Determination of Hydroxyoctadecadienoic Acids

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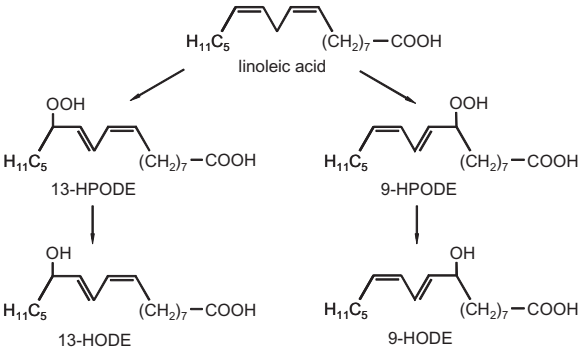
Oxidation of low-density lipoproteins (LDL) plays a crucial role in inflammatory diseases and aging. The main oxidation products of LDL are stereoisomeric 9-hydroxy-10,12-octadecadienoic acids (9-HODEs) and 13-hydroxy-9,11-octadecadienoic acids (13-HODEs). Nevertheless the content of HODEs in natural oxidized LDL is low compared to other components, thus determination of HODEs requires a sample enrichment in most cases. Big losses are encountered during the necessary processing due to the instability of HODEs against acidic conditions. Therefore the use of labeled standards is required. Standards with an ^{18}O label in the carboxylic group used previously may partly suffer a loss of the label by exchange with water. In this paper we describe an improved work-up procedure and the preparation of standards labeled with ^{18}O in the hydroxylic group which is not exchangeable.

Key words: Lipid Peroxidation, Hydroxyoctadecadienoic Acids, Inflammatory Diseases

Introduction

Lipid peroxidation (LPO) of polyunsaturated fatty acids (PUFAs) plays a dominant role in the genesis of inflammatory diseases, such as atherosclerosis (Lusis, 2000). Moreover, LPO processes are involved in the deteriotion of fat by storage (Frankel 1998), since PUFAs are extremely sensitive to oxidation by air (for a recent review see Spiteller 2002). The most abundant PUFA in tissue and blood of mammalians is linoleic acid (Esterbauer *et al.*, 1989) Its peroxidation generates stereoisomeric 9-hydroperoxy-10,12-octadecadienoic acids (9-HPODEs) and 13-hydroperoxy-9,11-octadecadienioc acids (13-HPODEs). These lipidhydroperoxides (LOOHs) are readily reduced in biological surrounding (Wang and Powell, 1991; Wang *et al.*, 1992) to the corresponding alcohols, (LOHs), 9-hydroxy-10,12-octadecadienoic acids (9-HODEs) and 13-hydroxy-9,11-octadecadienoic acids (13-HODEs) as outlined in Scheme 1.



Scheme 1. Transformation of linoleic acid to HODEs.

Since HODEs are the most abundant LPO products in blood their determination allows insight into the extend of LPO processes. Nevertheless HODEs are present – compared to other blood and tissue components – even in severe diseased states – only in low amounts. Thus their detection requires the use of sensitive and specific identification methods and also usually the application of enrichment procedures.

Sensitive immunological methods, based on antibodies raised against oxidatively damaged LDL have been found to be not specific enough (Esterbauer *et al.*, 1992). A fast and simple method for determination of all LOHs and LOOHs – in free form or esterified – uses the typical UV absorption of the diene system adjacent to a CH(OH) or a CH(OOH) group at 234 nm. Since compounds with conjugated double bonds are rare in nature (exceptions are trans fatty acids) (Zock and Katan, 1997) quantification of oxidized LDL samples

is achieved by separation on reversed phase HPLC (Ahotupo and Vasankari, 1999). Although this procedure is fast, it suffers from the missing ability to detect and determine single components. The separation in single components requires a second HPLC step using normal phase columns (Wu *et al.*, 1995).

Alternatively LOHs are determined by gas chromatography combined with mass spectrometry (GC/MS). This method needs sample enrichment. Enrichment is achieved by chromatography on silica gel originally applied to investigate hydroxy derivatives of arachidonic acid by GC/MS (Hamberg and Samuelsson, 1967). This step is combined with sample losses due to adsorption of free HODEs on silica gel. Therefore solid phase extraction on C-18 cartridges is recommended (Powell, 1982).

The isolated fraction of hydroxy acids is then derivatized to transform LOHs to volatile derivatives – necessary for separation by GC: The carboxylic groups are either protected by methylation or transformed to their pentafluorbenzylesters (Strife and Murphy, 1984). Finally the OH groups are subjected to trimethylsilylation (Boeynaems *et al.*, 1980; Aisen *et al.*, 1985; Wang *et al.*, 1992; Lehmann *et al.*, 1992). Thus generated samples are separated by GC and characterized either by registration of electron impact (EI) mass spectra (MS) (Wang and Powell, 1991; Wang *et al.*, 1992) or negative ion (NI) respectively chemical ionization (CI) mass spectra (Strife and Murphy, 1984). Since the EI/MS of thus derivatized saturated hydroxy fatty acids are more specific than those of LOHs usually a hydrogenation step is included before derivatization (Hamberg and Samuelsson, 1967; Lehmann *et al.*, 1992).

In this procedure standards are used for quantification, for instance, saturated or unsaturated hydroxy acids – not occurring in the biological material (Wang *et al.*, 1992; Jira *et al.*, 1996), – but better is the application of hydroxy acids labeled with isotopes. Usually labeled HODEs are prepared by exchange of the carboxylic oxygen by ^{18}O (Strife and Murphy, 1984). Nevertheless this method was also criticized since $^{18}O/^{16}O$ exchange occurs in biological media (Gleispach *et al.*, 1985).

Considering these difficulties we exchanged the standards labeled with ^{18}O in the carboxylic group by HODE standards labeled with ^{18}O in the hydroxy groups. By checking these standards we recognized severe sample losses during the different steps of sample processing. In this paper we describe the preparation of the new standard and report on a separation procedure which minimizes the losses of HODEs.

Experimental

Materials, chemicals, and reagents

HCl aqueous solution 32% (puriss *p. a*), MeOH, hexane, 2-propanol, THF, $CHCl_3$ (HPLC-grade), ethyl acetate, HOAc, NaOH, KOH and $SnCl_2$ were obtained from Merck (Darmstadt, Germany); PtO_2, linoleic acid (99% GC), BHT (= 2,6-bis-tertiary-butyl-4-methyl-phenol), Na_2HPO_4, NaH_2PO_4, heparin sodium salt (porcine-intestinal mucosa), and lipoxidase = lipoxygenase (soybean, 12.4 U/mg) were bought from Fluka (Buchs, Switzerland). 5% rhodium alumina powder was obtained from Aldrich (sample number: 21, 285–7). The $^{18}O_2$ (99% ^{18}O isotope) was purchased from Linde AG (Unterschleissheim, Germany). Nitrogen, argon, and oxygen were from Riessner Gase (Lichtenfels, Germany). The H_2O was purified with an MILLI-Q_{RG} apparatus (Millipore, Milford, MA, USA).

Equipment

HPLC: Beckman 112 Solvent Delivery System, Beckman System Gold detector module 167, injection volume 10 µl. Preparative HPLC: Beckman 112 solvent delivery system, Beckman System Gold detector module 167, injection volume 2 ml.

GC: HRGC Model 416 (Carlo Erba Strumentazione, Milano, Italy), carrier gas: hydrogen (pressure 0.5 bar); capillary fused silica DB-5 column J&C Scientific (Mainz-Kastel, Germany) length 30 m; splitting: 1:30; injection temperature: 270 °C. FID detector temperature: 290 °C.

GC-MS: Hewlett-Packard 5890 Series II; carrier gas: hydrogen (pressure 0.5–0.8 bar); no splitting; capillary column: fused silica DB-5 (J&C Scientific), length: 30 m, *i. d.* 0.25 mm; film thickness: 0.1–0.4 µm; injection temperature 270 °C. Finnigan MAT 95 mass spectrometer with inverse Nier-Johnson geometry and EI-CI ion source. ESI-MS: Finnigan TSQ 700 mass spectrometer, equipped with a Finnigan electrospray ionization (ESI) ion source.

Chromatographic conditions

Reversed phase preparative HPLC:

LiChrosorb RP-18 (250 × 25 mm, 7 μm, Merck) C_{18} column; mobile phase: isocratic: H_2O-MeOH (1:9) and 0.2% HOAc; flow 20 ml/min; detection at 234 nm.

Reversed phase analytical HPLC:

The attempts to separate 9- and 13-HODE on different endcapped and not endcapped C_{18}, C_8 and C_2 columns with different polar mobile phases were not successful, but separation was achieved using a ET 250/4 Nucleosil 100-5 C_4 column (Macherey-Nagel) and as gradient mobile phase: H_2O-MeOH 0.2% aqueous solution of HOAc, gradient: 0 min – (30:60:10, *v/v*) → 50 min – (5:85:10); flow rate 1 ml/min, injection volume 10 μl, detection at 234 nm. Validation: the calibration curves show linearity in the interval 30 ng – 3 μg/l. The differences for back calculated concentrations did not exceed 5% on average. The detection limit was 5 ng at a signal to noise ratio 3:1.

Normal phase preparative HPLC:
(Wu and Robinson, 1995)

Ultrasep FS 100 (250 × 25 mm, 6 μm) column Bischoff (Leonberg, Germany); mobile phase: isocratic: hexane-2-propanol-HOAC (986:12:2, *v/v*); flow: 20 ml/min; detection: 234 nm.

GC-MS:

Hewlett-Packard 5890 Series II; carrier gas: hydrogen (pressure 0.5–0.8 bar); no splitting; capillary column fused silica DB-5 (J&C Scientific), length: 30 m, *i.d.* 0.25 mm; film thickness: 0.1–0.4 μm; injection temperature 270 °C. Finnigan MAT 95 mass spectrometer with inverse Nier-Johnson geometry and EI-CI ion source. Temperature gradient: 120 °C→300 °C with 3 °C/min.

Mass spectrometry

GC-MS:

Carrier gas: hydrogen (pressure 0.5–0.8 bar); no splitting; capillary column fused silica DB-5 (J&C Scientific), length: 30 m, *i.d.* 0.25 mm; film thickness: 0.1–0.4 μm; injector temperature 270 °C.

Standard preparation

^{18}O (9S, 10E, 12Z)-9-hydroxy-10,12-octadecadienoic acid and ^{18}O (13S, 9Z, 11E)-9-hydroxy-9,11-octadecadienoic acid (analogous to enzymatic preparation of HP^{16}ODEs, (Gardner, 1989):

A solution of 0.3 g linoleic acid in 90 ml phosphate buffer (0.1 M, pH 8) was filled in a three-neck flask, the air was removed by evacuation. The flask was ventilated three times with Ar. 40 mg soybean lipoxidase = lipoxygenase (12.4 U/mg) were added under a constant Ar flow. After additional evacuation and Ar ventilation the reaction system was filled with $^{18}O_2$. The solution was stirred for 1 h at 25 °C. An additional portion of $^{18}O_2$ was added and the reaction mixture was stirred for another 1 h. The closed system was opened and 1.5 g $NaBH_4$ were carefully added (foam) under Ar ventilation. After 2 h the reaction mixture was acidified with 1 M aqueous solution of HCl (pH 3–3.5), saturated with NaCl, extracted three times with 100 ml $CHCl_3$-MeOH (3:1) and finally two times with 75 ml $CHCl_3$. In order to avoid losses of the product during the solvent evaporation 10 ml H_2O were added (see discussion). The solvents were removed in vacuum (temperature below 30 °C). The obtained colorless oil was separated by preparative normal phase HPLC: *Ultrasep* FS 100 (250 × 25 mm, 6 μm) column (Bischoff, Leonberg, Germany); mobile phase: isocratic hexane-2-propanol-HOAc (986:12:2, *v/v*); flow: 20 ml/min; detection: 234 nm. The isotopic purity of the obtained 9-HP^{18}ODE and 13-HP^{18}ODE was determined by ESI-MS to be 95%. 9-H^{18}ODE and 13-H^{18}ODE were used as internal standards for quantification. Yield: 40 and 35 mg respectively.

LDL sample preparation and work up procedure

Fresh blood samples (10 ml) from patients were transferred in ice to the laboratory. After coagulation (ca 30 min, 4 °C) the sample was centrifuged for 10 min at 9300 × *g*/min. The LDL-fraction was isolated according to the method of Leiß (Leiß *et al.*, 1979): briefly the precipitating reagent 1 – in an amount of 10% v/v in respect to the serum volume – was added to the separated blood serum (30–40% *v/v* of the blood sample). The mixed sample was kept for 1 h in the dark (ice bath) and centrifuged (30 min, 4 °C, 20,900 × *g*). The pellet

(VLDL) was separated, then the supernatant was mixed with the precipitating reagent 2, cooled for 1 h in the dark (ice bath) and centrifuged (30 min, 4 °C, 20,900 × *g*). The pellet (LDL) was stored at – 20 °C under Ar.

The LDL pellet was dissolved in a mixture of 20 ml phosphate buffer (0.1 M, pH 7.4) and 100 µl BHT solution. Then 10 µl of the standard solution, containing an exactly weighted amount of 10 µg of 9-HODE and 13-HODE labeled with ^{18}O in the hydroxylic group was added. The pellet was dissolved by stirring applying ultrasound. The solution was transferred to a funnel, 75 ml of a solution consisting of $CHCl_3$-MeOH (1:2) were added. The mixture was shaken. Then 5 ml $CHCl_3$ were added, the solution was shaken again. 20 ml of a 0.5 m NaH_2PO_4 solution were added and shaken again. The solution separated into two layers. The water layer was extracted three times with 50 ml $CHCl_3$. The collected organic extracts were evaporated in vacuum (room temperature). The raw lipid was then dried (protected against light) in vacuum. The residue was weighted (crude LDL, 5–30 mg).

Lipid hydrolysis: the obtained crude LDL was dissolved in 6 ml 1 M KOH/MeOH-H_2O (9:1) solution and stirred under Ar for 1.5 h at 50 °C (Ulberth and Kamptner, 1992). The cooled solvent was neutralized with 24.5 ml 1 M aqueous solution of mono-sodium dihydrogen phosphate (pH 5.8–6). 64 ml $CHCl_3$ and 58 ml MeOH were added to the neutralized solution. The mixture was shaken. Layer separation was achieved by addition of 32 ml H_2O. The H_2O phase was extracted additionally three times with 50 ml $CHCl_3$. ~ 10% H_2O *v/v* was added to the collected extracts and the mixture was evaporated in vacuum keeping the temperature below 30 °C. The residue was dissolved in 3 ml $CHCl_3$ and loaded into a home-made appropriately equilibrated SPE cartridge. The cartridge was washed with 4 ml $CHCl_3$. The acidic fraction was eluted with 4 ml 2% HOAc in $CHCl_3$ and flushed with nitrogen. Solvents were removed from the eluate by continuous nitrogen flow without heating. The samples were then dissolved in 0.5 ml $CHCl_3$ and derivatized by addition of 1 ml 4% solution of diazomethane in Et_2O, containing 5% of methanol. Then the samples were dissolved in AcOEt, about 10 mg of a 5% Rh catalyst on alumina powder were added and the samples were hydrogenated at 1 bar H_2 pressure for 10 min (Wang *et al.*, 1992). After removal of the catalyst and the solvent unsubstituted methylesters were separated from hydroxylated ones by silicagel flash chromatography. The residue obtained after hydrogenation was weighted again in order to determine the amount of HODE in LDL. Then the sample was dissolved in 0.5 ml $CHCl_3$ and treated with MSTFA (100 µl) for 1 h at 40 °C. The solvents were removed by continuous nitrogen flow. The dried samples were dissolved in a definite volume $CHCl_3$ (concentration 10 mg/ml). An equivalent of the solution was injected into the GC-MS for quantification.

Preparation of precipitation reagent 1:

5 ml solution of heparin sodium salt (2500 IU/ml) are mixed with 5 ml 2 M aqueous solution of $MgCl_2$ (Leiß *et al.*, 1979).

Preparation of precipitation reagent 2:

5 ml 2M aqueous solution of $MgCl_2$ are mixed with 5 ml aqueous solution of dextrane sulfate sodium salt 20 g/l (Leiß *et al.*, 1979).

Preparation of the home-made SPE cartridges:

A 2 ml glass syringe was filled with 0.4 g aminopropyl-modified silica gel CROMABOND LV-NH_2 and filter elements from filter paper 595 Schleicher & Schüll GmbH (Dassel, Germany). The packed cartridges were equilibrated with 4 ml hexane and 1 ml $CHCl_3$.

Esterification with diazomethane (esterification of -COOH groups (Hamberg and Fahlstadius, 1990).

The samples are dissolved in 0.5 ml $CHCl_3$ and 1 drop of methanol (addition of methanol traces accelerate the reaction, private communication by Dr. Gardner), then 1 ml 4% solution of diazomethane in Et_2O is added. After 3 min the solvents are removed by a continuous nitrogen flow.

Catalytic hydrogenation of the double bonds analogous to Wang (Wang *et al.*, 1992).

The samples are dissolved in 6 ml methanol. After addition of 5 mg 5% rhodium alumina powder the suspension is shaken under hydrogen (pressure 1 bar) for 10 min. The catalyst is filtered off and the solvent is evaporated (continuous nitrogen flow).

Separation of hydroxy acids from unsubstituted acids:

The residue obtained after hydrogenation is dissolved in 2 ml cyclohexane-EtOAc (98:2). This solution is brought onto a silicagel column (5 g) equilibrated with cyclohexane-EtOAc (98:2). The column is washed with 80 ml of the same solvent mixture. Hydroxylated methylates are eluted by rinsing the column with 80 ml EtOAc.

Trimethylsilylation with N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA, protection of hydroxy groups)

The samples obtained after removal of the solvent are dissolved in 0.5 ml $CHCl_3$ and heated with MSTFA (100 μl) for 1 h (40 °C). The solvents are removed by a continuous nitrogen flow.

Blank recovery experiments

Blank experiment 1:

In order to check the loss of HODEs (by application of previous separation procedures) 20 μg 9- and 13-HODE are dissolved in a mixture of 20 ml phosphate buffer (0.1 M, pH 7.4) and 200 μl BHT solution (2% in ethanol). 100 ml $CHCl_3$-MeOH *(v/v)* are added and the mixture is shaken. Separation in two layers is achieved by addition of 23 ml H_2O and 2 ml 1 M aqueous solution of HCl. The organic layer is separated and the water layer is extracted three times with 50 ml $CHCl_3$. The collected extracts are evaporated *in vacuo*. The residue is dissolved in a mixture of 1 ml THF and 2 ml 1 M NaOMe methanolic solution and stirred under Ar 1 h at room temperature. The solvent is acidified with 1 M aqueous solution of HCl (pH 2). The THF is evaporated and the residue is dissolved in 50 ml water and extracted three times with 50 ml $CHCl_3$. The collected extracts are dried with Na_2SO_4 and then subjected to evaporation in vacuum. Then the residue is taken up in CH_3OH, hydrogenated (as described above but using PtO_2 as catalyst) and derivatized with diazomethane and MSTFA. The dried samples are dissolved in a definite volume $CHCl_3$ and are analyzed by GC-MS. 97% ± 2 (average value from 10 samples) of HODEs were transformed to stearic acid.

Blank experiment 2:

The same HODE samples are processed as described above but the collected $CHCl_3$ extracts are not dried. The HODE losses are found to amount to 90–95% (average of ten samples).

Blank experiment 3:

is carried out like blank experiment 2, but instead 1 N HCl, a 1 M aqueous solution of NaH_2PO_4 is used for neutralization and the catalyst is substituted by rhodium on alumina. The losses were found to be less than 10% (average of ten samples).

Hydrogenation experiments

Hydrogenation experiment 1:

0.8 mg PtO_2 catalyst are added to 48 μg of 13-HODE dissolved in 4 ml of methanol. 5 ml of $CHCl_3$ are added. The solution is shaken for 30 min in a H_2 atmosphere at a pressure of 1 bar. The products are investigated after trimethylsilylation by GC-MS. Two peaks of equal height are recognized. The first corresponds to the TMS derivative of 13-hydroxystearic acid, the second to stearic acid.

Hydrogenation experiment 2:

Repeating of the experiment by hydrogenation in 10 ml AcOEt instead of CH_3OH-$CHCl_3$ results in an improvement of the yield (ratio of 13-hydroxystearic acid to stearic acid 4:1).

Hydrogenation experiment 3:

In a next experiment hydrogenation in CH_3OH-$CHCl_3$ is carried out with the methylate of 13-HODE. The yield improves considerably compared to hydrogenation of the free acid (ratio 13-hydroxystearic acid methylate to stearic acid methylate 3:1). 13-Methoxystearic acid methylate is recognized as byproduct.

Hydrogenation experiment 4:

Hydrogenation of the methylate of 13-HODE by exchange of PtO_2 versus 5% rhodium alumina powder as catalyst in AcOEt yields only traces of stearic acid methylate, but results also in partial transformation of the hydroxylic group to the oxo

group generating 13-oxostearic acid methylate as byproduct (ratio 13-oxostearic acid methylate: trimethylsilylated 13-hydroxystearic acid methylate 1:10).

Results and Discussion

Establishment of the extraction method

Already nearly four decades ago it has been recognized that homogenation of mammalian (Wills, 1966) or plant (Galliard, 1970) tissue in aqueous solutions – even at 0 °C (Galliard, 1970) – is inevitably connected with massive LPO processes. Thus large amounts of HODEs are generated artificially and consequently determination of LPO products after homogenation of tissue in aqueous solvents does not reflect the original extend of LPO processes in physiological and diseased states. Nevertheless often homogenation is still carried out in buffer solutions (Sohal *et al.*, 1994; Wu *et al.*, 1995). This artificial generation of LPO products can be prevented substantially when enzymes are destroyed before homogenation by boiling of the sample or by paralyzing their action using for extraction an organic solvent. In spite of these precautions a moderate generation of LPO products can not be excluded completely by processing of tissue samples due to their high water content combined with the immediately activation of enzymes by cell injury which might allow to induce LPO for a short time before the enzymes are inactivated. More reliable results are obtained if tissue samples are subjected to deep freezing (Lehmann *et al.*, 1992). Nevertheless already the removal of organs might be connected with partial injury and thus induction of LPO processes. The danger to initiate LPO processes is much lower when biological liquids, *e.g.* blood or liquor, are analyzed because mechanical destruction is avoided.

As mentioned above, unambiguous identification of HODEs requires a GC/MS analysis after appropriate enrichment and derivatization (Aisen *et al.*, 1985; Kühn *et al.*, 1989; Wang and Powell, 1991; Lehmann *et al.*, 1992; Jira *et al.*, 1996). When we checked the validity of the previously widely applied methods by use of ^{18}O labelled standards (see below) we observed great sample losses. Therefore we investigated each step of the sample processing by GC/MS analysis. Thus we recognized that predominantly traces of acid are responsible for loss of samples:

LOHs are secondary allyl alcohols, their sensitivity to acids is well known. Therefore LOH esters are saponified usually in alkaline media. The separation of the acids from the neutral compounds requires acidification. In previous investigations samples have been acidified to a p_H 2 (Wu *et al.*, 1995; Jira *et al.*, 1996) or even to p_H 3.1–3.2 either by addition of HCl (Gleispach *et al.*, 1985) or an ammonium formate buffer (Lehmann *et al.*, 1992). We observed extreme sample losses by acidifying the organic extract with HCl to p_H 3, especially when the extracts were dried before solvent removal: HCl and other acids are transferred by extraction partly into the organic layer. If the organic extract is dried acid is accumulated in the residue. The acid catalyzes elimination of water, transferring HODEs to a mixture of trienoic acids which are transformed to steric acid by catalytic hydrogenation. The latter is easily detectable after derivatization by GC/MS. The yield of HODEs is improved if the organic solvents are not dried because then acids are removed by azeotropic distillation when the organic solvent is distilled off. Therefore addition of 10–15% (*v/v*) of water to the organic solvents before distillation prevents at least partly the decomposition of LOHs.

The observation that weakly acidified solutions induce water elimination of HODEs is also important for their separation on normal phase HPLC columns (necessary for instance for standard preparation) where separations require addition of acetic acid (various amounts of acetic acid between 6% (Gleispach *et al.*, 1985) and 0.001% (Clare *et al.*, 1991) have been recommended). The lower the amount of acetic acid added the lower are the losses.

Nevertheless accumulation of acetic acid by removal of solvent can not be avoided completely. Therefore we neutralized the basic solutions obtained after saponification with a 1 M NaH_2PO_4 solution only to p_H 5.8. This improved the yield of HODEs dramatically.

The fraction of acids is then separated from unpolar products by SPE (Solid Phase Extraction) on a weekly basic ion exchange column (aminopropyl-modified silica gel). Since elution of acids requires addition of 2% of acetic acid to the chloroform solution, evaporation of the solvent must

be carried out carefully on a water bath not exceeding a temperature of 30 °C and after addition of approximately 10% of water (necessary for azeotropic removal of acid).

The tendency for water elimination in further processing is reduced if the double bonds are hydrogenated. Moreover derivatives of saturated hydroxy acids show very characteristic electron impact mass spectrometric fragmentation patterns (Lehmann *et al.*, 1992; Wu *et al.*, 1995).

The hydrogenation experiments revealed that hydrogenation of free HODEs by use of PtO_2 as catalyst and methanol as solvent generated large amounts of stearic acid, the sample losses were reduced when the free acids were converted to their methylates and even more by exchange of the solvent to ethylacetate. Best results were obtained by exchange of the PtO_2 catalyst by a 5% rhodium catalyst on alumina powder as recommended by Wang (Wang *et al.*, 1992) and reducing the time of hydrogention. Nevertheless we recognized also in this case a side reaction, the generation of saturated keto-acids.

The exchange of the OH group versus hydrogen observed by catalytic hydrogenation is thus apparently caused by a slightly acidic solvent and prolonged exposure to too large amounts of catalyst in respect to the sample amounts.

Polar groups cause partial sample decomposition on GC columns, moreover hydroxy-compounds are partly adsorbed. Therefore the free hydroxygroups must be converted to their trimethylsilylethers by treatment with N-methyl-N-trimethylsilyl-trifluroacetamide (MSTFA) or another silylating reagent before GC/MS investigation of the samples is started.

Although samples are ready for GC/MS analysis after derivatization it might be sometimes useful to remove the main amount of less polar saturated fatty acid methylates by flash chromatography on silicagel. Since free acids are adsorbed on silicagel columns to a much higher extend than methylates also this step requires preparation of esters.

Choice of the internal standards

The inevitable losses in the multistep procedure of sample preparation for quantification of HODEs require the application of internal standards. When saturated hydroxyacids of unusual chain length are used for this purpose (Wang and Powell, 1991; Jira *et al.*, 1996) losses occurring due to water elimination of HODEs and during hydrogenation escape partly detection. Better internal standards for quantification of HODEs are isotopically labeled analogues of HODEs. In previous investigations hydroxy acids derived by LPO processes were labeled at the carboxyl group with ^{18}O (Strife and Murphy, 1984), but exact quantification might be aggravated due to partial exchange of the ^{18}O to ^{16}O in biological media (Gleispach *et al.*, 1985). In contrast exchange of the ^{18}O labeled oxygen atom in the hydroxygroup of secondary alcohols versus ^{16}O is impossible under normal circumstances. Therefore we synthesized $H^{18}ODEs$ in analogous manner as described for $H^{16}ODEs$ (Gardner, 1989) by reacting linoleic acid with soybean lipoxygenase in an $^{18}O_2$ atmosphere. The ob-

Table I. Statistical validation for quantitation of 9-HODE using isotopically labeled 9-H^{18}ODE as an internal standard. 9-HODE was monitored by measuring the ion intensities at *m/z* 229 and the internal standard (9-H^{18}ODE by measuring the ion intensities at *m/z* 231 respectively. Ion currents at the appropriate GC retention times were integrated. The ratios of the peak integrals reflect the target analyte divided by that for the internal standard.

9-^{16}O-HODE [μg]	9-^{18}HODE as an internal standard [μg]	*m/z* 229/231 theoretical peaks ratio*	Number of experiments	Experimental *m/z* 229/231 average peaks ratio*	Standard deviation	Standard error	95.0% confidence intervals lower limit	95.0% confidence intervals upper limit	RSD %	Accuracy %
30	10	3.0	5	3.002	0.0432	0.0193	2.948	3.056	1.4	97
20	10	2.0	5	2.000	0.0387	0.0173	1.952	2.048	1.9	98
10	10	1.0	5	1.001	0.0106	0.0047	0.988	1.014	1.1	98
5	10	0.5	5	0.500	0.0061	0.0027	0.492	0.507	1.2	98
3	10	0.3	5	0.331	0.0045	0.0020	0.326	0.337	1.4	98
2	10	0.2	5	0.1998	0.0058	0.0026	0.193	0.207	2.9	96
1	10	9,1	5	0.0998	0.0040	0.0017	0.095	0.104	4.0	95

tained [^{18}O]-HODEs were determined to have an isotope purity greater than 95% (by ESI/MS). HODEs proved to be stable if stored without solvent in an argon atmosphere.

Determination of LDL

An aliquot of LDL samples processed as described in the experimental section was dissolved in MSTFA and then subjected to separation by GC/MS.

GC peaks corresponding to derivatized 9- and 13-hydroxystearic acid and their ^{18}O labeled isomers were recognized by their typical EI/MS spectra. Quantification was achieved by measuring the ion currents of the α-fragments as described (Wang and Powell, 1992; Lehmann *et al.*, 1995) by registering the ion currents of the α-fragments found at *m/z* 173 and *m/z* 315 in the not labeled and at *m/z* 175 resp. 317 in the labeled 13-HODE spectra (Fig. 1). Likewise the fragments of 9-HODE were registered at *m/z* 229 and *m/z* 259, resp. at *m/z* 231 and *m/z* 261. The ion currents of these fragments can be measured with great accuracy (see Table I).

Conclusions

The reported improved processing for HODE quantification minimizes the sample losses during HODE extraction from biological samples caused by presence of strong acids. SPE is a quick and easy method for preliminary separation of the acidic sample fraction from the matrix and allows to increase the sensitivity. Quantification was achieved with ^{18}O isotopically labeled internal standards, containing the label in the hydroxylic group. Application of the method will be published elsewhere.

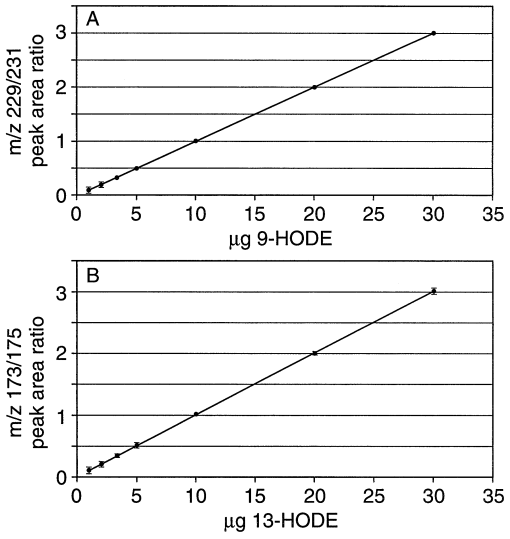


Fig. 1. Standard curves for (A) 9-HODE and (B) 13-HODE. Isotopically labeled 9-H^{18}ODE and 13-H^{18}ODE were used as internal standards (10 µg). 9-HODE was monitored by measuring the ion intensities at *m/z* 229 (A) and *m/z* 259 (data not shown), 13-HODE was monitored by measuring the ion intensities at *m/z* 173 (B) and *m/z* 315 (data not shown). The internal standard: 9-H^{18}ODE was monitored by measuring the ion intensities *m/z* 231 and *m/z* 261 and 13-H^{18}ODE was monitored by measuring the ion intensities at *m/z* 175 and *m/z* 317. Ion current peaks at the appropriate GC retention times were integrated. The ratios of the peak integrals reflecting the target analyte divided by that of the internal standard were plotted as a function of the amounts of (A) 9-HODE and (B) 13-HODE.

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