paper on methodology

Monitoring of lipoprotein oxidation by gas chromatographic analysis of hydroxy fatty acids

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Abstract We describe a method developed for the quantitative analysis of hydroxy fatty acids derived from fatty acid monohydroperoxides formed during lipoprotein oxidation. The procedure starts with catalytic hydrogenation of the lipid extract, whereby hydroperoxyl groups are converted to hydroxyl groups and double bonds are eliminated, and the risk for lipid oxidation during the rest of the procedure is eliminated. The fatty acids are converted to methyl esters, which are fractionated by gas chromatography on a nonpolar column. The major differences to existing methods are that a mass spectrometer is not required and that the specificity thus lost is replaced by gas chromatography before and after acetylation of the hydroxyl groups. This changes the retention times of the hydroxyacids with respect to the unsubstituted fatty acids moving them to positions usually occupied by trace components only. The method allows quantification of monohydroxy fatty acids derived from 18-, 20- and 22-carbon polyunsaturated fatty acids. Positional isomers are separated from each other to some extent. The method has been mainly used for analysis of hydroperoxides in human low density lipoprotein preparations and for following lipoprotein oxidation in vitro.—**Nikkari, T., U. Malo-Ranta, T. Hiltunen, O. Jaakkola, and S. Ylä-Herttuala.** Monitoring of lipoprotein oxidation by gas chromatographic analysis of hydroxy fatty acids. *J. Lipid Res.* 1995. **36:** 200–207.

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The primary products of lipid oxidation are monohydroperoxides (1, 2). Gas chromatography–mass spectrometry methods have been used for analysis of individual hydroxy fatty acids derived from hydroperoxides of polyunsaturated fatty acids in lipid extracts of biological specimens (3–10). The first step in these methods is usually the reduction of hydroperoxyl groups to hydroxyl groups by borohydride or triphenylphosphine treatment followed by liberation of the fatty acids and their conversion to suitable esters. After derivatization of the hydroxyl groups, the fatty acids are separated by gas chromatography and the individual hydroxyacids are detected by mass spectrometry on the basis of their specific fragmentation patterns. Thomas et al. (8, 9) have simplified the method by including catalytic hydrogenation of the sample at the start of the procedure; during hydrogenation, fatty acid hydroperoxides are reduced to hydroxy fatty acids and the double bonds are removed, which gives the advantage of eliminating the risk of lipid oxidation during the analytic procedure. Positional isomers of the monohydroxy fatty acids can still be separated from each other by gas chromatography, but information about the number and position of double bonds is naturally lost. However, as the majority of the 18-carbon hydroxy fatty acids in biological samples are derived from oxidation of linoleic acid and those with 20 carbons from arachidonic acid, the extent of oxidation of these fatty acids can be evaluated.

Because a mass spectrometer is costly, we have developed a method for analysis of fatty acid hydroperoxide-derived hydroxyacids without this instrumentation. The method involves catalytic hydrogenation of a lipid extract, conversion of the fatty acids to methyl esters, and fractionation of the methyl esters by gas chromatography. Specificity is gained by gas chromatography of the sample both before and after acetylation of the hydroxyl groups, which changes the retention times of the hydroxy acids with respect to the unsubstituted fatty acids to positions usually occupied only by trace components.

MATERIALS AND METHODS

Materials

All organic solvents were redistilled. Chloroform, methanol, toluene and pyridine (all analytic grade) were purchased from Merck (Darmstadt, Germany). Petroleum

Abbreviations: BHT, butylated hydroxytoluene; HETE, hydroxyeicosatetraenoate; HODE, hydroxyoctadecadienoate; LDL, low density lipoprotein; 18:0, octadecanoate; 20:0, eicosanoate; 22:0, dodecanoate; OH-18, hydroxyoctadecanoate; OH-20, hydroxyeicosanoate; OH-22, hydroxydodecanoate; R_t, retention time.

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spirit (b.p. 68–69°C) was from Neste Oy (Finland) and ethanol (spectrophotometric grade) was from Alko (Finland). Platinum(IV)oxide and acetic anhydride were from Fluka (Buchs, Switzerland). Hydroxyoctadecadienoic acids (9- and 13-HODE) and hydroxyeicosatetraenoic acids (5-, 8-, 9-, 11- and 12-HETE) were from Cayman Chemical Co. (Ann Arbor, MI). Triheptadecanoin and fatty acid standards, including 2-hydroxyhexadecanoic, 2- and 12-hydroxyoctadecanoic, 2-hydroxyeicosanoic, and 15-hydroxyeicosatetraenoic acids, as well as 13(S)-HODE (lot 81H3397) and 15(S)-HETE (lot 60H38663) used for recovery experiments, were from Sigma Chemical Company (St. Louis, MO). Hydroperoxy derivatives of arachidonic and 7,10,13,16-docosatetraenoic acids were prepared according to Boeynaems (11).

Isolation and oxidation of LDL

LDL (d 1.019–1.063 g/ml) was isolated by sequential ultracentrifugation from pooled plasma of fasting healthy humans in the presence of 0.01% EDTA and a combination of protease inhibitors and antioxidants as described (12). LDL was dialyzed at 4°C against 0.15 M NaCl, pH 7.40, containing 0.01% EDTA and sterilized passing through a 0.45-μm filter. LDL protein was determined (13) using bovine serum albumin (Sigma Chemical Co.) as standard.

Before oxidation, EDTA was removed from the LDL preparation by dialysis against 0.15 M NaCl in 10 mM Na_2HPO_4, pH 7.4. LDL (500 μg/ml) was oxidized for indicated times at 37°C in Optimem-1 (Gibco, Paisley, Scotland) containing penicillin-streptomycin (Gibco) and 20 μM $CuSO_4$. Aliquots (usually 0.5 ml) and 50 μg BHT (in ethanol) were added to chloroform–methanol at suitable intervals.

Extraction of lipids

EDTA-plasma (200–500 μl) or LDL containing 25–500 μg protein were immediately mixed with 50 μg BHT and 30–70 μg of the internal standard triheptadecanoin and extracted with 6 ml chloroform–methanol 1:1 in a ground glass-stoppered test tube. The supernatant after centrifugation was diluted with 3 ml chloroform and treated according to Folch, Lees, and Sloane Stanley (14) as previously described (15). Usually, half of the chloroform phase was esterified, the fatty acid methyl esters were purified with thin-layer chromatography and analyzed for the composition of unsubstituted fatty acids. The other half was used for analysis of the hydroxyacids.

Hydrogenation

The aliquot for hydroxyacid analysis was evaporated to dryness in a stream of nitrogen in a conical tube and immediately dissolved in 0.5 ml methanol by sonication. $Pt(IV)O_2$ (2 mg) was prereduced by bubbling hydrogen gas through a suspension in 1 ml methanol for 2–3 min and added to the sample tube. The tube was filled with hydrogen and allowed to stand at room temperature for 30 min with occasional shaking. This gentle hydrogenation step for reduction of compounds with allylic hydroperoxyl and hydroxyl groups was followed by a more vigorous hydrogenation step in order to convert all unsaturated fatty acids to their saturated derivatives: 1.5 ml chloroform was added to the sample tube and hydrogen gas was led from a glass capillary through the solution for 5 min. The catalyst was centrifuged off and the sample was transferred to another tube for esterification.

Extraction and hydrogenation were carried out during a single working day, usually within 1–2 h, to reduce the risk of lipid oxidation during the procedure.

Saponification

The lipid was dissolved in 200 μl toluene, and 2.5 ml ethanol, 250 μl 33% aqueous KOH, and a boiling chip were added. The mixture was refluxed at 85°C for 1–2 h in a ground glass-stoppered tube. After cooling, 2.5 ml water was added and the unsaponifiable matter was extracted twice (5 ml and 3 ml) with petroleum spirit; the extracts were discarded. After acidification with 200 μl concentrated HCl, the fatty acids were extracted thrice (5, 4, and 2 ml) with petroleum spirit.

Esterification

After evaporation of the solvent in a stream of nitrogen, the sample was dissolved in 200 μl toluene, and 2.5 ml of a solution of 2% H_2SO_4 in dry methanol and a boiling chip were added. The solution was refluxed in a ground glass-stoppered tube at 80°C for 1–2 h. After addition of 2.5 ml water, fatty acid methyl esters were extracted thrice (5, 4, and 2 ml) with petroleum spirit.

The sample for unsubstituted fatty acid analysis was further purified by thin-layer chromatography on Silica Gel G using petroleum spirit–diethyl ether 4:1 as the developing solvent. The fatty acid band was scraped off and dissolved in chloroform.

Gas chromatography

Hydroxy fatty acids. The hydrogenated fatty acid methyl esters were dissolved in petroleum spirit and fractionated in a 30-m × 0.5-mm i.d. fused silica capillary column coated with DB1 (J&W Scientific, Inc., Folsom, CA) in a Hewlett-Packard 5890 Series II gas chromatograph with an on-column injector (Hewlett-Packard Co., Palo Alto, CA). The temperature was programmed 25°/min from 50°C to 170°C and 4°/min from 170°C to 300°C. The response of a flame ionization detector was monitored with a Hewlett-Packard HP 3365 ChemStation program (Version A.03.21) running in a 486DX microcomputer connected to a Laserjet IIP printer.

Usually, each sample was chromatographed both before and after derivatization of the hydroxyl groups by

acetylation in 200–300 μl acetic anhydride–pyridine 4:1 at room temperature overnight. The reagent was evaporated in a stream of nitrogen and the sample was dissolved in petroleum spirit for gas chromatography.

Unsubstituted fatty acids. The (unhydrogenated) methyl esters purified by TLC were dissolved in petroleum spirit and fractionated in a 25-m × 0.32-mm i.d. fused silica capillary column coated with NB-351 (HNU-Nordion Ltd., Helsinki, Finland) in a Hewlett-Packard 5890 gas chromatograph with an on-column injector. The temperature was programmed from 50°C to 230°C. The peaks generated by a flame ionization detector were quantified by a Hewlett-Packard 3396A integrator or the reports were converted by the Hewlett-Packard Peak96 program to a format that could be analyzed with the ChemStation program.

RESULTS

As acetylation plays a central role in the identification and quantification of the hydroxy fatty acids in the present method, we examined the carbon numbers of C_{18}-, C_{20}-, and C_{22}-monohydroxy fatty acid methyl ester standards both before and after acetylation (**Table 1, Fig. 1**). The hydroxyl group per se lengthens the retention times (R_t) of most of the fatty acids by 1.85 to 1.90 carbon units, causing OH-18:0, OH-20:0, and OH-22:0 to emerge from the column just before the unsubstituted 20:0, 22:0, and 24:0, respectively. Acetylation of the hydroxyl group further increased the R_t values and the acetylated OH-18, OH-20, and OH-22 had values 0.33–0.54 carbon units longer than the unsubstituted 20:0, 22:0, and 24:0, respectively. Unacetylated 2- and 5-hydroxyacids had R_t values quite different from those of the other positional isomers. Acetylated 2-hydroxy isomers migrated just in front of the peak containing most of the other acetylated positional isomers of the same chain length except acetyl-13-OH-18 and acetyl-15-OH-20, which gave rise to their own peaks behind that of the others.

TABLE 1. Relative retention times (carbon numbers) of the hydroxy fatty acid methyl ester standards before and after acetylation

	Before Acetylation	After Acetylation
Hydroxy 18:0		
2-OH	19.24	20.38
9-OH	19.85	20.46
12-OH	19.86	20.51
13-OH	19.85	20.55
Hydroxy 20:0		
2-OH	21.25	22.33
5-OH	22.08	22.43
8-OH	21.87	22.43
9-OH	21.88	22.43
11-OH	21.88	22.44
12-OH	21.88	22.45
15-OH	21.87	22.54
Hydroxy 22:0[a]		
	23.90	24.42 and 24.52

Column of DB-1 (30-m × 0.5 mm i.d.) programmed from 50 to 300°C.
[a]OH-22:0 was prepared from 22:4 n-6 (11). Positions of the hydroxyl groups were not determined.

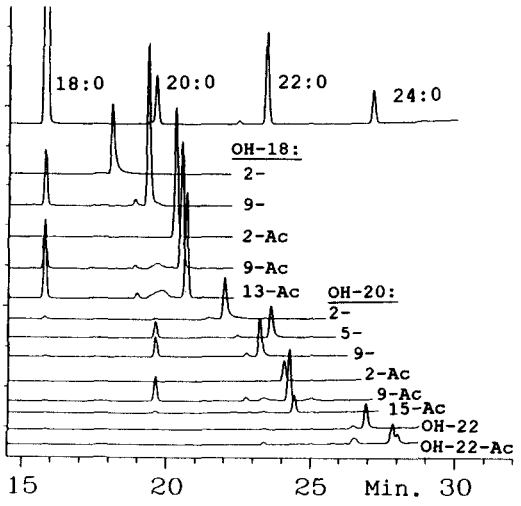

Fig. 1. Gas chromatography of standards of saturated fatty acid methyl esters in a 30-m × 0.5-mm i.d. column of DB-1 programmed from 50 to 290°C. The uppermost chromatogram is from unsubstituted fatty acids 18:0 to 24:0, the rest of the chromatograms are from hydroxy fatty acid standards. The numbers to the right of each hydroxyacid curve refer to the position of hydroxyl group; Ac after the number indicates that the hydroxyl is acetylated. Many of the OH-18 preparations also contained (unsubstituted) 18:0, and OH-20 standards contained 20:0. The positions of hydroxyl groups in the OH-22 standard were not determined. Curves of the isomers with the same R_t as 9-OH-18 (12-OH-18 and 13-OH-18), 9-OH-20 (8-OH-20, 11-OH-20, 12-OH-20 and 15-OH-20), or acetylated 9-OH-20 (5-OH-20, 8-OH-20, 11-OH-20 and 12-OH-20) are not shown.

Figure 2 shows the analysis of fatty acid hydroperoxy-derived hydroxyacids from an EDTA-plasma sample stored for 7 months at 5–8°C. All double bonds in the fatty acids were reduced by hydrogenation and, consequently, all unsaturated fatty acids were converted to their saturated parent fatty acids. In the unacetylated sample (upper curve), the methyl esters of OH-18, OH-20, and OH-22 moved immediately before the unsubstituted fatty acid methyl esters two carbon atoms longer. After acetylation (middle curve), the R_ts of the hydroxyacids were shifted to positions (marked with letters A, B, and C in Fig. 2) occupied by only minor or no components before acetylation. With the aid of an internal standard (triheptadecanoin), added at the stage of lipid extraction, the concentrations of the acetylated hydroxyacids could be determined by integration of the peaks of in the regions A, B, and C. Integration of these areas was carried out both before and after acetylation, the unacetylated sample serving as a blank; any peaks that the unacetylated sam-

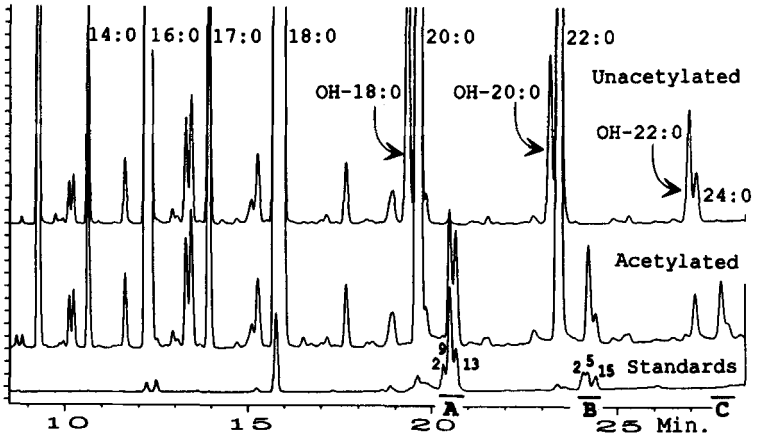

Fig. 2. Gas chromatography of hydrogenated fatty acid methyl esters of a plasma sample kept for 7 months at 5–8°C. Conditions as in Fig. 1. The positions of the unsubstituted fatty acids 14:0 to 24:0 are indicated in the upper part of the figure. The hydroxy fatty acid peaks (marked with arrows) in the unacetylated sample (upper curve) have moved to regions A, B, and C in the acetylated sample (middle curve). The lowest curve is a chromatogram of acetylated 2-, 9-, and 13-OH-18:0 (A) and 2-, 5-, and 15-OH-20:0 (B) standard mixtures. Region C contains acetylated OH-22:0 peaks. The plasma had 42.1, 19.8, and 11.5 μmol/l of OH-18, OH-20, and OH-22, respectively. They amounted to 5.0, 2.6, and 1.6 μg/mg total fatty acids, respectively, whose contents were measured from the same chromatogram.

ple showed in the areas A, B, and C were subtracted from peaks with the corresponding R_ts in the acetylated sample. For instance, the unacetylated sample in the upper curve of Fig. 2 had small peaks in areas A, B, and C corresponding to 125 ng, 19 ng, and 5 ng/sample, respectively. These blank values were subtracted from the contents calculated from the middle curve for the acetylated hydroxyacids: OH-18 6320 ng, OH-20 3355 ng, and OH-22 2040 ng, respectively. In this case the blank values were insignificant, but as the sensitivity was increased they became more meaningful (see below).

The recovery of the method was tested by adding 100 ng to 5 μg 13-HODE and 50 ng to 2.5 μg 15-HETE to the lipid extract from 250 μl of normal plasma together with the internal standard, triheptadecanoin. The samples were hydrogenated and either *a)* saponified followed by H_2SO_4-methanol esterification or *b)* esterified directly with H_2SO_4-methanol without saponification (**Fig. 3**). Assuming that the contents of HODE and HETE in the commercial products were those given by the manufacturer, the mean recoveries through the longer procedure (*a*) were 80% for HODE and 74% for HETE. The shorter procedure (*b*) gave yields of 88% for HODE and 86% for HETE.

When 100 μg of chromatographically purified linoleate and arachidonate were passed through the longer procedure, their chromatograms represented less than 20 ng of compounds migrating with R_ts corresponding to OH-18 and OH-20, indicating that these major polyunsaturated fatty acids present in biological samples do not give rise to hydroxyacids during the procedure.

The method was used for monitoring fatty acid hydroperoxide formation during in vitro LDL oxidation. **Figure 4** shows a typical series of chromatograms obtained during Cu^{2+}-catalyzed oxidation of a pool of human LDL. The concentration of OH-18 rose strongly reaching a maximum in the 24-h sample, while the contents of OH-20 and OH-22 only increased up to 6 h. The 24-h sample contained many unidentified peaks apparently arising from degradation of the polyunsaturated fatty acids. The large peak with R_t of 18.95 is not a hydroxyacid as its R_t did not change after acetylation. The nearest match in gas chromatography–mass spectrometry is 9-oxo-octadecanoic acid.

Figure 5 shows plots of the decrease in the contents of the unsaturated (unsubstituted) fatty acids (panels A and

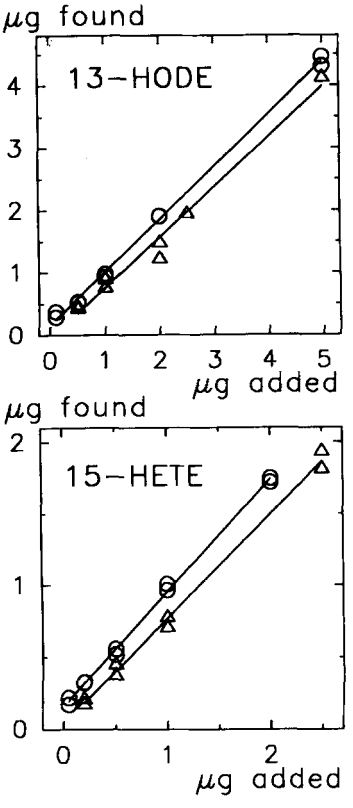

Fig. 3. Recoveries of 13-HODE and 15-HETE through the whole procedure including (△) or excluding (○) saponification. The compounds were added to lipids extracted from 250 μl normal human plasma containing 37 μg of the internal standard triheptadecanoin. The quantities of added HODE and HETE are based on the content given by the manufacturer. Duplicate determinations were carried out at each concentration except for the two highest △-points for 13-HODE.

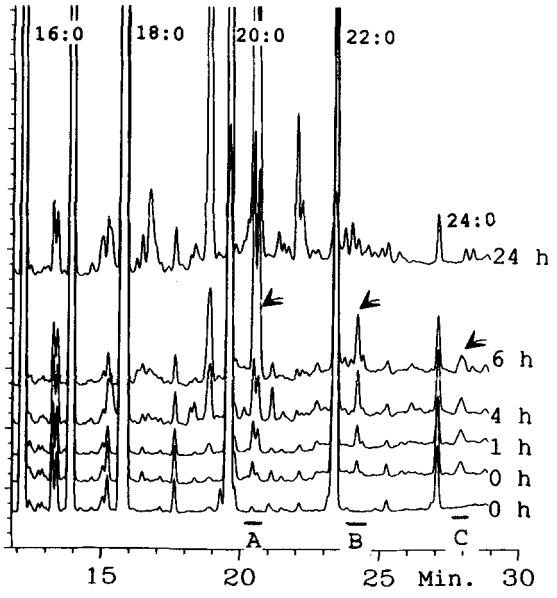

Fig. 4. Gas chromatographic tracings of aliquots of hydrogenated fatty acid methyl esters obtained from a pool of human LDL oxidized by Cu^{2+} at 37°C. Aliquots corresponding to 250 μg LDL protein were taken at indicated times. The positions of the unacetylated fatty acids 16:0 to 24:0 are indicated in the upper part of the figure. The lowest 0-h curve is from the unacetylated LDL pool sample and the other curves are from acetylated samples. Letters A, B, and C indicate regions corresponding to acetylated OH-18, OH-20, and OH-22, respectively. In addition, the acetylated hydroxyacid peaks are marked with arrows in the 6-h curve. The concentrations of OH-18, OH-20, and OH-22 in the 0-h sample were 3.1, 2.1, and 2.4 nmol/mg LDL protein (0.58, 0.52, and 0.63 μg/mg total fatty acids), respectively, and rose to 58, 9.8, and 4.7 nmol/mg LDL protein after a 6-h oxidation. OH-18 increased further to 112 nmol/mg in the 24-h sample.

B) and increase of the hydroxyacids (panels C and D) during Cu^{2+}-catalyzed oxidation of another pool of LDL. Roughly the same information as that shown in panels A and B, which required separate runs of the unhydrogenated fatty acid methyl esters in a polar gas chromatography column, can be calculated from the chromatogram used for hydroxyacid analysis (panels E and F).

Figure 6 shows representative chromatograms of an aliquot of isolated LDL pool used for in vitro oxidation studies. The mean concentrations of OH-18 and OH-20 in this pool were 5.76 ± 0.42 (SD) nmol/mg LDL-protein and 2.50 ± 0.27 nmol/mg, respectively, as determined from nine samples each containing 25 μg LDL-protein and going through the whole procedure. Thus, the reproducibilities (coefficients of variation) for the determination of OH-18 and OH-20 were 7.3% and 10.6%, respectively.

The smaller the concentration of the hydroxyacids, the greater becomes the proportion of other compounds migrating in the same region to which the hydroxyacids move after acetylation, rendering their assay less accurate. This is exemplified by gas chromatograms of hydrogenated fatty acids of total lipids from a sample of fresh normal human plasma (**Fig. 7**), which is one from a series of seven plasmas from healthy adult humans that we assayed for hydroxyacids by this method. The mean concentration of OH-18 was 1.15 ± 0.28 μmol/l (range 0.80–1.48) and that of OH-20 was 0.45 ± 0.21 μmol/l (range 0.11–0.78).

DISCUSSION

The present paper introduces a new principle for gas chromatographic analysis of hydroperoxide-derived hydroxy fatty acids in biological samples, affording the possibility of quantifying these early products of lipid peroxidation without the use of a mass spectrometer. The key steps in the procedure are (A) catalytic hydrogenation of the lipid extract and (B) gas chromatography of the fatty acid methyl esters both before and after acetylation. The hydrogenation step reduces the double bonds, and only saturated fatty acids are present in the sample after

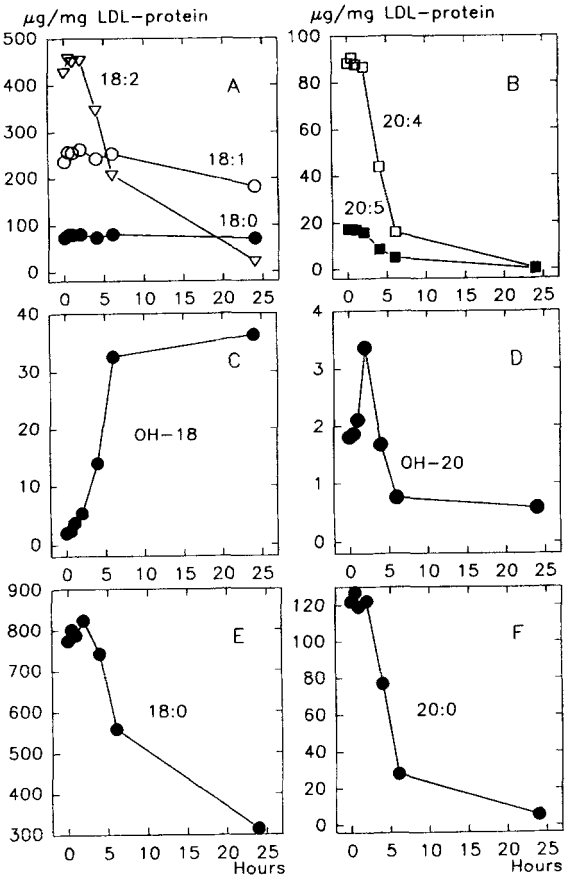

Fig. 5. Plots of the decrease of polyunsaturated fatty acids during a Cu^{2+}-catalyzed oxidation of an LDL pool (panels A and B), analyzed by gas chromatography in a polar column, and simultaneous increase of the hydroxy fatty acids (panels C and D), analyzed by the present method. Panels E and F show the decrease of the hydrogenated unsubstituted fatty acids, analyzed from the same chromatograms as the data shown in panels C and D.

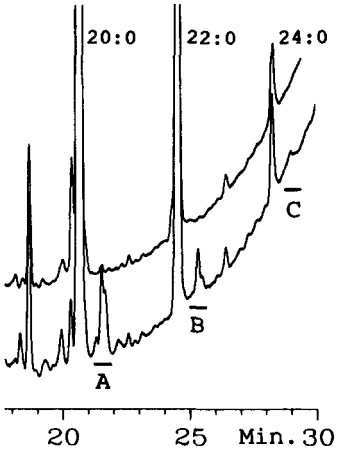

Fig. 6. Gas chromatograms of hydrogenated fatty acids obtained from 25 μg of an LDL pool. A, B, and C indicate the positions of peaks of acetylated OH-18, OH-20, and OH-22, respectively. The quantities of compounds in regions A, B, and C in the unacetylated sample (upper curve) were relatively small (0.22 ng, 0.06 ng, and 0.00 ng per injection, respectively) compared with the acetylated sample (lower curve: 2.89 ng, 1.11 ng, and 0.34 ng, respectively). The calculated contents of OH-18, OH-20, and OH-20 were 1.93, 0.80, and 0.24 μg/mg total fatty acids, respectively.

the treatment, eliminating the danger of oxidation taking place during the procedure. As BHT is added to the sample before lipid extraction, which can be accomplished within a few minutes, the likelihood of oxidation during handling of the specimens is minimized.

For optimum results it is essential that all unsaturated fatty acids be completely hydrogenated. We chose chloroform–methanol as the solvent for hydrogenation, because it dissolves all plasma lipids and allows their thorough hydrogenation. However, some of the allylic hydroperoxyl and hydroxyl groups are lost by hydrogenolysis under these conditions, giving rise to corresponding unsubstituted fatty acids and lowering the yield. To avoid hydrogenolysis, our procedure starts with treatment of the sample with prereduced PtO_2 in methanol, which gave complete hydrogenation of the HODE and HETE standards with less than 5% conversion to 18:0 and 20:0, respectively.

As fatty acid hydroperoxides are unstable in gas chromatography, they have to be reduced to hydroxy derivatives before analysis. Catalytic hydrogenation accomplishes this task (8). For gas chromatography, the fatty acids were esterified in acid dry methanol, converting both free and esterified fatty acids to methyl esters. The saponification step preceding transesterification is for removal of the nonsaponifiable lipid constituents, most of which is cholesterol. The nonsaponifiable lipids move in gas chromatography after the fatty acid methyl esters. The most recent version of the method omits the saponification step. This lengthens the time taken by a gas chromatography run to 50 min, but even with the saponification step included, an increase in sensitivity during the analysis of the smallest concentrations of hydroxyacids will necessitate long runs in order to clean the column for the next analysis.

In addition to reducing the number of extractions, the omission of saponification has the advantage of increasing the yield of the method. The recoveries of HODE and HETE added to a plasma lipid sample increased from 80 and 74%, respectively, to 88 and 86%, respectively, when the saponification step was omitted. The most probable cause for the loss of the hydroxyacids (saturated at this stage) during the saponification step is that their solubility in petroleum spirit differs from that of heptadecanoic acid, used as the internal standard. A more ideal internal standard would be a hydroxyacid. The only commercially available compound that we have found suitable for the purpose is 2-hydroxy-hexadecanoic acid, which has an R_t of 14.2 min (after 17:0) and moves to R_t 16.5 (after 18:0) after acetylation. However, its use did not improve recoveries in comparison to 17:0.

The recovery of the method was assayed by using commercial HODE and HETE from freshly opened ampoules. As we did not have methods other than gas chromatography for the quantitative determination of these compounds, we used the concentrations given by the manufacturer in our calculations of recovery. Esterification of the compounds by diazomethane followed by gas chromatography gave a maximum content similar to the present method. It is possible that some portion of the standards may have deteriorated during transportation and storage. In that case the percentage recoveries are higher than the maximum 86–88% that we now report.

Acetylation of the hydroxyl groups lends specificity to the method by translocating the hydroxyacids to regions

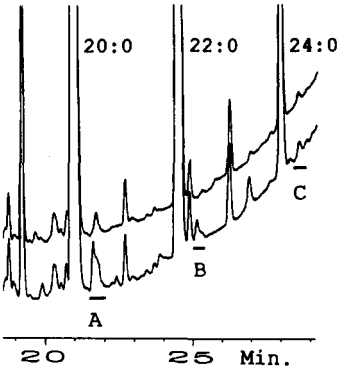

Fig. 7. Gas chromatograms of hydrogenated fatty acids obtained from 250 μl of whole plasma of a healthy person. A, B, and C indicate the regions of the peaks of acetylated OH-18, OH-20, and OH-22, respectively. The unacetylated sample (upper curve) has unidentified peaks in the regions A to C (A 0.83 ng; B 0.13 ng per injection), hampering quantification of the peaks in the acetylated sample (lower curve: A 2.20 ng; B 0.99 ng). The calculated contents of OH-18 and OH-20 were 0.144 and 0.090 μg/mg total fatty acids, respectively.

where there are usually only trace components. Prior gas chromatography of the sample in unacetylated form thus serves as an individual blank run for each hydroxyacid analysis. When hydroxyacids are in high concentration, the blank runs are actually unnecessary and the hydroxyacids can be quantified from a single run of the acetylated sample. Acetylation was the derivatization of our choice, because it increases the retention times of the hydroxyacids more than trimethylsilylation, for example, which leaves the hydroxyacid peaks too close to the large unsubstituted fatty acid peak two carbon atoms longer.

The sensitivity of the method is not so much dependent on the sensitivity of gas chromatography per se than on the presence of unknown components with the same R_ts as the acetylated hydroxyacids. The proportion of these components becomes higher as the concentration of the hydroxyacids decreases. It is likely that the problem can be overcome by the choice of another derivative and/or gas chromatography column, causing the unknown compound and hydroxyacids to migrate with different R_ts.

To test the sensitivity of the method, we analyzed the contents of hydroxyacids in freshly taken EDTA-plasmas of seven healthy laboratory workers. The concentration of OH-18 ranged from 0.80 to 1.48 μmol/l. From the gas chromatography–mass spectrometry results of Wang and Powell (7) it can be calculated that the mean content of 9-OH-18 + 13-OH-18 in LDL of plasma of normal New Zealand White rabbits was 0.043 μmol/l and rose to 0.54–0.63 μmol/l after 8–15 weeks of cholesterol feeding, when serum cholesterol increased to 4.2–5.6 mmol/l. Because our values comprise all plasma lipoproteins, they are not much different from those found in hypercholesterolemic rabbits. The concentration of OH-18 + OH-20 in our subjects ranged from 0.92 to 2.25 μmol/l. The content of lipid peroxides in normal human plasma ranged from 0.26 to 0.73 μmol/l in the study of Marshall, Warso, and Lands (16), who used a method based on cyclooxygenase activity. On the other hand, by using a chemiluminescence assay, Frei et al. (17) concluded that human plasma from healthy subjects does not contain lipid hydroperoxides in concentrations greater than 0.03 μmol/l. It is obvious that only a fraction of the hydroxyacids in our samples were in the form of hydroperoxides.

During lipid peroxidation, 9- and 13-hydroperoxides are formed from linoleic acid and 5-, 8-, 9-, 11-, 12- and 15-hydroperoxides from arachidonic acid (1). A portion of these hydroperoxides is spontaneously reduced to corresponding hydroxy fatty acids. In the first phase of the present method, the hydroxy- or hydroperoxy-derivatives of linoleic acid are converted to 9- and 13-OH-octadecenoic acid (OH-18) and those of arachidonic acid to 5-, 8-, 9-, 11-, 12- and 15-OH-eicosanoic acid (OH-20). In our gas chromatograms, 9-OH-18 and 13-OH-18 were separated from each other by about 50%, and 15-OH-20 was partly separated from the rest of the OH-20 isomers, allowing a semiquantitative evaluation of proportions of the various isomers. To achieve better separation of the isomers and also those arising from other polyunsaturated fatty acids and oleic acid (10), we are testing more polar gas chromatography phases.

The content of OH-18 in our LDL preparations ranged from 2.6 to 6.2 nmol/mg LDL protein and that of OH-20 from 1.4 to 2.7 nmol/mg LDL protein. In isolated human LDL analyzed by Lenz et al. (6), the concentrations of each of four different positional and *cis, trans*-isomers of OH-18 were below the detection limit of the method (< 0.3 nmol/mg LDL protein), as were the contents of each of the four positional isomers of OH-20. Wang, Yu, and Powell (10) report mean concentrations of 0.9 and 1.5 nmol/mg LDL protein for OH-18 and OH-20, respectively, in their "native" LDL. In LDL isolated from normal and hypercholesterolemic New Zealand White rabbits, the mean contents of OH-18 were 0.21 and 0.42 nmol/mg LDL protein, respectively, as calculated from the results of Wang and Powell (7). Our high values are apparently due to oxidation of LDL during the long dialysis for removal of EDTA from the samples before the in vitro oxidation studies.

In our Cu^{2+}-catalyzed oxidations, the content of OH-18 rose to 58–113 nmol/mg LDL protein in 6 h and to 93–114 nmol/mg in 24 h. The 6-h results agree with the 5-h value of Wang et al. (10), whose oxidation conditions resembled ours. Our 6-h values were appreciably higher and 24-h values lower than the corresponding mean values of Lenz et al. (15.6 and 178 nmol/mg, respectively) (6), who used milder conditions. In mild conditions the monohydroperoxides may last longer before breakdown into shorter chain compounds. This is probably why OH-20 in our experiments and also in those of Wang et al. (10) only increased during 5–6 h, whereas Lenz et al. (6) recorded a continuous rise up to a mean of 18.8 nmol/mg in 24 h.

In conclusion, we have introduced a technique for the assay of fatty acid hydroperoxide-derived hydroxyacids formed during lipid oxidation. The method involves catalytic hydrogenation of the lipid extract, conversion of the resulting saturated hydroxy and unsubstituted fatty acids to methyl esters, and analysis of these by gas chromatography both before and after acetylation of the hydroxyl groups. Acetylation moves the hydroxyacids to positions usually occupied by trace components, whose contents can be assayed from the chromatogram obtained from the unacetylated sample. The method has been mainly used for analysis of hydroperoxides in isolated LDL and monitoring in vitro LDL oxidation. Its applicability for quantification of fatty acid oxidation products formed in vivo is currently being assessed.

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