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Lipid oxidation in blood plasma of patients with neurological disorders

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ABSTRACT: Nuclear magnetic resonance (NMR) spectra of blood plasma lipids from lyophilized plasma samples from patients with neurological disorders stored for several weeks in an evacuated exsiccator show characteristic differences compared to freshly lyophilized plasma samples. The main differences concern the unsaturated fatty acids, e.g., the extent of unsaturation and their structural composition. The total amount of double bond signals of unsaturated fatty acids are noticeably reduced in intensity and new signals arise from conjugated double bonds. These signals can be assigned to keto-octadecadienoic acid (KODE) or hydroxy-octadecadienoic acid (HODE). The proton and carbon NMR chemical shifts and their structural assignment to the main molecular components are given. Whereas the KODE and HODE signals occur only as storage artifacts in the spectra, we have found small amounts of 9,11-octadecadienoic acid also in fresh blood plasma of controls. Its concentration is about 60 μM. In two-dimensional H,H total correlation spectroscopy spectra also a very low amount (6–7 μM) of 13-HODE can be detected. © 2000 Elsevier Science Inc.

INTRODUCTION

Nuclear magnetic resonance (NMR) spectroscopy is an excellent analytical tool to study the complex composition of body fluids without prior separation into individual components for example by chromatography. Even the molecular structure of unknown metabolites can be elucidated within the mixture, provided the concentrations are above 10 μM. A collection of one and two dimensional NMR techniques is available to solve these problems. As the composition of body fluids reflects the metabolic state of healthy or pathological situations, fast and efficient analytical methods are required. In particular the study of structurally very similar lipids within a lipid fraction is still a challenge. As the lipid composition and concentration in blood and brain changes in the course of several neurological disorders, it is important to know about possible storage artefacts. NMR spectra of lipid extracts show a normally indistinguishable overlaps of numerous resonance lines because of the structural similarities of the involved fatty acids and their derivatives. Only the highest magnetic field strength (currently 18.8 Tesla, which corresponds to a resonance frequency of 800 MHz) are capable to disperse coinciding signals. In addition, two-dimensional (2D) NMR detection methods [1], which correlate the resonance line of carbons and their directly attached hydrogen, are able to supply sufficient information for a reliable peak assignment [9], also see below.

During routine blood plasma analysis we found characteristic lipid peroxidation products of unsaturated fatty acids in human blood plasma lipids. We identified these lipid oxidation products as conjugated dienes. Signals of conjugated fatty acid dienes in blood plasma are normally near or below the detection limit of NMR spectroscopy. In some samples however their concentration is increased by a factor of 10–30. These oxidized lipids are formed during long time storage of lyophilized blood plasma. Normally lyophilization is used to prevent enzymatic and other reactions in body fluids. However, the unsaturated fatty acids of lyophilized, dry blood plasma are oxidized at room temperature in the presence of oxygen.

The formation of conjugated dienes is an oxidation process initiated by free radical species [5] (see Scheme 1). Oxygen is added to form a dienylperoxyl radical, which is subsequently transformed into a hydroperoxid. The final reaction products are ketodienoic acid and hydroxydienoic acid. Depending upon the starting material several isomers are possible. For example from linoleic acid (18:2), the secondary reaction products are 13-keto-9,11-octadecadienoic acid (13-KODE), 9-keto-10,12-octadecadienoic acid (9-KODE), (Z,E and E,E) 13-hydroxy-9,11-octadecadienoic acid (13-HODE) and (Z,E and E,E) 9-hydroxy-10,12-octadecadienoic acid (9-HODE).

EXPERIMENTAL

All experiments were performed on Bruker DRX 600 or 800 MHz spectrometers at 300 K using a 5-mm H,C,N inverse triple resonance probe with actively shielded field gradient coils. Gradients were shaped by a waveform generator and amplified by a Bruker Acustar amplifier. Sinusoidal three-axis gradients of 1 ms duration and a recovery time of 100 μs were used for the echo/antiecho gradient selection. Fine tuning of the gradient amplitude ratios (40:10.08) resulted in optimum signal intensities. Low power adiabatic composite pulse decoupling with WURST [4] has been used for C13-decoupling. A sensitivity-improved heteronuclear single quantum correlation (HSQC) [3,6] in the version described in [8] with a relaxation delay of 1 s was used. The semi-selective HSQC experiments have been acquired with 1-k increments in F1 and 96 scans per increment and a total spectral recording time of 17 h to obtain a resolution of 5 Hz in F1. An

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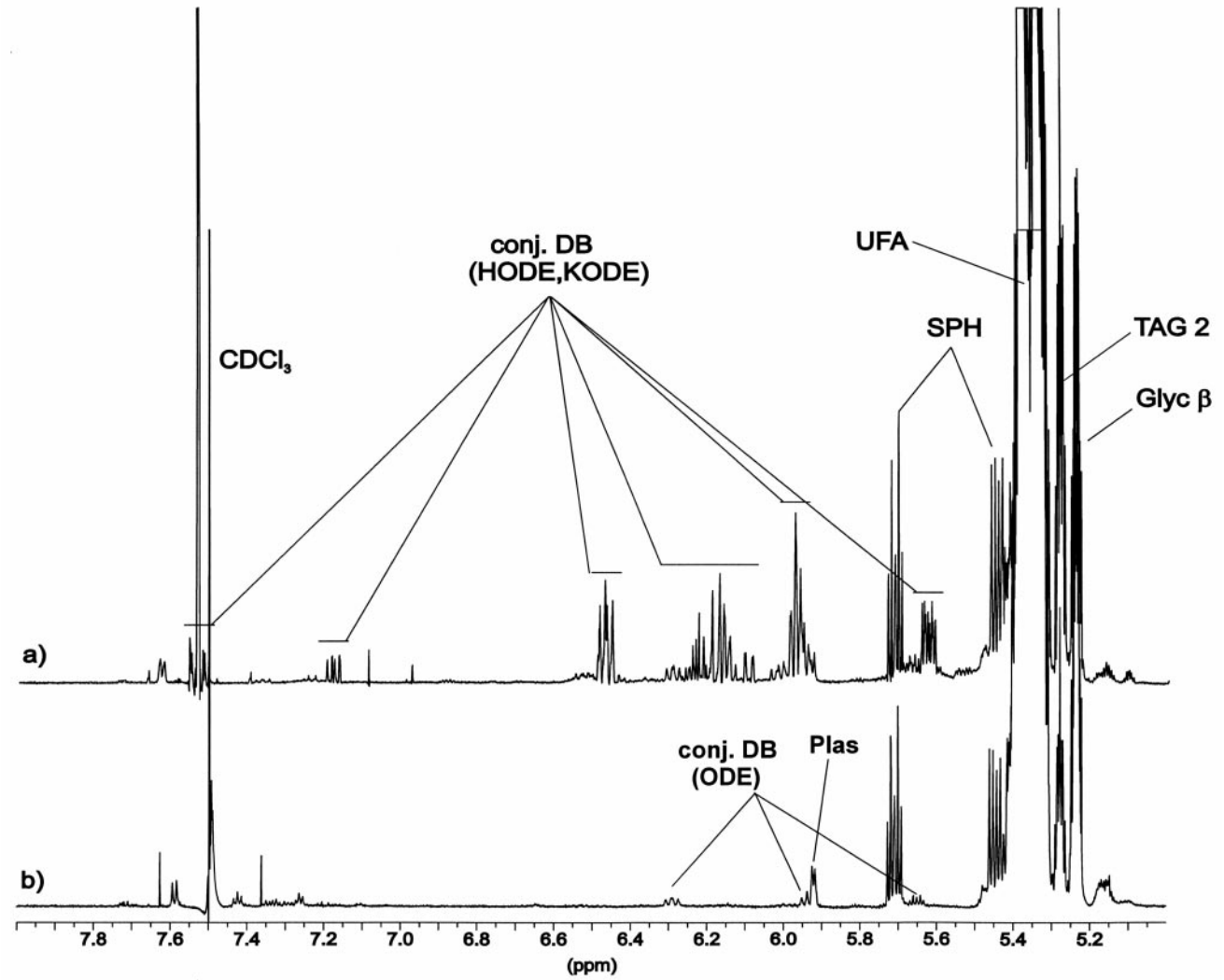


FIG. 1. Comparison of the low field sections of 800 MHz proton nuclear magnetic resonance spectra of human blood plasma lipids. (a) 1-year storage. (b) Fresh sample. Abbreviations: conj. DB; conjugated double bond signals; Glyc, glycerol backbone; HODE, hydroxy-octadecadienoic acid; KODE, keto-octadecadienoic acid; ODE, octadecadienoic acid; Plas, plasmalogen; SPH, sphingomyelin; TAG, triacyl glycerol; UFA, unsaturated fatty acid.

acquisition time of 426 ms has been used to acquire 2048 data points for a spectral width of 3 ppm in the proton dimension. Folded signals have been suppressed using digital quadrature detection (DQD, Bruker). For details of the semi-selective HSQC see [7,8]. All spectra are pure absorption mode spectra and were processed with a $\pi/2$ shifted squared sine bell in F2 (1H) and a $\pi/4$ shifted squared sine bell in F1 (13C).

Simultaneous Lipid/Water Extraction

Ten milliliters blood were collected in a heparinized centrifugal tube and centrifuged at 4000 rpm for 15 min. Three milliliters plasma were taken from the supernatant and were lyophilized. The lyophilized plasma was transferred into a glass centrifugal tube and was mixed with 2 ml $CHCl_3$ and 2 ml MeOH. The mixture was carefully shaken until the suspension was homogeneous (1–2 min). The mixture was then centrifuged at 4000 rpm for 15 min. The supernatant has been removed into a clean tube. The residual pellet was suspended in 1 ml $CHCl_3$ and 1 ml MeOH, shaken and centrifuged again. The lipophilic supernatants were combined and washed with 1 ml of H_2O. After careful shaking the mixture was centrifuged at 4000 rpm for 5 min. The upper H_2O phase was collected and combined with the solid residue. The CHCl3/MeOH phase was cooled for 15 min at -20°C to allow for a better phase separation. The residual water is removed and the $CHCl_3$/MeOH solvent is evaporated in a stream of dry nitrogen. The lipids were redissolved in 0.6 ml $CDCl_3$/MeOD (2:1) and transferred into a NMR tube. The solid residue with the 1 ml of water was carefully stirred and shaken until the suspension was homogeneous. Then the mixture was centrifuged at 4000 rpm for 15 min. The solid residue was suspended again with another 1 ml of H_2O, shaken and centrifuged. The combined water phases were lyophilized. The water phase still contains considerable amounts of methanol, which evaporates in the beginning (good cooling is recommended!). The water phase contains small amounts of ethanol also, which is generally used as a stabilizer

TABLE 1

CHEMICAL SHIFTS OF 9,11-OCTADECADIENOIC ACID FOUND IN ALL BLOOD PLASMA SAMPLES

Position	^{1}H (ppm)	^{13}C (ppm)
A-2	1.40	
A-1	2.11	27.12 (?)
A	5.65	134.7
B	6.30	125.7
C	5.94	128.9
D	5.28	129.7
D-1	2.17	27.92
D-2	1.37	

All shifts are measured at 300 K and are referenced to MeOD = 3.349 ppm/48.93 ppm (^{1}H/^{13}C).

TABLE 3

ASSIGNMENT OF 13-HYDROXY-9,11-OCTADECADIENOIC ACID IN BLOOD PLASMA LIPIDS AFTER LONG STORAGE

Position	^{1}H (ppm)	^{13}C (ppm)
C15	1.33	
C14	1.53	37.52
C13	4.10	72.82
C12	5.63	136.1–136.3
C11	6.47	126.0
C10	5.97	128.2–128.3
C9	5.42	132.5–132.8
C8	2.18	28.0
C7	1.39	

All shifts are measured at 300 K and are referenced to MeOD = 3,349 ppm/48.93 ppm (^{1}H/^{13}C).

for $CDCl_3$. To remove it completely, the lyophilization should last at least 24 h. The water soluble phase is redissolved in 0.6 ml D_2O and transferred into a NMR tube.

The lyophilized lipid samples which contained the artefacts had been stored at ambient temperature in an exsiccator under vacuum for weeks or months.

Concentrations

The lipid concentration was quantified using a trimethylsilylpropionic-acid capillary with a reference concentration of triacylglycerol. A concentration of 0.65 mM was estimated for sphingomyelin (SPH) in normal blood (This value was used to calculate the absolute concentration of the oxidized lipids).

RESULTS AND DISCUSSION

Figure 1 shows a comparison of the low field section of 800 MHz proton NMR spectra of blood plasma lipids extracted from a long term stored, e.g., several weeks, (a) and fresh plasma sample (b). High concentrations (0.3–0.5 mM) of fatty acids with conjugated double bonds can be seen in the spectrum of the long term stored sample.

However, only one conjugated fatty acid is detectable in both samples: It is a fatty acid with two conjugated double bonds, but without either a neighboured hydroxyl or a keto group. This fatty acid is present in all of our spectra of human blood plasma lipids. It is assigned to 9,11-octadecadienoic acid (9-ODE). Its concentration in fresh, control blood plasma is about 60 μM. The peak assignments are given in Table 1. Additionally, a very low amount (6–7 μM) of 13-HODE can be detected in 2D NMR spectra (see below) of normal blood also.

To assign the individual signals to the different oxidized lipid species, we have performed several 2D experiments (H,H- total correlation spectroscopy [TOCSY], HSQC, HSQC-TOCSY and heteronuclear multiple bond correlation [HMBC]). The resulting assignments of various lipid compounds are listed in Tables 1–4. For the general lipid assignment see [9].

TABLE 2

ASSIGNMENT OF THE MAIN KETO-OCTADECADIENOIC ACID IN BLOOD PLASMA LIPIDS AFTER LONG TERM STORAGES

Position	^{1}H (ppm)	^{13}C (ppm)
CO-3	1.34	31.73
CO-2	1.63	24.55
CO-1	2.59	41.08
Co	–	203.1
A	7.53	138.25
B	6.181	129.53
C	6.156	127.23
D	5.96	143.6–143.3
D-1	2.34	28.67
D-2	1.46	
D-3	1.35	

All shifts are measured at 300 K and are referenced to MeOD = 3.349 ppm/48.93 ppm (^{1}H/^{13}C).

TABLE 4

MINOR HYDROXY-OCTADECADIENOIC (HODE) AND KETO-OCTADECADIENOIC ACID (KODE) COMPONENTS PRESENT IN BLOOD LIPIDS AFTER LONG TERM STORAGE

	Position	^{1}H (ppm)	^{13}C (ppm)
KODE-2	A	7.17	144.44
	B	6.09	127.92
	C	6.23	146.93
	D	6.23	129.22
	D-1	2.20	33.39
HODE-2	A-1	4.05	72.77
	A	6.15	131.11
	B	5.54	133.98
	C	6.02	130.06
	D	5.67	135.38
	D-1	2.07	32.83
HODE-3	A-1	3.58	
	A	6.69	
	B	6.43	
	C	5.97	
	D	5.72	

All shifts are measured at 300 K and are referenced to MeOD = 3.349 pm/48.93 ppm (^{1}H/^{13}C).

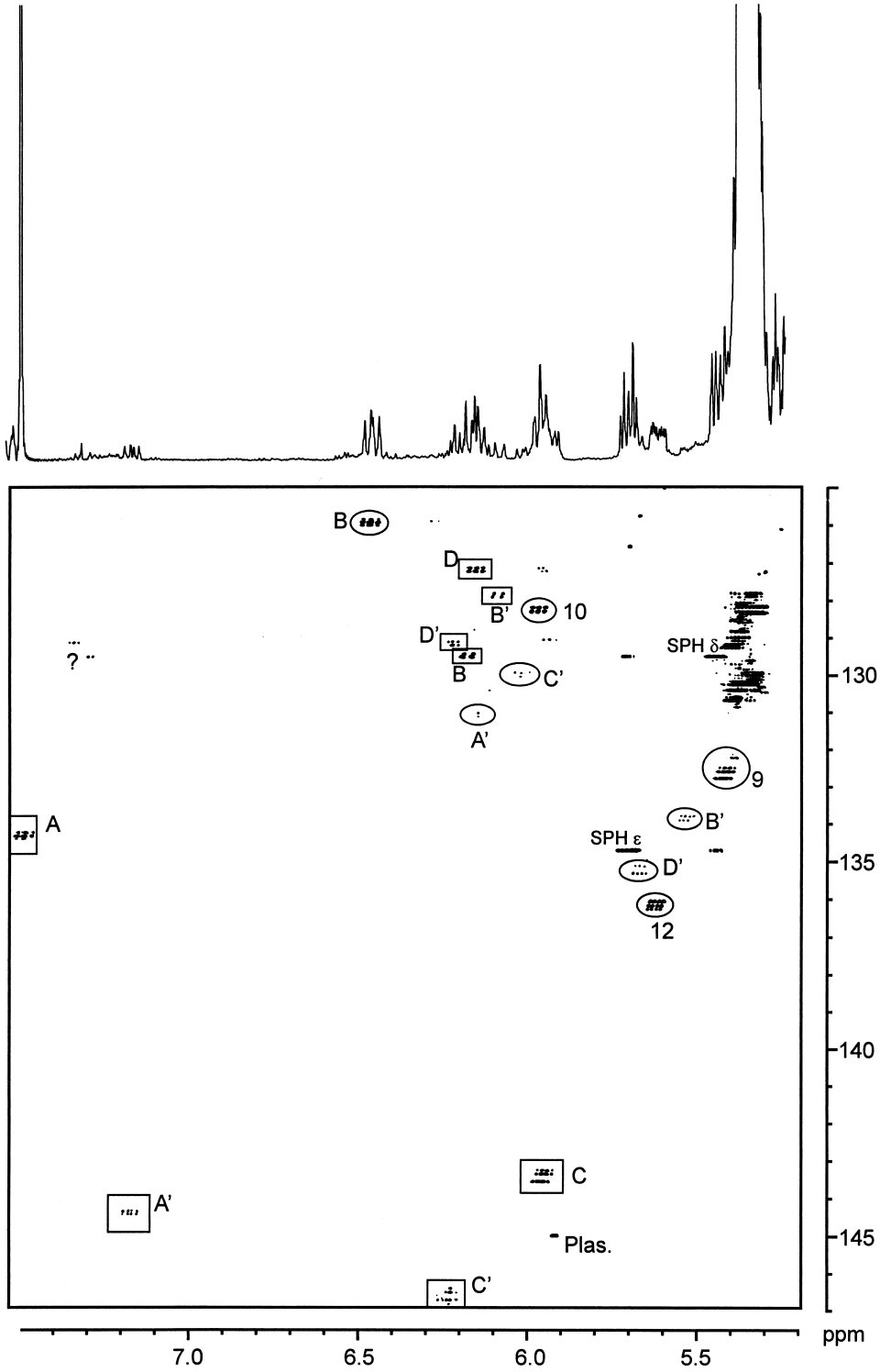


FIG. 2. Lowfield region of a heteronuclear single quantum correlation spectrum of human blood plasma lipids from a long-storage-sample. Signals arising from conjugated double bonds are encircled. Squares indicate keto-octadecadienoic acid signals, and ellipsoids indicate hydroxy-octadecadienoic acid (HODE). The letters indicate the position in the coupling network with respect to the keto or hydroxyl function at position A-1. The numbering gives the position in 13-HODE. Other signals are Plas, plasmalogen; SPH, sphingomyelin and the normal double bond signals in the upper right.

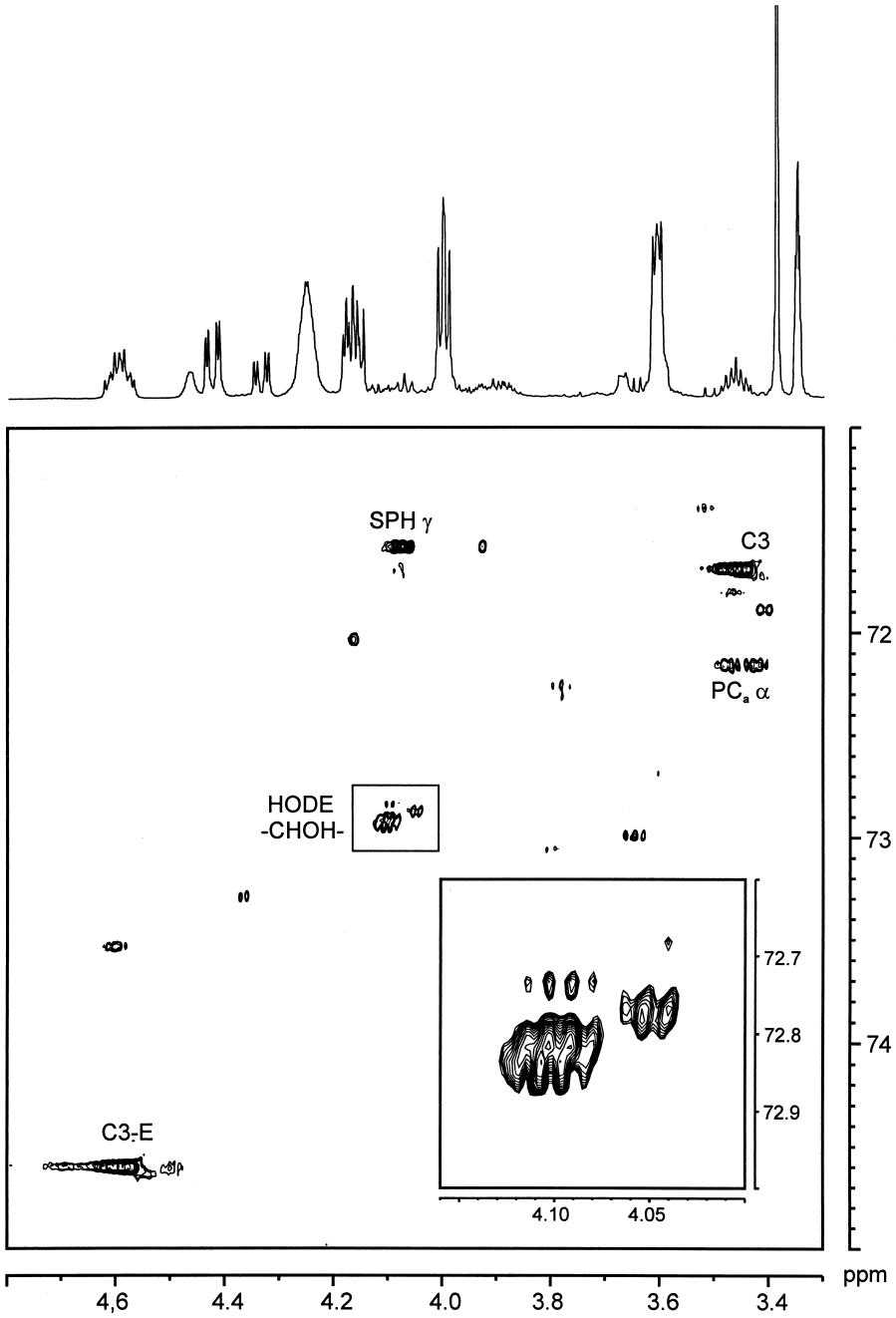


FIG. 3. Midfield region of a heteronuclear single quantum correlation spectrum of human blood plasma lipids from a long-storage-sample. The encircled -CHOH- signals of hydroxy-octadecadienoic acid (HODE) compounds are enlarged in the inset. Four different HODE signals can be distinguished. Abbreviation: SPH, sphingomyelin.

The HSQC experiment correlates the hydrogen (proton) chemical shift in the first dimension with the carbon chemical shift of the directly attached carbon in the second dimension of the spectrum (see Figs. 2–4). As the proton chemical shifts of the double bond protons are often very similar, the much better separation in the carbon dimension is utilized to separate these superimposed signals of protons attached to double bond carbons. As a result one obtains the chemical shifts of individual proton/carbon pairs from a HSQC spectrum. TOCSY experiments are now used to correlate a proton with its neighboured protons to which it is connected within a scalar spin-spin-coupling network. The H,H TOCSY experiment is the most sensitive 2D experiment. High magnetic fields (600 and 800 MHz) are needed to separate the conjugated double bond signals with very similar chemical shifts. Even then

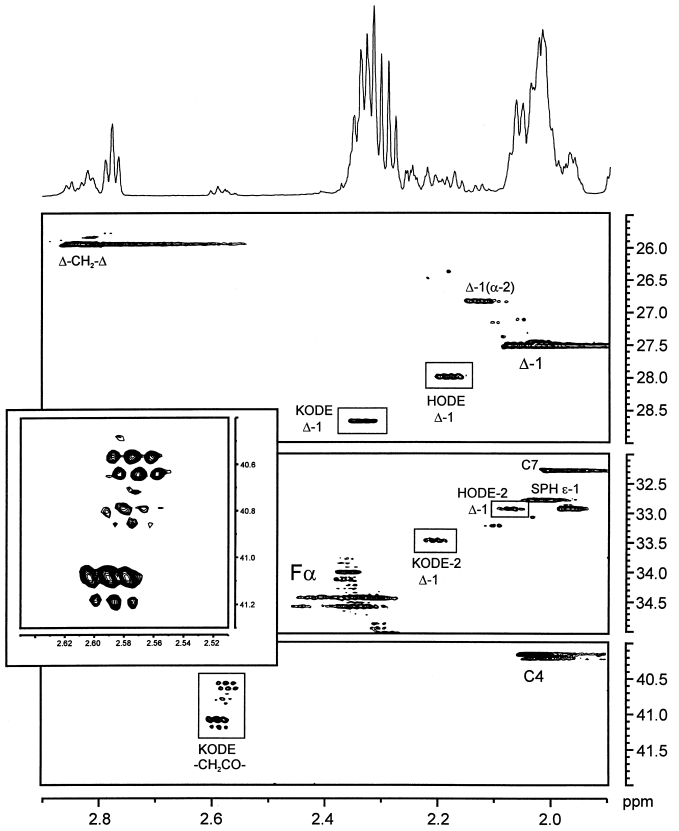


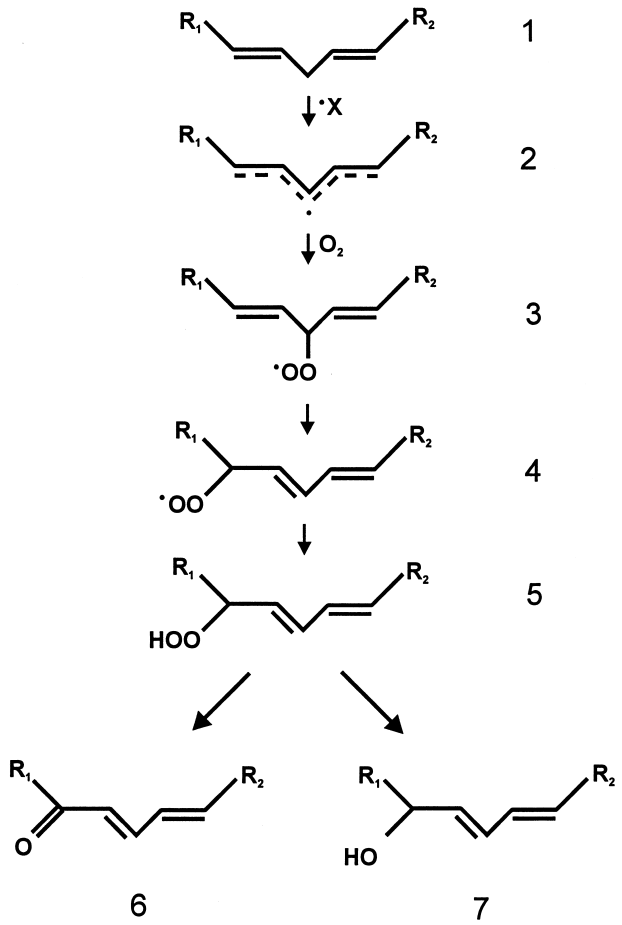
FIG. 4. Highfield region of a heteronuclear single quantum correlation spectrum of human blood plasma lipids from a long-storage-sample. The new signals from keto-octadecadienoic acid (KODE) and hydroxy-octadecadienoic acid (HODE) are encircled. The inset shows the enlarged -C<u>H</u>$_2$CO- signals of KODE components. Six signals can be distinguished. Abbreviation: SPH, sphingomyelin.

they are unable to provide a sufficient resolution for some double bond protons. In this case it is necessary to use the HSQC-TOCSY experiment. It combines the HSQC experiment with a TOCSY correlation step. Again the good chemical shift dispersion of carbon is used for signal separation. TOCSY correlations to characteristic high field proton signals, like the Δ-1 (e.g., protons attached to the allylic carbon next to the double bond) or the CO-CH position are used for an unequivocal assignment. For a detailed description of this method for the identification of lipids, see [9]. Finally, the quaternary C═O carbons have to be assigned, which is achieved with the HMBC experiment. In this experiment only protons coupled coupled via two or more chemical bonds to a carbon are correlated and give rise to peaks at the corresponding carbon position. It is possible to see correlations of the carbonyl CO carbon with the CO-1 protons ($3J_{CH}$) or with the CO-2 protons ($^3J_{CH}$). Although it is the most sensitive experiment to establish assignments of quarternary carbons, it is too insensitive to get signals from the minor KODE components in plasma spectra. Only the carbonyl carbon shift of the main KODE component (at 203 ppm) could be detected.

Figure 2 shows the HSQC low field region of the blood plasma lipids. The assignment of 13-HODE is in agreement with Feussner et al. [2]. Whether the other signals have the keto or hydroxyl group at position 9 or at position 13 is not clear so far. The signals are labeled A, B, C and D to indicate a coupling network with the keto or hydroxyl group at position A-1. There is one main HODE species (13-HODE) and one main KODE species (13-KODE). With sufficient resolution these signals can be further resolved. Every encircled signal consists of at least two different components. These different components represent different phosphatidyl choline species, e.g., a fatty acid bound at the glycerol β-position or bound at glycerol γ-position. Alternatively, the position of the unsaturated fatty acid is the same, but different saturated fatty acids like 18:0 or 16:0 are bound to the glycerol subunit. One minor HODE and one minor KODE component can be detected in the lowfield region of the HSQC spectrum (HODE-2, KODE-2, labelled with ′). Another minor component can be assigned in H,H-TOCSY spectra (HODE-3), see Table 4. HODE-2 is tentatively assigned to 9-HODE, but no reference material was available for further proof.

Figure 3 shows the midfield region of the HSQC spectrum with the -C<u>H</u>OH- proton signals of HODE components. In contrast to the lowfield region these signal are not very clearly identifiable in one-dimensional ^{1}H spectra, because of overlap with other strong signals (SPH). However, the signal are well separated in the 2D HSQC spectrum and at least four components can be distinguished. The left part at 4.05 ppm represents HODE-2.

Figure 4 shows the corresponding highfield region, where new signals arise from the Δ-1 protons of HODE and KODE components and from the -C<u>H</u>$_2$-CO- protons of KODE compounds. The



SCHEME 1.

inset shows the enlarged KODE -$C\underline{H_2}$-CO- signals. At least six different signals can be distinguished. HODE-2 and KODE-2 show a distinct lowfield shift of the Δ-1 signals.

In conclusion, we have shown that lyophilized blood plasma samples are very sensitive to oxygen exposure and form increasing amounts of peroxydation products with long term storage. Several of these species have been assigned to fatty acids with α-hydroxyl or α-keto dienon substructures. A complete assignment of their relevant carbon and proton chemical shifts has been given. For the analysis of blood plasma lipids of patients with neurological disorders we recommend to store the native samples frozen and not lyophilized, because the lipids are better protected from oxygen in the native state.

In fresh blood samples we have identified also two fatty acids with conjugated double bonds but without an oxygen function in the α-position to the dien group: 9-ODE (about 60 μM). Additionally a very small amount of 13-HODE (6–7 μM) has been found. These signals appear in all measured samples and obviously belong to the normal composition of human blood.

Oxidation is prevented if blood plasma is not stored in the lyophilized state but as immediately frozen blood plasma. It is suggested to perform the lyophilization only shortly before the extraction procedure. Lipids in a chloroforme/methanol mixture underwent no measurable oxidation after extraction.

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