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DETERMINATION BY CAPILLARY GAS-LIQUID CHROMATOGRAPHY OF THE ABSOLUTE CONFIGURATIONS OF UNSATURATED FATTY ACID HYDROPEROXIDES FORMED BY LIPOXYGENASES

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Summary

The absolute configurations of a number of unsaturated hydroperoxy fatty acids obtained by lipoxygenase catalysis were investigated by capillary gas-liquid chromatography after proper derivatization. To this end the hydroperoxy groups were reduced and the resulting hydroxyl groups acetylated. Oxidative ozonolysis of the acetylated methyl esters yielded acetylated 2-hydroxycarboxylic acids, which were converted into *R*-(—)-2-butyl esters and then reacetylated. The ratio of the resulting diastereomers, which reflects the optical purity of the chiral centers in the parent hydroperoxy fatty acids, was determined by capillary gas-liquid chromatography. Application of this simple method to a number of mono- and dihydroperoxy fatty acids obtained by incubation with soybean lipoxygenase-1 or -2, or by corn-germ lipoxygenase yields enantiometric compositions which are in good agreement with results obtained by other methods.

For the characterization of lipoxygenases (linoleate:oxygen oxido reductase EC 1.13.11.12), which convert polyunsaturated fatty acids containing a 1,4-*cis*,*cis*-pentadiene system into hydroperoxides, product analysis in terms of regio- and stereoselectivities of the oxygenation is necessary. It has been shown that depending on the source of the enzyme and the incubation conditions different isomeric hydroperoxides can be produced from one substrate fatty acid [1]. The recent progress in the separation by means of high-performance liquid chromatography of hydroperoxy fatty acids or the corresponding hydroxy compounds enables the fast determination of the regio-

specificity. Furthermore, high-performance liquid chromatography gives access to these isomers on such a scale that these compounds become available for the analysis of the stereospecificity of their formation. As pointed out earlier, measurement of the optical rotation has inherent limitations in the determination of enantiomeric compositions. Recently, we described a lanthanide-induced ^{1}H-NMR shift method for this purpose, which makes use of diastereomeric (+)-α-methoxy-α-trifluoromethylphenylacetate esters of the unsaturated hydroxy fatty acid methyl esters [2]. Hamberg [3] developed a gas-liquid chromatographic method which is based on the analysis of fragment diastereomeric menthyloxyformates of 2-hydroxycarboxylic acid methyl esters obtained after ozonolysis of the (—)-menthyloxyformates of the methyl-hydroxydienoates (see also Refs. 4, 5). The present paper presents a more simple gas-liquid chromatographic method for the determination of the absolute configurations of 2-hydroxycarboxylic acids obtained after ozonolysis of the acetylated methyl-hydroxydienoates. Diastereomers are then formed by conversion into *R*-(—)-2-butyl esters, and the determination of the composition of the diastereomeric mixture is performed with capillary gas-liquid chromatography on an non-chiral phase.

D_RL_S-Malic acid, L_S-malic acid and D_RL_S-2-hydroxyheptanoic acid were obtained from commercial sources*. D_RL_S-2-Hydroxyadipic acid and D_RL_S-2-hydroxysebacic acid were synthesized from adipic acid and sebacic acid, respectively [6]. L_S-2-Hydroxyadipic acid was prepared from L_S-2-aminoadipic acid. This reaction, which is carried out with aqueous nitrous acid, has been reported to proceed with retention of configuration [7]. A racemic mixture of 2-aminosebacic acid was enriched in the L enantiomer by treatment with a crude D-amino acid oxidase preparation from hog-kidney which was obtained from Sigma. The amino acid (22 mM) was incubated with enzyme (74 μM) in 0.02 M sodium pyrophosphate buffer (pH 8.3) at 37°C under an oxygen atmosphere and in the presence of catalase (2 nM). The reaction could be monitored by the measurement of the optical rotation. After separation from the other products by preparative thin-layer chromatography (eluent: *n*-butanol/pyridine/H_2O, 1:1:1, v/v/v), the amino acid was converted into the corresponding hydroxy compound [7]. The enantiometric composition of the thus obtained 2-hydroxysebacic acid was L_SD_R = 57:43.

Methyl-13-L_S-hydroxy-9-*cis*,11-*trans*-octadecadienoate (I), methyl-9-D_S-hydroxy-10-*trans*,12-*cis*-octadecadienoate (II), methyl-9-L_R-hydroxy-10-*trans*,12-*cis*-octadecadienoate (IIa), methyl-15-L_S-hydroxy-5-*cis*,8-*cis*,11-*cis*,13-*trans*-eicosatetraenoate (IV), methyl-8-D_S,15-L_S-dihydroxy-5-*cis*,9-*trans*,11-*cis*,13-*trans*-eicosatetraenoate (V) and methyl-5-D_S,15-L_S-dihydroxy-6-*trans*,-8-*cis*,11-*cis*,13-*trans*-eicosatetraenoate (VI) were prepared as described before [2, 8, 9]. It should be noted that the indicated absolute configurations refer to the main constituent in the mixture of stereoisomers.

Methyl-13-L_S-hydroxy-9-*cis*,11-*trans*,15-*cis*-octadecatrienoate (III) was prepared from α-linolenic acid (0.36 mM) by incubation with soybean lipoxygenase-1 (10 nM) at 4°C in 0.1 M sodium borate buffer (pH 9.0)

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*L and D refer to the nomenclature according to the Fischer convention; subscripts *S* and *R* refer to the nomenclature according to Cahn, Ingold and Prelog.

flushed with oxygen. After the reaction was completed, the hydroperoxide was reduced with a 10-fold molar excess of sodium borohydride. Subsequently, the reaction mixture was acidified with HCl to pH 3.0 and the hydroxy fatty acid extracted with diethyl ether, esterified with diazomethane and purified by thin-layer chromatography on precoated plates (Merck 60F-254 silicagel 0.5 mm, eluent: hexane/diethyl ether, 70:30, v/v). *R*-(—)-2-Butanolic 1 M HCl was prepared by bubbling dry HCl through *R*-(—)-2-butanol (Fluka AG; percentage of *R* enantiomer approx. 94% [10]) and stored at −18°C in a dessicator.

Preparation of acetylated R-(—)-2-butyl esters of model compounds

To an ampoule containing 5 mg of 2-hydroxycarboxylic acid 0.5 ml of *R*-(—)-2-butanolic 1 M HCl was added. After heating for 2 h at 100°C the solvent was evaporated under reduced pressure. The residue was acetylated in 1 ml of pyridine/acetic anhydride (1:1, v/v) for 30 min at 100°C. After evaporation to dryness the residue was dissolved in $CHCl_3$ and analysed by capillary gas-liquid chromatography.

Ozonolysis

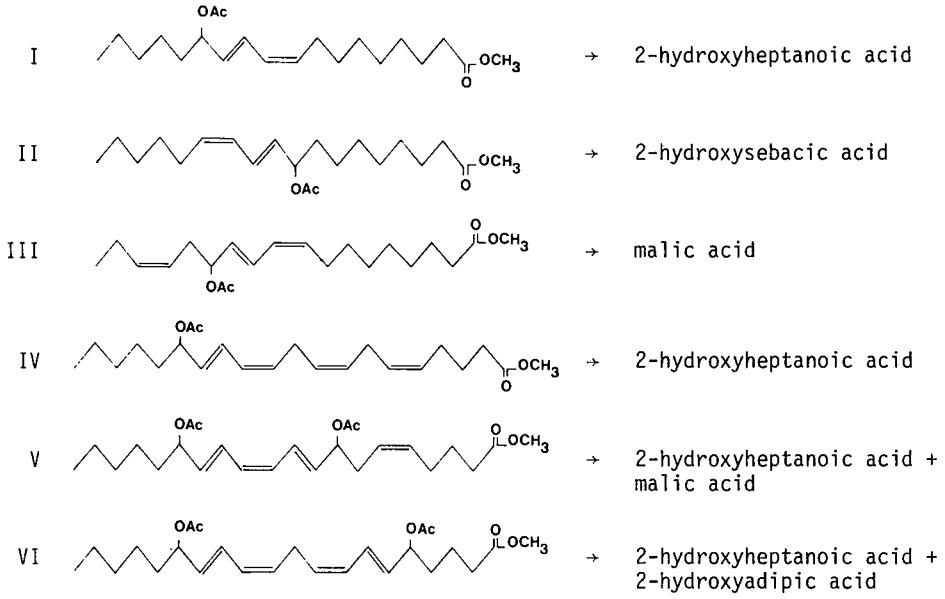
1 mg portions of the unsaturated hydroxy fatty acid methyl esters (I-VI) were acetylated in 2 ml of pyridine/acetic anhydride (1:1, v/v) for 30 min at 100°C. The reaction mixture was diluted with 5 ml diethyl ether and washed with 2 M HCl, 8% $NaHCO_3$ and finally with redistilled water. After evaporation of the organic phase to dryness the residue was dissolved in 2 ml chloroform, and oxygen containing approx. 0.5% (v/v) ozone was bubbled through the solution for 10 min at −15°C. The solvent was removed in vacuo and the residue dissolved in 0.5 ml CH_3COOH and 0.1 ml H_2O_2 (30%). This mixture was kept for 16 h at 50°C and then evaporated to dryness. Finally, the residue was treated with *R*-(—)-2-butanolic-HCl and pyridine/acetic anhydride as described for the model compounds.

Capillary gas-liquid chromatography

The acetylated *R*-(—)-2-butyl esters of 2-hydroxycarboxylic acids were analysed by capillary gas-liquid chromatography on SP-1000 (LKB Stockholm) as a non-chiral stationary phase either isothermally at different temperatures or with a temperature program from 110 to 200°C at 2°C/min. The column dimensions were 25 m × 0.3 mm inner diameter.

Ozonolysis of compounds I–VI gives rise to a number of 2-hydroxycarboxylic acids as depicted in Scheme I.

The gas chromatogram of the acetylated *R*-(—)-2-butyl esters of the relevant model compounds: D_RL_S-2-hydroxyheptanoic acid, D_RL_S-malic acid, D_RL_S-2-hydroxyadipic acid and D_RL_S-2-hydroxysebacic acid is shown in Fig. 1. The peaks belonging to the L_S- or D_R-2-hydroxy-dicarboxylic acids have been assigned by co-chromatography with the acetylated di-*R*-(—)-2-butyl esters of the corresponding, pure or enriched, L_S-2-hydroxy acids. For the identification of the peaks of D_RL_S-2-hydroxyheptanoic acid the ozonolysis product of 13-L_S-hydroxyoctadecadienoate (I) is used, because its absolute configuration has been well established [11]. It should be noted, that in spite



Scheme I. Formation of 2-hydroxycarboxylic acid derivatives from acetylated unsaturated hydroxy fatty acid methyl esters by oxidative ozonolysis.

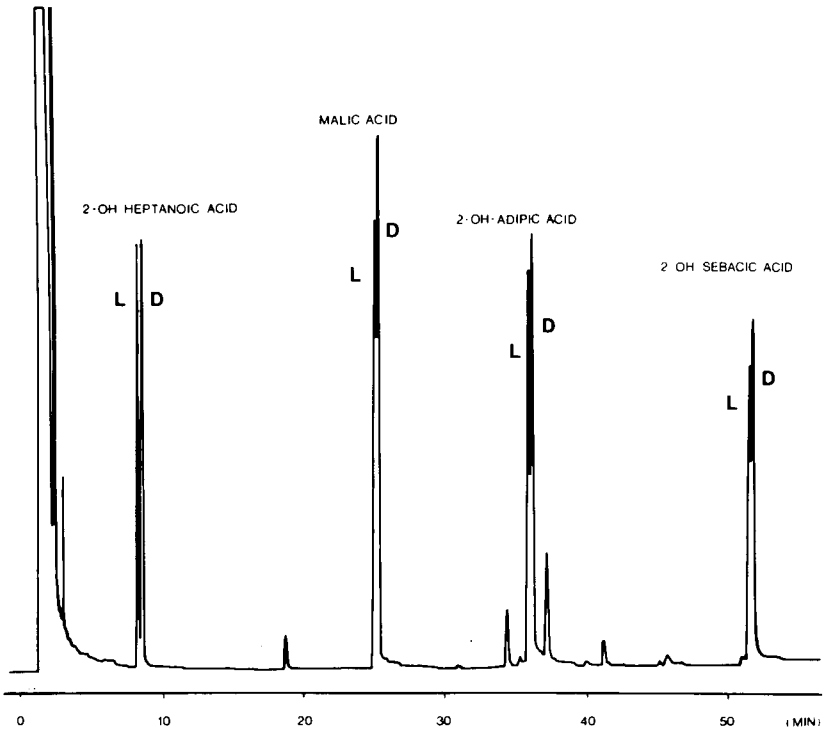


Fig. 1. Capillary gas-liquid chromatogram of acetylated *R*-(—)-2-butyl esters of racemic model compounds. Temperature program: 120°C isothermal for 10 min then 2°C/min to 220°C. Separation factors R_D/R_L: 2-OH-heptanoic acid 1.07; malic acid, 2-OH-adipic acid and 2-OH-sebacic acid 1.01.

of the contamination of the *R*-(−)-2-butanol with 6% of the *S* enantiomer the $D_R L_S$-2-hydroxycarboxylic acid derivatives give rise to only two peaks in the gas chromatogram. Apparently, the chirality of the carboxyl ester remote from the acetyl group does not cause the appearance of additional peaks. Generally, the *R*-(−)-2-butyl ester derived from the L_S-2-hydroxy acid is eluted before the *R*-(−)-2-butyl ester of the corresponding D_R-2-hydroxy acid as is evident from Fig. 1. The identity of the derivatives has been confirmed by gas-liquid chromatography-mass spectrometry using a packed OV-225 column. The 2-hydroxy fatty acids obtained after oxidative ozonolysis of compounds I—VI have been analysed in this way after esterification with *R*-(−)-2-butanol and reacetylation. Some typical gas chromatograms are shown in Fig. 2. The compositions of the diastereomeric mixtures as derived from integration of the peak areas in the gas chromatograms are given in Table I. For comparison the data obtained with the ^{1}H-NMR shift method for the intact unsaturated hydroxy fatty acids are included [2, 8, 9]. From this table it can be seen that all 2-hydroxycarboxylic acids possess mainly the *S* configuration with the exception of the 2-hydroxysebacic acid derived from IIa which has mainly the *R* configuration. The parent hydroperoxy fatty acids possess the corresponding chiralities in terms of the Cahn-Ingold-Prelog convention. As is evident from Table I, the gas-liquid chromatographic method

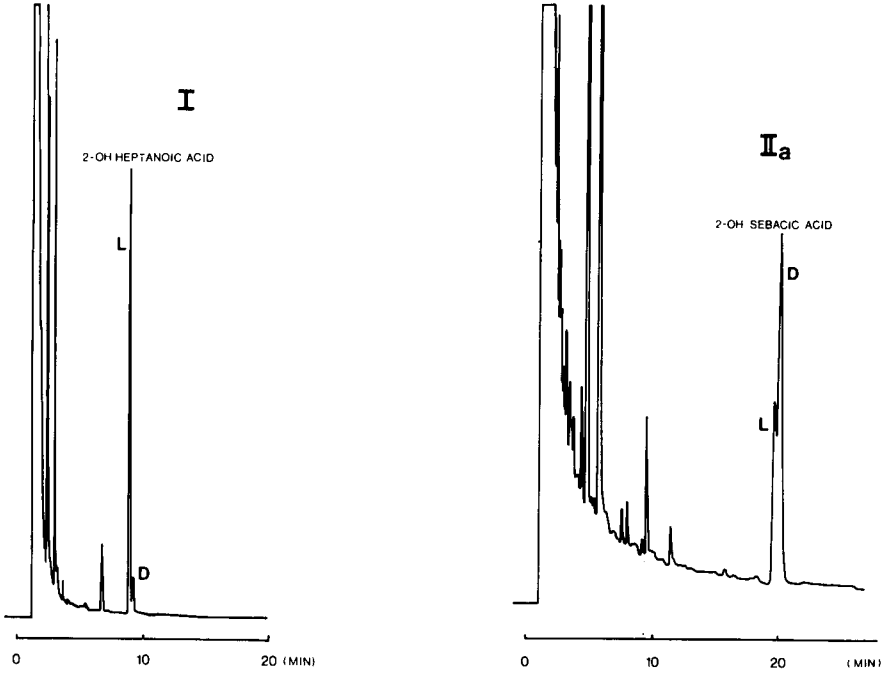


Fig. 2. Capillary gas-liquid chromatograms of the properly derivatized reaction mixtures obtained after oxidative ozonolysis of compounds I and IIa (cf. Scheme I) Conditions: I, isothermal at 120°C; IIa, isothermal at 190°C. Peaks designated D are at least in part due to contamination of the *R*-(−)-2-butanol with 6% of the *S*-(+) enantiomer. NB: On a non-chiral phase the *R*-(−)-2-butyl ester of a D_R-2-hydroxy acid can not be separated from the *S*-(+)-2-butyl ester of the corresponding L_S-2-hydroxy acid.

TABLE I

COMPARISON OF THE ENANTIOMERIC COMPOSITIONS OBTAINED BY THE CAPILLARY GAS-LIQUID CHROMATOGRAPHY METHOD WITH THOSE FROM THE ^{1}H-NMR SHIFT METHOD

Compound	Enantiomeric composition		Chiral center
	GLC method* $S:R$	^{1}H-NMR method $S:R$	
I	98 : 2	97 : 3	(C 13)
II	94 : 6	94 : 6	(C 9)
IIa	29 : 71	30 : 70	(C 9)
III	$S > 92$**	93 : 7	(C 13)
IV	96 : 4	97 : 3	(C 15)
V	94 : 6	93 : 7***	(C 15)
	$S > 95$**	93 : 7***	(C 8)
VI	96 : 4	92 : 8***	(C 15)
	$S > 91$***	90 : 10***	(C 5)

* The values are corrected for the presence of 6% of the *S*-(+) enantiomer in the *R*-(—)-2-butanol.
** More accurate data can not be given due to the incomplete separation of the gas-liquid chromatographic peaks.
*** The accuracy of these values is believed to be ± 5%.

as well as the ^{1}H-NMR method give rise to similar enantiomeric compositions of the hydroperoxy fatty acids.

The present method is generally applicable to the determination of the enantiomeric compositions of chiral 2-hydroxycarboxylic acids. Therefore, the stereospecificity of the introduction of hydroperoxy groups in unsaturated fatty acids by lipoxygenase can be examined by this method. The derivatives used are relatively volatile which enables the analysis by gas-liquid chromatography of rather long-chain fatty acids. Furthermore, it requires only minute amounts of material and can be applied to mixtures of positional isomers, provided that ozonolysis yields different 2-hydroxycarboxylic acids.

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