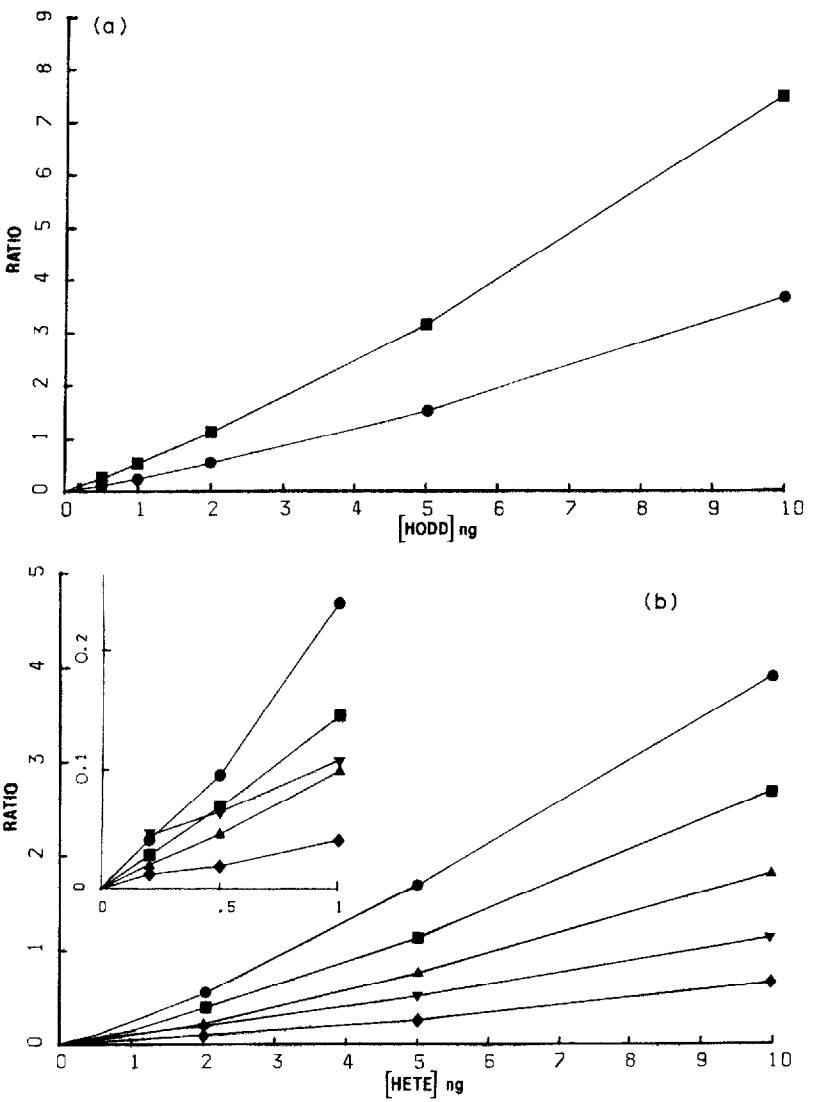

Fig. 9. GC—MS—SIM calibration curves obtained using post-chromatographic reduction (Fig. 2, configuration 2). Curves were obtained from duplicate solutions containing the methyl ester TMS ether derivatives of 14-HETE (10 ng) per injection (3 μl) against (a) 9- (■) and 13-HODD (●), 0.2—10 ng and (b) 5- (◆), 8- and 12- (▲), 9- (●), 11- (■) and 15-HETE (▼), 0.2—10 ng. The ions were monitored after a single injection of each solution as indicated in Table I. A trace amount of 15-HETE in the internal standard accounts for the interception of the curve for 15-HETE on the y axis.

this step to be retained. We therefore constructed calibration curves relating responses to varying amounts of each HODD and HETE derivative with responses to a constant amount of 14-HETE derivative (Fig. 9a and b).

Selectivity

The selectivity of the assay is improved by HPLC purification of the metabolites after derivatisation, as this procedure tends to select only those com-

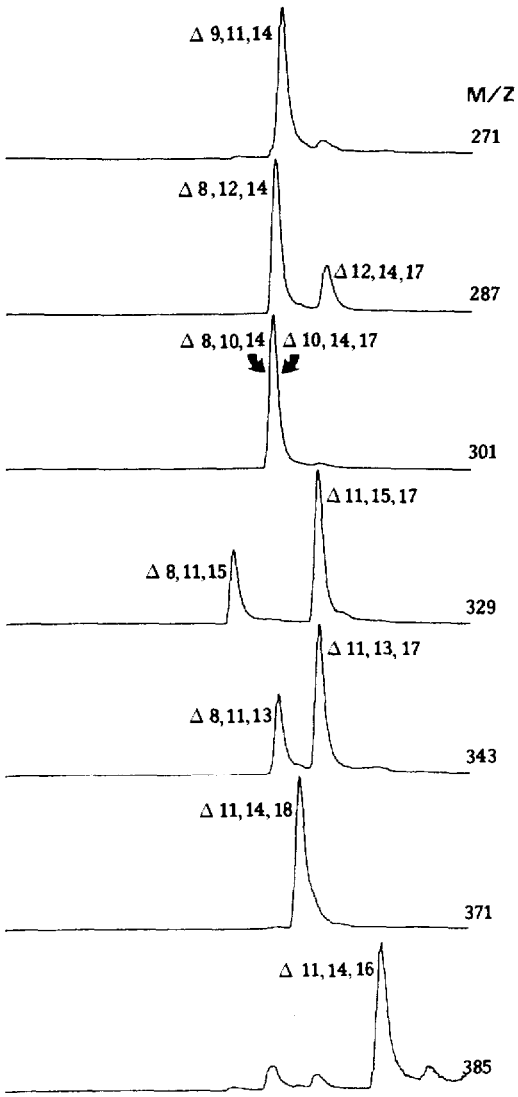

Fig. 10. GC—MS—SIM of the double bond isomers of $C_{20:3}$ hydroxy fatty acid, methyl ester TMS ether derivatives (single injection). GC with post-chromatographic reduction was carried out in configuration 1 (Experimental). The ions monitored correspond to the α-cleavage fragments of compounds in Table I and the labelling of the peaks indicates the positions of the double bonds.

pounds able to form methyl esters and tBDMS ethers. While the HPLC and GC—MS—SIM steps are complimentary in differentiating between degree of unsaturation and positional hydroxyl isomers respectively, the discrimination between double bond isomers hydroxylated in an identical position is dependent on their separation by GC and therefore on the equivalent chain length of their derivatives (Table I). Pre-chromatographic reduction therefore, would not distinguish between them.

Post-chromatographic reduction of those hydroxy fatty acid derivatives

resulting from photo-oxidation of 8,11,14- and 11,14,17-eicosatrienoic acids, as described (Experimental), is illustrated (Fig. 10). In a single injection all the ether derivatives substituted in identical positions were separated by GC with one exception. In this instance, separation was not achieved between the 12-hydroxy-8,10,14- and 12-hydroxy-10,14,17-eicosatrienoic acid derivatives.

Limits of detection

For any given recovery during sample preparation the minimum possible amount of analyte monitored will depend on the signal-to-noise (S/N) ratio for the final detection. As nine or ten ions were monitored in any one injection the signal was attenuated to approximately 1/3 of that value obtained had only one

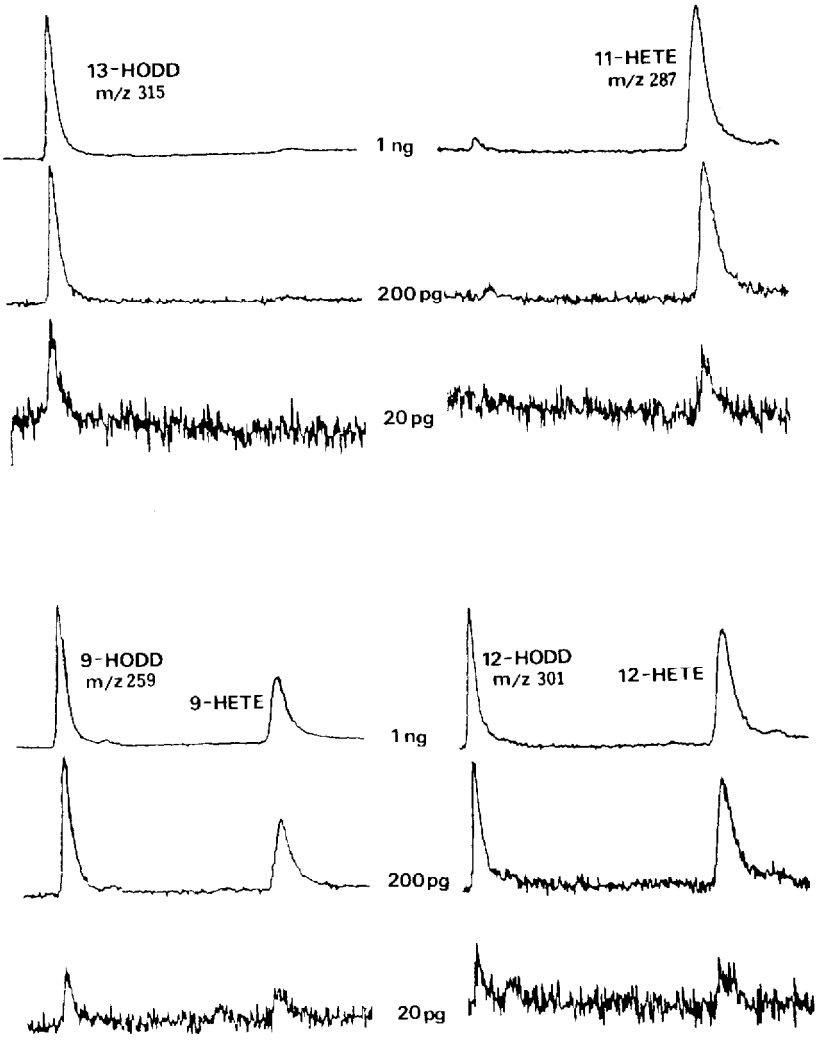

Fig. 11. GC—MS—SIM analysis of the methyl ester TMS ether derivatives of the compounds indicated. GC with post-chromatographic reduction was carried out as in Fig. 2 (configuration 2).

ion been monitored. However, all the compounds examined were detectable at amounts below 200 pg per injection while S/N ratios of approximately 3:1 were obtained for injection of 20 pg of 9- and 13-HODDs and 9-, 11- and 12-HETEs (Fig. 11).

CONCLUSIONS

The ability of the assay to measure the in vivo concentrations of a wide range of metabolites may provide a means of assessing the specificity and efficacy of potential lipoxygenase inhibitors. The predictability inherent in the HPLC purification of the methyl ester tBDMS ether derivatives facilitates screening for particular monohydroxy metabolites or lipoxygenase pathways. In addition, the batchwise collection of groups of derivatives allows the analysis, from a single biological sample, of both the precursor fatty acids and dihydroxy metabolites, such as leukotriene B_4.

This pathway however, presents special problems as demonstrated by the anomolous behaviour of the 5-HETE derivative. The application of this method, in our hands, to the measurement of this compound awaits the successful hydrolysis of the tBDMS ether group. The 5-lipoxygenase pathway is particularly important due to the potent leukocyte stimulating properties of leukotriene B_4 [16], which is derived from 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid, and its association with the disease, psoriasis [17].

Vapour-phase reactions in combination with GC have been exploited in the past as a means of identification. For quantification, this technique may find uses in the analysis of an increasing number of unsaturated compounds, especially where hydrogenation simplifies the mass spectra yielding useful structural information or intense ions suitable for selected ion monitoring detection. In addition on-column catalytic reactions are not limited to hydrogenation, as demonstrated by the vapour-phase oxidation of alcohols to aldehydes [18].

The identity and concentrations of hydroxy fatty acids present in psoriatic skin are at present under investigation. Whereas it has been shown that some monohydroxy eicosatetraenoic acids have chemokinetic and chemotactic properties, it will be of interest to evaluate the biological activity of other monohydroxylated metabolites.

ACKNOWLEDGEMENTS

The authors would like to thank Mr. Roy Clare for his help with the mass spectrometry and acknowledge the Medical Research Council of Great Britain for the purchase of the VG 305 mass spectrometer. In addition thanks are also due to our clinical colleagues for the collection of the psoriatic scale.

REFERENCES

1 M. Hamberg and B. Samuelsson, Proc. Nat. Acad. Sci. U.S., 71 (1974) 3400.
2 J. Turk, R.L. Maas, A.R. Brash, L.J. Roberts, II and J.A. Oates, J. Biol. Chem., 257 (1982) 7068.

3 E.J. Goetzl and F.F. Sun, J. Exp. Med., 150 (1979) 406.
4 P. Borgeat, M. Hamberg and B. Samuelsson, J. Biol. Chem., 251 (1976) 7816.
5 S. Hammarstrom, M. Hamberg, B. Samuelsson, F.A. Duell, M. Stawiski and J.J. Voorhees, Proc. Nat. Acad. Sci. U.S., 72 (1975) 5130.
6 R.D.R. Camp, A.I. Mallet, P.M. Woollard, S.D. Brain, A. Kobza Black and M.W. Greaves, Prostaglandins, 26 (1983) 431.
7 R.D.R. Camp, Clin. Exp. Dermatol., 7 (1982) 435.
8 O. Chowaniec, S. Jablonska, E.H. Beutner, M. Proniewska, M. Jarzabek-Charzelska and G. Rzesa, Dermatologica, 163 (1981) 42.
9 D.I. Wilkinson, Lipids, 7 (1972) 544.
10 N.A. Porter, J. Logan and V. Kontoyiannidou, J. Org. Chem., 44 (1979) 3177.
11 E.J. Corey and A. Venkateswarlu, J. Amer. Chem. Soc., 94 (1972) 6190.
12 M. Beroza and R. Sarmiento, Anal. Chem., 35 (1963) 1353.
13 P.M. Woollard, Biomed. Mass Spectrom., 10 (1983) 143.
14 M. Claeys, M.C. Coene, A.G. Herman, G.H. Jouvenaz and D.H. Nugteren, Biochim. Biophys. Acta, 713 (1982) 160.
15 J.M. Boeynaems, A.R. Brash, J.A. Oates and W.C. Hubbard, Anal. Biochem., 104 (1980) 259.
16 A.W. Ford Hutchinson, M.A. Bray, M.V. Doig, M.E. Shipley and M.J.H. Smith, Nature (London), 286 (1980) 264.
17 S.D. Brain, R.D.R. Camp, P.M. Dowd, A. Kobza Black, P.M. Woollard, A.I. Mallet and M.W. Greaves, Lancet, ii (1982) 762.
18 A.I. Mikaya, V.I. Smetanin, V.G. Zaikin, A.V. Antonova and N.S. Postakov, Org. Mass Spectrom., 18 (1983) 99.