14

Determination of the Absolute Configuration of Chiral Hydroxy Acids by GLC

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I. INTRODUCTION

In biochemical studies on chiral organic compounds, it is often of great importance to know the absolute configurations of the chiral centers. This information can give insight into the stereospecific course of certain enzymatic reactions. In clinical practice it can indicate specific enzyme deficiencies in inborn errors of metabolism.

The absolute configuration of a chiral compound can be determined in different ways. Generally applied techniques include measurements based on the optical activity (polarimetry, optical rotation dispersion, circular dichroism), enzymatic analysis, nuclear magnetic resonance spectroscopy, and chromatography (gas-liquid chromatography, high-pressure liquid chromatography, etc.).

During the last 20 years significant progress has been made in the application of gas-liquid chromatography (GLC) for the determination of the absolute configuration of biochemically interesting chiral substances. Comprehensive reviews on this subject have been published by Gil-Av and Nurok [1] and Hušek and Macek [2]. The GLC separation of racemic mixtures can be effected in two ways: (1) separation of the enantiomers (if necessary, after protection of polar groups) on a chiral stationary phase; and (2) conversion of enantiomers into diastereomers using a chiral reagent, followed by separation of the diastereomers on a nonchiral stationary phase. After assignment of the GLC peaks by cochromatography with enantiopure substances, the method can be applied for chirality determinations.

The great advantages of the GLC determination of the absolute configurations are:

1. It is a highly sensitive method with a generally applicable detection system.
2. It is not necessary to purify the compound under investigation to a great extent, which facilitates the analysis of material obtained from biological sources.
3. Mixtures of chiral substances can be analyzed without preceding separation.
4. The enantiopurity of chiral compounds can be determined very easily.
5. A coupling with mass spectrometry for identification and verification is possible.

The GLC method has already been applied to a wide range of natural compounds, for example, amino acids [2–9], monosaccharides [10–12], and chiral organic acids. This chapter will deal with the application of capillary and packed column GLC for the analysis of the absolute configuration of chiral hydroxy acids, using diastereomeric derivatives and nonchiral stationary phases. In particular, emphasis is given to chiral hydroxy acids which occur in metabolic pathways.

II. CLINICAL CHEMICAL ASPECTS

In the last decennia, biochemical investigations in relation to inborn errors of metabolism have become very important in human health and disease. Nowadays, many clinical laboratories apply a large number of screening procedures for amino acids, organic acids, steroids, purines, pyrimidines, monosaccharides, oligosaccharides, (glyco)peptides, mucopolysaccharides, etc. In the study of metabolites, GLC in combination with mass spectrometry (MS) has been shown to be a powerful technique for getting definite answers.

GLC-MS analysis of organic acids from physiological fluids has led to the detection and identification of many acids which were not known before [13–15]. A number of new inborn errors of metabolism can be diagnosed, especially in relation with amino acid catabolism [16,17].

The investigation of organic acids from physiological fluids has now reached a stage wherein it has become significant to determine the absolute configuration of the chiral acids as well. Because different stereoisomers will be formed along different metabolic routes, the absolute configuration of the chiral centers might give a clue to the origin of these metabolites.

III. FATTY ACID OXIDATION

In plant and animal systems a wide variety of hydroxy fatty acids may occur [18], which differ with regard to the positions of the hydroxyl groups as well as the chain lengths of these compounds.

A number of metabolic pathways may lead to the formation of hydroxy fatty acids. In the framework of this review the attention has been restricted to the analysis of some products obtained from α and ω2 hydroxylation of a few fatty acids. Furthermore, the determination of the enantiopurity and the absolute configurations of hydroxy fatty acids, which can be prepared through reduction of hydroperoxides formed by the action of lipoxygenases [EC 1.13.11.12], will be discussed. The latter enzyme is capable of oxygenating polyunsaturated fatty acids containing a 1,4-*cis*,*cis*-pentadiene system [19]. In this way conjugated hydroperoxy fatty acids are formed. Apparently the source of the enzyme as well as the incubation conditions affect the regio- and stereospecificities of the reaction [20].

IV. CARBOHYDRATES

The determination of the absolute configuration of monosaccharides is required for the structural determination of polysaccharides, oligosaccharides, glycoproteins, glycolipids, and other carbohydrate-containing materials. Also, in several biosynthetic processes dealing with carbohydrates, it can be of importance to know the absolute configuration of the monosaccharides. The introduction of GLC has made it possible to analyze mixtures of monosaccharides directly.

Nowadays, two GLC approaches exist for the determination of the absolute configuration of monosaccharides. The first method, described by Pollock and Jermany [21,22], is based on the oxidation of the aldehyde function and analysis of the formed aldonic acids. In this way the absolute configuration of the substituents at carbon-2 is determined. From the known relative positions of the hydroxyl groups in the parent sugar, it is possible to deduce the absolute configuration of the substituents at the chiral carbon atom adjacent to the primary hydroxyl group, which indicated the D- or L-monosaccharide. The second technique, described by Gerwig et al. [10,11] and Leontein et al. [12], consists of the glycosidation of the monosaccharides with a chiral alcohol and analysis of the formed alkyl glycosides. Although the latter approach is much more convenient, only the analysis of aldonic acids will be included in this review.

V. GAS-LIQUID CHROMATOGRAPHIC DATA

A. Lactic Acid

The enantiomeric forms of lactic acid can be separated on nonchiral stationary phases after acylation with a chiral acid chloride or after esterification with a chiral alcohol. Westley and Halpern [23] have reported the GLC separation of DL-lactic acid on 5% QF-1 (5 ft × 1/8 in.; N_2 flow, 30 ml/min) using the *O*-(-)-menthyloxycarbonyl methyl ester derivatives (R_D/R_L = 1.09; T = 170°C). As shown by Pollock and Jermany [21], D-lactic acid gives rise to two peaks on a Carbowax 20 M capillary column (150 ft × 0.02 in.) after esterification with

(±)-2-butanol, (±)-3-methyl-2-butanol, or (±)-3,3-dimethyl-2-butanol, followed by *O*-acetylation. In relation to these data it has to be noted that on nonchiral stationary phases the combinations D-enantiomer/(-) chiral reagent and L-enantiomer/(+) chiral reagent are eluted together, as are the combinations D-enantiomer/(+) chiral reagent and L-enantiomer/(-) chiral reagent. Kamerling et al. [24] used the *O*-acetylated (-)-menthyl ester derivatives for the resolution of DL-lactic acid.

O-Acetylated L-lactic acid has been employed for the resolution of a large number of (±) chiral alcohols. Capillary columns and packed columns with both polar and nonpolar stationary phases have been applied [1]. Gil-Av et al. [6] demonstrated that increasing chain lengths of 2-*n*-alkanols (4–10 carbon atoms) result in increasing resolution factors. Karger et al. [25] reported on the separation of the L-lactic acid esters of the branched aliphatic alcohols 3-methyl-2-butanol, 3,3-dimethyl-2-butanol, 2-methyl-3-pentanol, and 2,2-dimethyl-3-pentanol. The separation of the L-lactic acid ester of (±)-menthol on a packed column with 1,2,3,4-tetrakis(2-cyanoethoxy)butane has been shown by Nurok [26].

In Figure 14.1a, the separation of the *O*-acetylated DL-lactic acid (-)-menthyl ester on a SP-1000 capillary column is presented [24]. The two peaks were identified by cochromatography with the diastereomeric derivatives of D- or L-lactic acid. To ensure that the menthylation procedure did not lead to racemization, D- and L-alanine were tested. In both cases only the enantiopure substances were found. Commercially available D-lactic acid consisted of

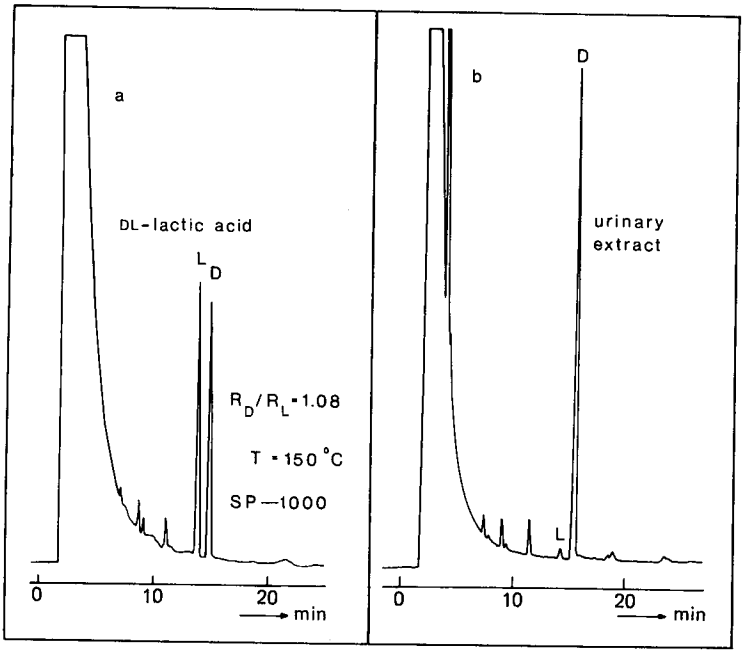

Figure 14.1 Gas chromatograms on a SP-1000 WCOT capillary column (25 m × 0.3 mm I.D.; N_2 flow, 1 ml/min) of the *O*-acetylated (-)-menthyl ester derivatives of (a) commercial DL-lactic acid, and (b) D-lactic acid from an urinary ethyl acetate extract of a patient with D-lactic aciduria. (Data from Refs. 24 and 27.)

98.4% D and 1.6% L, and L-lactic acid of 99.4% L and 0.6% D. Since it was shown that treatment with menthol/HCl did not lead to racemization, the contaminating optical antipodes were considered as intrinsic.

In the screening for inborn errors of metabolism the accumulation of lactic acid in the urine of patients is a relatively often occurring phenomenon. In almost all cases the absolute configuration of the excreted substance has been shown to be L (see Section V.C). However, the excretion of high levels of D-lactic acid, not derived from the intestinal bacterial flora, has been observed by Duran et al. [27]. In the latter case, the absolute configuration was determined using the *O*-acetylated (-)-menthyl ester derivative. Figure 14.1b presents the gas chromatogram of the urinary ethyl acetate extract after derivatization with (-)-menthol/HCl and acetic anhydride. No additional purification was carried out. D-lactic acid was also found to occur in the blood and CSF.

B. Glyceric Acid

Pollock and Jermany [21] reported that the *O*-acetylated (±)-2-butyl, (±)-3-methyl-2-butyl, or (±)-3,3-dimethyl-2-butyl esters of D-glyceric acid give rise to two peaks on a Carbowax 20 M capillary column (150 ft × 0.02 in.). As has been shown by Kamerling et al. [24], the enantiomers of DL-glyceric acid can be resolved as the *O*-acetylated (-)-menthyl ester diastereomeric derivatives. The latter separation on a SP-1000 capillary column is presented in Figure 14.2a. The two peaks were identified by cochromatography with the diastereomeric derivatives of D- or L-glyceric acid.

Patients with hyperoxaluria type II excrete high amounts of oxalic acid and L-glyceric acid [28]. However, glyceric acid accumulation is not restricted to those patients only. It has also been found in patients with other clinical symptoms. Brandt et al. [29] described a patient with D-glyceric acidemia in conjuction with hyperglycinemia. Wadman et al. [30] reported on a patient showing D-glyceric acidemia and aciduria, but without hyperglycinemia. Figures 14.2b and 14.2c show the analyses of the *O*-acetylated (-)-menthyl esters of the urinary organic acids obtained from a patient with L-glyceric aciduria (hyperoxaluria type II), as reported by Kamerling et al. [31], and one with D-glyceric aciduria [30], respectively. In both cases urinary ethyl acetate extracts were used without further purification.

C. Short-Chain Hydroxy Acids

The resolution of the *O*-acetylated (-)-menthyl esters of DL-2-hydroxybutyric acid and DL-3-hydroxybutyric acid have been described by Kamerling et al. [32]. Figure 14.3a shows the separation of these diastereomers on a SE-30 capillary column; the separation of the DL-lactic acid derivatives has also been included. The different peaks were assigned by cochromatography with the derivatives of L-lactic acid, D-2-hydroxybutyric acid, and D-3-hydroxybutyric acid. It has to be mentioned that the diastereomeric derivatives of 3-hydroxybutyric acid elute in the reversed order with respect to those of lactic acid and 2-hydroxybutyric acid.

Patients with lactic acidemia and ketosis regularly show urinary excretion of increased amounts of 2-hydroxybutyric acid. As shown in Figure 14.3b, the absolute configuration of this 2-hydroxy acid and those of lactic acid and 3-hydroxybutyric acid can be determined in one gas chromatographic run. For 2-hydroxybutyric acid, the L configuration was found. Lactic acid occurred in the L configuration, and 3-hydroxybutyric acid in the D configuration.

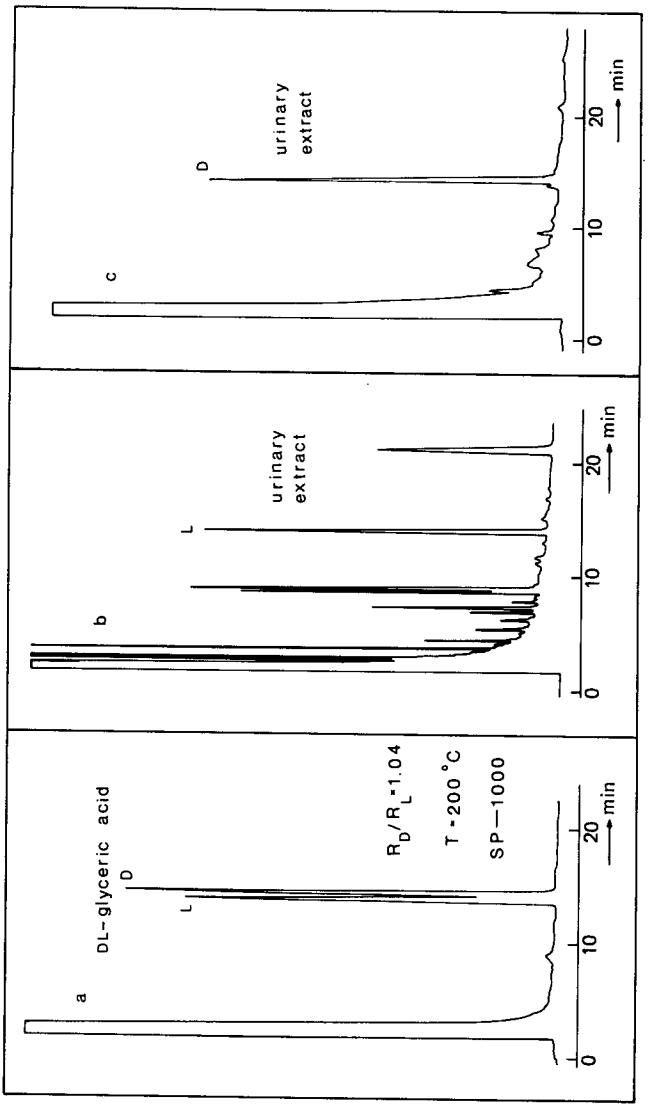

Figure 14.2 Gas chromatograms on a SP-1000 WCOT capillary column (25 m × 0.3 mm I.D.; N_2 flow, 1 ml/min) of the O-acetylated (-)-menthyl ester derivatives of (a) commercial DL-glyceric acid, (b) L-glyceric acid from an urinary ethyl acetate extract of a patient with hyperoxaluria type II, and (c) D-glyceric acid from an urinary ethyl acetate extract of a patient with D-glyceric aciduria. (Data from Refs. 24, 30, and 31.)

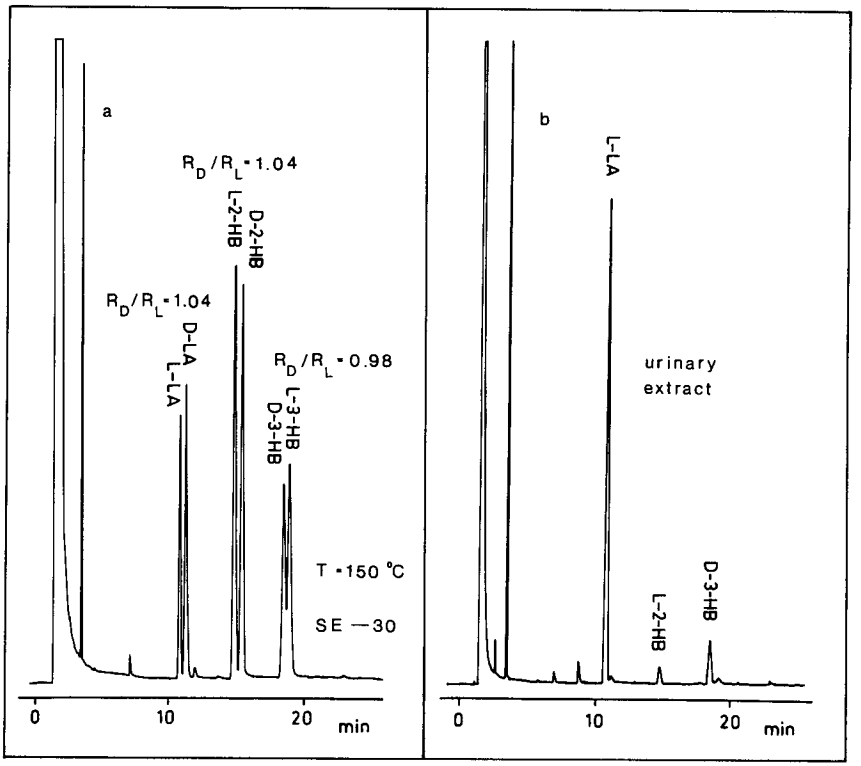

Figure 14.3 Gas chromatograms on a SE-30 WCOT capillary column (25 m × 0.3 mm I.D.; N_2 flow, 1 ml/min) of the *O*-acetylated (-)-menthyl ester derivatives of (a) a mixture of commercial DL-lactic acid (LA), DL-2-hydroxybutyric acid (2-HB), and DL-3-hydroxybutyric acid (3-HB); and (b) the urinary organic acids in a patient with lactic acidemia due to deficiency of cytochromes aa_3 and *b*. (Data from Ref. 32.)

The diastereomeric separation of the *O*-(-)-menthyloxycarbonyl methyl esters of DL-2-hydroxyisovaleric acid and DL-2-hydroxylisocaproic acid on 5% QF-1 (5 ft × 1/8 in.; N_2 flow, 30 ml/min) have been reported by Westley and Halpern [23]. Resolution factors at 170°C were found to be 1.14 and 1.15, respectively. The DL enantiomers of these acids and those of 2-hydroxy-3-methylvaleric acid can also be resolved as the *O*-acetylated (-)-2-butyl ester derivatives (Kamerling and Wadman, unpublished results). In Figure 14.4a, the separation of the *O*-acetylated (-)-2-butyl esters of DL-2-hydroxyisovaleric acid, DL-2-hydroxyisocaproic acid, and DL-2-hydroxy-DL-3-methylvaleric acid on a SP-1000 capillary column are presented. The observed resolution of the diastereomeric derivatives of the latter hydroxy acid was influenced only by the absolute configuration of the substituents at carbon-2. The peak assignments were carried out by cochromatography.

Maple syrup urine disease is attended with the urinary accumulation of the branched-chain amino acids valine, leucine, isoleucine, and *allo*-isoleucine, as well as of the corresponding oxo and hydroxy acids. The concentration of 2-hydroxyisovaleric acid is relatively high in comparison to the other 2-hydroxy acids. The analysis of the urine of such a patient is shown in Figure 14.4b,

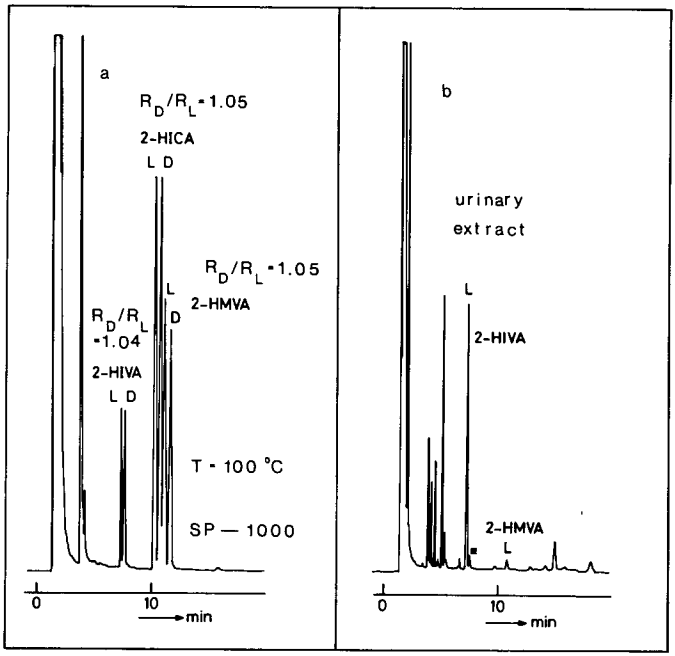

Figure 14.4 Gas chromatograms on a SP-1000 WCOT capillary column (25 m × 0.3 mm I.D.; N_2 flow, 1 ml/min) of the *O*-acetylated (-)-2-butyl esters of (a) a mixture of commercial DL-2-hydroxyisovaleric acid (2-HIVA), DL-2-hydroxyisocaproic acid (2-HICA), and DL-2-hydroxy-DL-3-methylvaleric acid (2-HMVA); and (b) the urinary organic acids in a patient with maple syrup urine disease (* indicates the small peak obtained from the contaminating (+)-2-butanol in the commercial (-)-2-butanol sample).

demonstrating relatively large amounts of L-2-hydroxyisovaleric acid and smaller amounts of L-2-hydroxy-3-methylvaleric acid (Kamerling and Wadman, unpublished results). Because of the very small quantity of 2-hydroxyisocaproic acid in the urinary extract, the absolute configuration of the latter acid could not be determined.

The resolution of the *O*-(-)-menthyloxycarbonyl methyl ester derivatives of DL-2-hydroxyheptanoic acid has been described by Hamberg [33] and that of DL-2-hydroxydecanoic acid by Nugteren [34] (see Section V.D.). As reported by van Os et al. [35], the *O*-acetylated (-)-2-butyl ester derivatives of DL-2-hydroxyheptanoic acid could be separated on a SP-1000 capillary column (25 m × 0.3 mm; N_2 flow, 1 ml/min; R_D/R_L = 1.04; T = 120°C).

D. Hydroxydicarboxylic Acids

The resolution of the *O*-acetylated di-(±)-2-butyl, (±)-3-methyl-2-butyl and (±)-3,3-dimethyl-2-butyl esters of D-malic acid and D-tartaric acid was carried out by Pollock and Jermany on a Carbowax 20 M capillary column (150 ft × 0.02 in.) [21]. D-malic acid gives rise to two peaks, whereas for D-tartaric acid, a triplet with a ratio 1:2:1 was obtained. As has been reported by Kamerling et al. [36] and van Os et al. [35], the *O*-acetylated di-(-)-2-butyl esters of DL-malic acid, DL-2-hydroxyglutaric acid, DL-2-hydroxyadipic acid, and

DL-2-hydroxysebacic acid can be separated on a SP-1000 capillary column (Figure 14.5). The different peaks were assigned L and D using the L enantiomers as reference compounds. In all cases the L-enantiomer derivative elutes earlier than the D-enantiomer derivative. Esterification of 2-hydroxyglutaric acid yields considerable amounts of the lactone form (see Figure 14.5). Gas-liquid chromatography of the *O*-acetylated di-(±)-2-butyl esters of the L-2-hydroxydicarboxylic acids gives rise to the same chromatographic peak pattern as presented in Figure 14.5. Therefore, the introduced chiral ester function not directly attached at the chiral carbon-2 center of the hydroxy acid does not influence the chromatographic behavior on SP-1000.

Relatively high excretions of malic acid, 2-hydroxyglutaric acid, or 2-hydroxyadipic acid have been observed in patients with different diseases (see Kamerling et al. [36]). From the urine of such patients, Figures 14.6a-e show gas chromatograms of ethyl acetate extracts, treated with (-)-2-butanol/HCl and acetic anhydride. Figure 14.6a shows the chromatogram of a patient with lactic acidemia who excreted among other organic acids L-malic acid [36]; Figure 14.6b represents the chromatogram of a patient with L-2-hydroxyglutaric aciduria [36], described by Duran et al. [37]; Figure 14.6c, the chromatogram of a patient with D-2-hydroxyglutaric aciduria [36], described by Chalmers et al. [38]; Figure 14.6d, the chromatogram of a patient excreting elevated amounts of a mixture of D- and L-2-hydroxyglutaric acid [36]; and

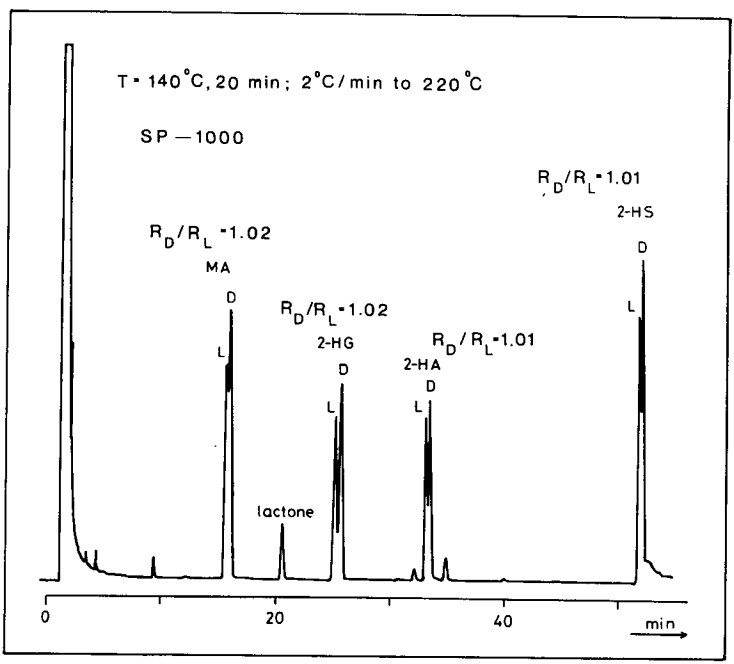

Figure 14.5 Gas chromatogram on a SP-1000 WCOT capillary column (25 m × 0.3 mm I.D.; N_2 flow, 1 ml/min) of the *O*-acetylated di-(-)-2-butyl esters of a mixture of commercial or synthetic DL-malic acid (MA), DL-2-hydroxyglutaric acid (2-HG), DL-2-hydroxyadipic acid (2-HA), and DL-2-hydroxysebacic acid (2-HS). (Data from Refs. 35 and 36.)

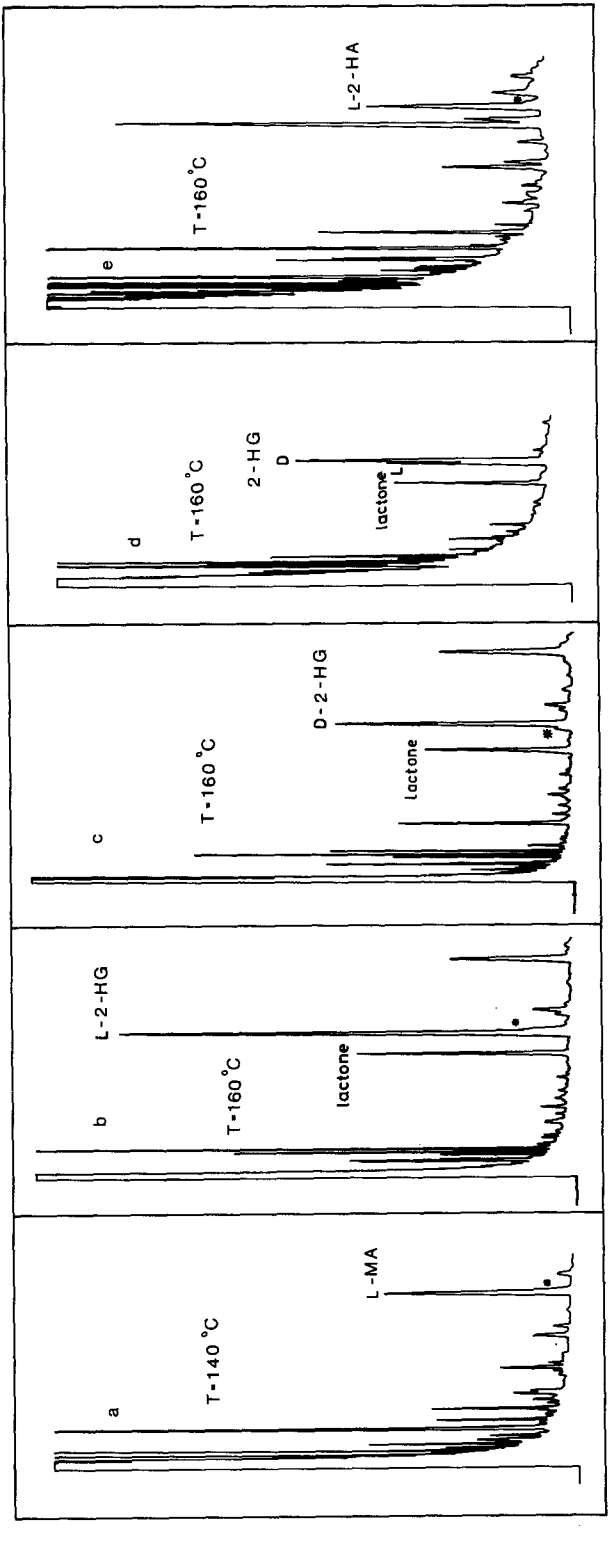

Figure 14.6 Gas chromatograms on a SP-1000 WCOT capillary column (25 m × 0.3 mm I.D.; N_2 flow, 1 ml/min) of urinary extracts of different patients derivatized with (-)-2-butanol and acetic anhydride: (a) L-malic acid; (b) L-2-hydroxyglutaric acid; (c) D-2-hydroxyglutaric acid; (d) D- + L-2-hydroxyglutaric acid; (e) L-2-hydroxyadipic acid (* indicates the shoulder or small peak obtained from the contaminating (+)-2-butanol in the commercial (-)-2-butanol sample). (Data from Refs. 36, 37, and 38.)

Figure 14.6e, the chromatogram of a patient with 2-aminoadipic aciduria, excreting relatively large amounts of L-2-hydroxyadipic acid [36].

Unsaturated fatty acids having a 1,4-*cis,cis*-pentadiene system can be converted into conjugated hydroperoxides by lipoxygenase oxygenation or by autoxidation (see Section III). The stereoisomeric composition of the formed hydroperoxides can not be determined directly by GLC. To obtain suitable 2-hydroxy (di)carboxylic acid derivatives, the hydroperoxides are reduced, and subsequently the prepared *O*-acylated unsaturated hydroxy fatty acid methyl esters are submitted to oxidative ozonolysis. When a chiral *O*-acyl group is present, the 2-hydroxy (di)carboxylic acid methyl ester derivatives can be analyzed directly by GLC. If not, chiral ester groups can be introduced.

Hamberg [33] analyzed the *O*-(-)-menthyloxycarbonyl (di)methyl ester derivatives of 2-hydroxysebacic acid (R_D/R_L = 1.10; T = 200°C) and 2-hydroxyheptanoic acid (R_D/R_L = 1.16; T = 141°C), formed from the *O*-(-)-menthyloxycarbonyl methyl ester derivatives of 9-hydroxy-10,12-octadecadienoic acid and 13-hydroxy-9,11-octadecadienoic acid, respectively (5% QF-1; helium flow, 65 ml/min). In this way it was possible to estimate indirectly the relative amounts of the four isomeric (9-D, 9-L, 13-D, and 13-L) hydroperoxy acids, obtained from linoleic acid by incubation with lipoxygenases from various sources (Table 14.1). Additional examples include the analysis of malic acid, 2-hydroxyadipic acid, 2-hydroxyazelaic acid, and 2-hydroxydecanoic acid.

Borgeat et al. [39,40] reported on the transformation of arachidonic acid into 5-D-hydroxy-6,8,11,14-eicosatetraenoic acid and of homo-γ-linolenic acid into 8-D-hydroxy-9,11,14-eicosatrienoic acid by rabbit polymorphonuclear leukocytes containing lipoxygenase-type enzymatic activities (intermediate hydroperoxides were not detected). GLC analysis of the 2-hydroxyadipic acid derivative (R_D/R_L = 1.10; T = 162°C; 2.2% QF-1), derived from the 5-hydroxy

Table 14.1 Analyses of Hydroperoxyoctadecadienoates Formed from Linoleic Acid on Incubation with Soybean and Corn Lipoxygenases

	Buffer	pH	9-D : 9-L : 13-D : 13-L
A, soybean	0.1 M sodium borate	9.0	4 : 3 : 3 : 90
B, soybean	0.1 M sodium borate	10.5	2 : 2 : 2 : 94
C, soybean	0.1 M sodium borate	9.0	2 : 2 : 3 : 93
D, soybean	0.04 M NH_4OH–NH_4Cl	9.0	6 : 4 : 4 : 86
E, corn	0.1 M potassium phosphate	7.4	86 : 5 : 4.5 : 4.5
F, corn	0.1 M potassium phosphate	6.5	89 : 4 : 3.5 : 3.5
G, autoxidation	—	—	25 : 25 : 25 : 25

A,B: Soybean lipoxygenase from Sigma Chemical Co.
C,D: Soybean lipoxygenase from Nutritional Biochemicals Corp.
E,F: Ammonium sulfate precipate (45-60% saturation) containing corn lipoxygenase.
Source: Data from Ref. 33.

Table 14.2 Comparison of the Enantiomeric Compositions of Hydroperoxides Obtained in Lipoxygenase-Catalyzed Oxygenation of Polyunsaturated Fatty Acids Containing a 1,4-*cis*,*cis*-pentadiene system[a]

Compound[b]	GLC[c]			^{1}H-NMR		
	Ozonolysis product(s)	L : D (hydroperoxide)		Chiral center	L : D (hydroperoxide)	
I	2-hydroxyheptanoic acid	98	2	C_{13}	97	3
II	2-hydroxysebacic acid	6	94	C_9	6	94
IIa	2-hydroxysebacic acid	71	29	C_9	70	30
III	malic acid	L>92	D< 8	C_{13}	93	7
IV	2-hydroxyheptanoic acid	96	4	C_{15}	97	3
V	2-hydroxyheptanoic acid	94	6	C_{15}	93	7
	malic acid	L< 5	D>95	C_8	7	93
VI	2-hydroxyheptanoic acid	96	4	C_{15}	92	8
	2-hydroxyadipic acid	L< 9	D>91	C_5	10	90

[a]The absolute configurations were determined in two ways: (1) Capillary GLC on SP-1000 of *O*-acetylated (di)-(-)-2-butyl ester derivatives (oxidative ozonolysis of the *O*-acetylated unsaturated hydroxy fatty acid methyl esters; van Os et al. [35]), and (2) ^{1}H-NMR spectroscopy of *O*-α-methoxy-α-trifluoromethylphenylacetylated methyl esters of unsaturated hydroxy fatty acids. The various unsaturated hydroxy fatty acid methyl esters were purified by high-pressure liquid chromatography (see van Os [20]).

[b]I = 13-hydroperoxy 9-*cis*,11-*trans*-octadecadienoic acid (from linleic acid by incubation with soybean lipoxygenase-1, 0.1 M sodium borate pH 9.0, 4°C, O_2 saturated; main product: 13-L_S).

II = 9-hydroperoxy 10-*trans*,12-*cis*-octadecadienoic acid (from linoleic acid by incubation with corn germ lipoxygenase, 0.1 M sodium phosphate pH 6.6, 4°C, O_2 saturated; main product: 9-D_S).

IIa = 9-hydroperoxy 10-*trans*,12-*cis*-octadecadienoic acid (from linoleic acid by incubation with soybean lipoxygenase-2, 0.1 M sodium borate pH 9.0, 4°C, O_2 saturated; main product: 9-L_R).

III = 13-hydroperoxy 9-*cis*,11-*trans*,15-*cis*-octadecatrienoic acid (from α-linolenic acid by incubation with soybean lipoxygenase-1, 0.1 M sodium borate pH 9.0, 4°C, O_2 saturated; main product: 13-L_S).

IV = 15-hydroperoxy 5-*cis*,8-*cis*,11-*cis*,13-*trans*-eicosatetraenoic acid (from arachidonic acid by incubation with soybean lipoxygenase-1, 0.1 M sodium borate pH 9.0, 4°C, O_2 saturated; main product: 15-L_S).

V = 8,15-dihydroperoxy 5-*cis*,9-*trans*,11-*cis*,13-*trans*-eicosatetraenoic acid (from arachidonic acid by incubation with soybean lipoxygenase-1, 0.1 M sodium borate pH 8.75, 4°C, O_2 saturated; main product: 8-D_S,15-L_S).

VI = 5,15-dihydroxy 6-*trans*,8-*cis*,11-*cis*,13-*trans*-eicosatetraenoic acid (from arachidonic acid by incubation with soybean lipoxygenase-1, 0.1 M sodium borate pH 8.75, 4°C, O_2 saturated; main product: 5-D_S,15-L_S).

[c]The values are corrected for the presence of 6% of the (+)-enantiomer in commercially available (-)-2-butanol. SP-1000 capillary column: 25 m × 0.3 mm I.D.; N_2 flow, 1 ml/min. Analysis conditions: 2-hydroxyheptanoic acid, 120°C; malic acid, 140°C; 2-hydroxyadipic acid, 160°C; 2-hydroxysebacic acid, 190°C.

Source: Data from Ref. 35.

unsaturated fatty acid, showed a L:D ratio of 83:17, indicating mainly the D-configuration for the 5-OH group. Analysis of the 2-hydroxyazelaic acid derivative (R_D/R_L = 1.10; T = 180°C; 2.2% QF-1), derived from the 8-hydroxy unsaturated fatty acid, showed a L:D ratio of 82:18, indicating mainly the D-configuration for the 8-OH group. Furthermore, Borgeat and Samuelsson [41] described a second product of arachidonic acid incubation, namely, 5-D,-12-D-dihydroxy-6,8,10,14-eicosatetraenoic acid. A small amount of the 5-L,-12-D stereoisomer appeared to be present. The absolute configurations were assigned on the basis of the GLC data of the degradation products 2-hydroxyadipic acid and malic acid.

As shown by Hamberg and Samuelsson [42], the incubation of arachidonic acid with human blood platelets leads to the formation of 12-L-hydroxy-5,8,10,-14-eicosatetraenoic acid (action of lipoxygenase), and 12-L-hydroxy-5,8,10-heptadecatrienoic acid, and the hemiacetal derivative of 8-(1-hydroxy-3-oxopropyl)-9,12-L-dihydroxy-5,10-heptadecadienoic acid (action of fatty acid cyclooxygenase). The absolute configuration of the first compound was established via the malic acid derivative. The other two compounds yielded 2-hydroxyheptanoic acid.

Nugteren [34] studied the incubation of all-*cis*-8,11-eicosadienoic acid, homo-γ-linolenic acid (all-*cis*-8,11,14-eicosatrienoic acid), and arachidonic acid (all-*cis*-5,8,11,14-eicosatetraenoic acid) with bovine blood platelets. The action of lipoxygenase gave rise to the formation of 12-hydroxy 8-*cis*,10-*trans*-eicosadienoic acid (88% L and 12% D; 2-hydroxydecanoic acid as degradation product), 12-hydroxy 8-*cis*,10-*trans*,14-*cis*-eicosatrienoic acid (91% L and 9% D; malic acid as degradation product), and 12-L-hydroxy 5-*cis*,8-*cis*,10-*trans*,-14-*cis*-eicosatetraenoic acid (see Hamberg and Samuelsson [42]), respectively. For the analysis of the absolute configuration of hydroperoxides a similar approach has been developed by van Os et al. [35], making use of the readily accessible *O*-acetylated (-)-2-butyl ester diastereomers. Table 14.2 summarizes the results of some studies, using the latter derivatives.

The absolute configuration of the nonhydroxy dicarboxylic acid methylsuccinic acid, isolated from normal human urine, has been studied by Zeitman and Lawless [43]. The di-(+)-2-butyl ester diastereomeric derivatives were separated on diethylene glycol succinate as nonchiral stationary phase. The urinary substance was found to have the D-configuration.

E. Long-Chain Hydroxy Acids

Hammarström and Hamberg [44] have reported the GLC resolution of a series of DL-hydroxyoctadecanoic acids with hydroxyl groups at different positions. They studied also the separation of homologous DL-3-hydroxy fatty acids (C_{10}-C_{18}). For this purpose the D-2-phenylpropionate diastereomeric derivatives of the hydroxy acid methyl esters were applied. The results are presented in Table 14.3. As nonchiral stationary phase, 5% QF-1 was employed. The diastereomeric derivatives of the methyl 4-, 7-, and 13-hydroxyoctadecanoates could not be resolved. In view of the latter results, it was stated that it is unlikely that any of the monohydroxyoctadecanoates substituted in the region from carbon-4 to carbon-13 could be resolved in this way. Concerning the homologous series of DL-3-hydroxy acids, it was found that in all cases the diastereomer derived from the D enantiomer has a shorter retention time than that from the L enantiomer (see also D-3-hydroxybutyric acid, Section V.C.).

The separation of the *O*-(-)-menthyloxycarbonyl methyl esters of homologous DL-2-hydroxy fatty acids (C_{14}-C_{26}) on 1.4% OV-210 have been described by

Table 14.3 Resolution of a Series of Long-Chain Hydroxy Fatty Acids on 5% QF-1 (Carrier Gas, He) Using the D-2-phenylpropionate Methyl Ester Derivatives

Hydroxy acid		Resolution factor	T(°C)
DL-17-hydroxy	18:0	1.08 (R_D/R_L)	230
DL-16-hydroxy	18:0	1.08 (R_D/R_L)	230
DL-15-hydroxy	18:0	1.05 (R_D/R_L)	230
DL-14-hydroxy	18:0	1.03	230
DL-13-hydroxy	18:0	—	230
DL-7-hydroxy	18:0	—	230
DL-4-hydroxy	18:0	—	230
DL-3-hydroxy	18:0	1.07 (R_L/R_D)	230
DL-2-hydroxy	18:0	1.03	230
DL-3-hydroxy	10:0	1.06	200
DL-3-hydroxy	13:0	1.08	200
DL-3-hydroxy	14:0	1.08 R_L/R_D	200
DL-3-hydroxy	16:0	1.08	200
DL-3-hydroxy	18:0	1.08	200

Source: Data from Ref. 44.

Hammarström [45] (Figure 14.7). It has been verified that for pure enantiomers the preparation of methyl esters with methanolic HCl (refluxing time, 5 hr) does not give rise to racemization. In all cases, the peak derived from the L enantiomer emerges earlier than that from the D enantiomer. This method has been applied for the determination of the absolute configuration of 2-hydroxy fatty acids from brain cerebrosides. The unsaturated acids were hydrogenated before the analysis was carried out. Only the D configuration was found to occur. Another application was published by Markovetz et al. [46] in relation to studies on fatty acid metabolism (α-oxidation) in peanuts. Enzymatic oxidation of palmitic acid led to the accumulation of D-2-hydroxypalmitic acid. It has to be noted that in contrast to the 2-hydroxy fatty acids, the 3- and ω2-hydroxy fatty acids could not be resolved as their *O*-(-)-menthyloxycarbonyl methyl ester derivatives on QF-1 nor on OV-210 as stationary phases [44].

The determination of the absolute configuration of the ω2-hydroxy acids 17-hydroxyoctadecanoic acid, 19-hydroxyeicosanoic acid, and 21-hydroxydocosanoic acid has been reported by Hamberg [47]. *R*-1-phenylethyl isocyanate was used as chiral reagent. The diastereomeric *N*-(1-phenylethyl)-urethane methyl esters could be separated on 1% QF-1 (1.8 m × 3.5 mm I.D.; carrier gas, helium; resolution factors, 1.08; T = 210°C). Only for the 17-hydroxy acid was the peak sequence estimated. The derivative obtained from the L enantiomer was found to have the smallest retention time. 2-Hydroxy

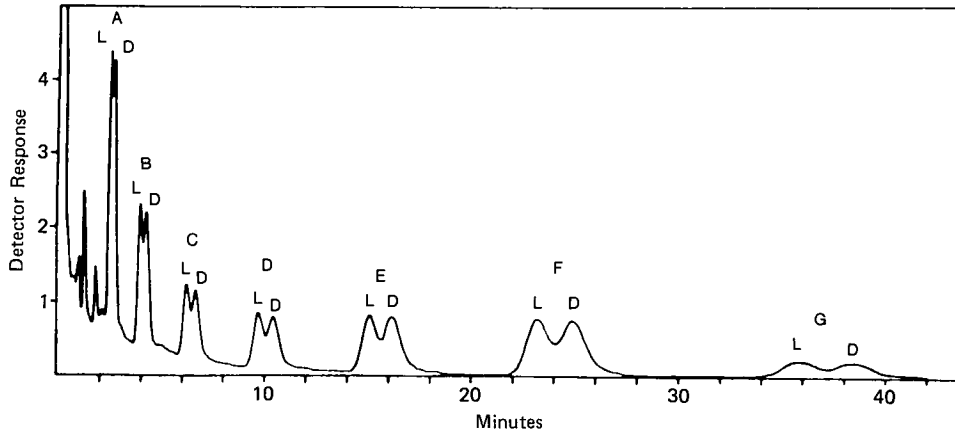

Figure 14.7 Gas chromatogram on 1.4% OV-210 (1.7 m × 3.5 mm I.D.; carrier gas, He) at 250°C of the *O*-(-)-menthyloxycarbonyl methyl ester derivatives of a series of long-chain DL-2-hydroxy fatty acids. A, DL-2-hydroxymyristic acid (C_{14}); B, DL-2-hydroxypalmitic acid (C_{16}); C, DL-2-hydroxystearic acid (C_{18}); D, DL-2-hydroxyarachidic acid (C_{20}); E, DL-2-hydroxybehenic acid (C_{22}); F, DL-2-hydroxylignoceric acid (C_{24}); and G, DL-2-hydroxycerotic acid (C_{26}). (From Ref. 45.)

acid methyl esters and 3-hydroxy acid methyl esters could not be resolved in this way [44]. Applications have been described in studies on the mechanism of microsomal ω2-hydroxylation of fatty acids. Björkhem and Hamberg [48] reported the ω2 hydroxylation of lauric acid to be 40–44% L- and 56–60% D-11-hydroxylauric acid. Hamberg and Björkhem found that with decanoic acid, 75% L- and 25% D-9-hydroxydecanoic acid were formed [49]. The same approach was used by Björkhem and Hamberg in studies on the enzymatic oxidoreduction of 17-hydroxystearic acid [50].

F. Aromatic Hydroxy Acids

Pollock and Jermany [21] reported the occurrence of two peaks for the *O*-acetylated (±)-2-butyl, (±)-3-methyl-2-butyl, and (±)-3,3-dimethyl-2-butyl esters of D-mandelic acid on a Carbowax 20 M capillary column (150 ft × 0.02 in.). Westley and Halpern demonstrated the resolution of DL-3-phenyllactic acid as their *O*-(-)-menthyloxycarbonyl methyl ester derivatives on 5% QF-1 (5 ft × 1/8 in.; nitrogen flow, 30 ml/min; R_D/R_L = 1.14; T = 200°C) [23]. Separations were also obtained for the *O*-acetylated (-)-2-butyl ester derivatives of DL-3-phenyllactic acid and DL-3-(4-hydroxyphenyl) lactic acid on a SP-1000 capillary column (Kamerling and Wadman, unpublished results) (Figure 14.8a,b). The two peaks in Figure 14.8a were assigned by cochromatography with the L enantiomer. The assignment of the peaks in Figure 14.8b was made on the basis of the results obtained with other *O*-acetylated (-)-2-butyl esters of 2-hydroxy acids, described in Section V.

Phenylketonuria and tyrosyluria lead to the urinary accumulation of 3-phenyllactic acid and 3-(4-hydroxyphenyl)lactic acid, respectively. Analysis of the *O*-acetylated (-)-2-butyl esters of the urinary organic acids shows in both cases the presence of only the L enantiomer (Figure 14.8c,d).

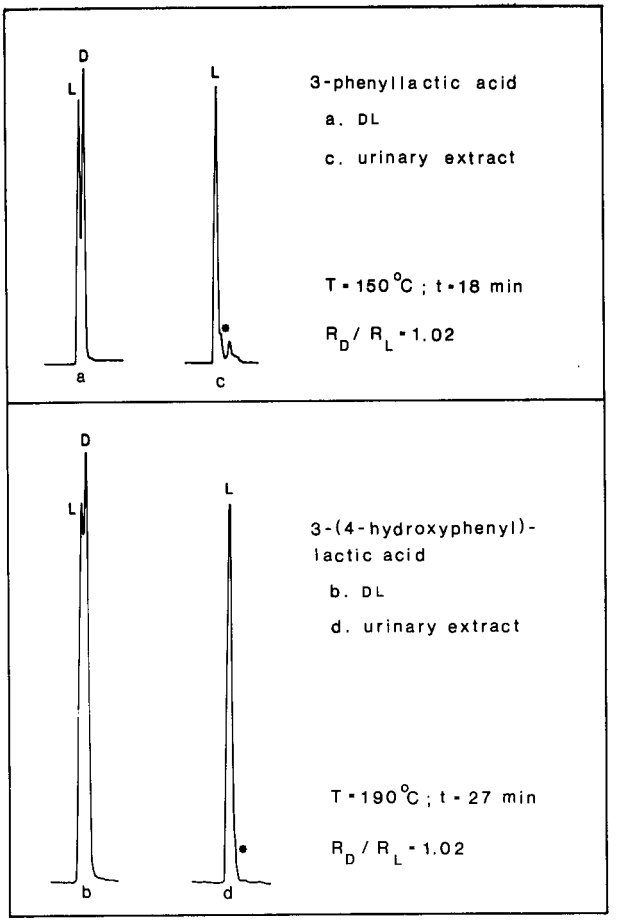

Figure 14.8 Partial gas chromatograms on a SP-1000 WCOT capillary column (25 m × 0.3 mm I.D.; N_2 flow, 1 ml/min) of the *O*-acetylated (-)-2-butyl esters of (a) commercial DL-3-phenyllactic acid, (b) commercial DL-3-(4-hydroxyphenyl)lactic acid, (c) L-3-phenyllactic acid from a urinary ethyl acetate extract of a patient with phenylketonuria, and (d) L-3-(4-hydroxyphenyl)lactic acid from a urinary ethyl acetate extract of a patient with tyrosyluria (* indicates the shoulder obtained from the contaminating (+)-2-butanol in the commercial (-)-2-butanol sample).

G. Aldonic Acids

The separation of the *O*-acetylated (±)-2-butyl, (±)-3-methyl-2-butyl, and (±)-3,3-dimethyl-2-butyl esters of a series of D-aldonic acids (in this case, D according to the sugar nomenclature; see Section IV) on Carbowax 20 M (150 ft × 0.02 in.; helium flow: 6.5–6.8 ml/min) or OV-225 (50 ft × 0.02 in.; helium flow: 6 ml/min) capillary columns has been reported by Pollock and Jermany [21,22]. The underivatized acids were prepared by oxidation of the aldehyde function of tetroses, pentoses, and hexoses. For peak assignments the derivatives obtained with the optically active alcohols were used. The data from the literature presented in Table 14.4 have been arranged in

Table 14.4 Separation Factors of Enantiomeric Aldonic Acids

O-Acetylated aldonic acid (-)-alkyl esters[a]	R_D/R_L[b]	T(°C)	Column material[c]
2B threonic acid	1.03	160	Carbowax 20 M
3M2B threonic acid	1.06	160	Carbowax 20 M
33D2B threonic acid	1.09	160	Carbowax 20 M
2B erythronic acid	1.02	160	Carbowax 20 M
3M2B erythronic acid	1.04	160	Carbowax 20 M
33D2B erythronic acid	1.06	160	Carbowax 20 M
2B xylonic acid	1.03	175	Carbowax 20 M
3M2B xylonic acid	1.05	175	Carbowax 20 M
33D2B xylonic acid	1.09	175	Carbowax 20 M
2B lyxonic acid	1.01	175	Carbowax 20 M
3M2B lyxonic acid	1.03	175	Carbowax 20 M
33D2B lyxonic acid	1.04	175	Carbowax 20 M
2B ribonic acid	1.02	175	Carbowax 20 M
3M2B ribonic acid	1.05	175	Carbowax 20 M
33D2B ribonic acid	1.08	175	Carbowax 20 M
2B arabonic acid	1.03	175	Carbowax 20 M
3M2B arabonic acid	1.06	175	Carbowax 20 M
33D2B arabonic acid	1.13	175	Carbowax 20 M
2B galactonic acid	1.05	180	OV-225
3M2B galactonic acid	1.08	180	OV-225
33D2B galactonic acid	1.16	180	OV-225
2B gluconic acid	1.02	180	OV-225
3M2B gluconic acid	1.04	180	OV-225
33D2B gluconic acid	1.05	180	OV-225

[a] 2B = (-)-2-butanol; 3M2B = (-)-3-methyl-2-butanol; 33D2B = (-)-3,3-dimethyl-2-butanol.

[b] Here D and L indicate the absolute configuration at C-2 and *not* that at the chiral atom adjacent to the primary hydroxyl function (see text).

[c] Carbowax 20M: 150 ft × 0.02 in.; helium flow, 6.5–6.8 ml/min. OV-225: 50 ft × 0.02 in.; helium flow, 6 ml/min.

Source: Data from Refs. 21 and 22.

such a way that D and L indicate the absolute configuration at C-2; the carboxyl function bears only a (-)-2-alkyl group. Using the latter chiral alcohols, it can be seen that L enantiomers are eluted earlier than D enantiomers. Furthermore it was found that a better resolution of diastereomers is obtained with the more sterically hindered alcohols.

ADDENDUM*

Recently, König and Benecke [51] have reported on the gas chromatographic resolution of the *O*-trifluoroacetylated and *O*-trimethylsilylated (+)-3-methyl-2-butyl esters of DL-lactic acid, DL-2-hydroxybutyric acid, DL-2-hydroxyisovaleric acid, DL-2-hydroxyisohexanoic acid, DL-2-hydroxyhexanoic acid, DL-2-hydroxyoctanoic acid, DL-2-hydroxydodecanoic acid, DL-2-hydroxytetradecanoic acid, DL-2-hydroxyhexadecanoic acid, DL-malic acid, DL-2-phenyllactic acid, DL-3-phenyllactic acid, DL-tropaic acid, DL-mandelic acid, DL-4-hydroxy-3-methoxymandelic acid, DL-3-hydroxy-4-methoxymandelic acid, and DL-4-hydroxymandelic acid, and the (+)-3-methyl-2-butyl esters of DL-2-methylsuccinic acid, DL-2-phenyl succinic acid, and DL-2-phenylbutyric acid on SE-30 and OV-17 capillary columns. A typical example of the separation is presented in Figure 14.9. The *O*-trifluoroacetylated derivatives are more

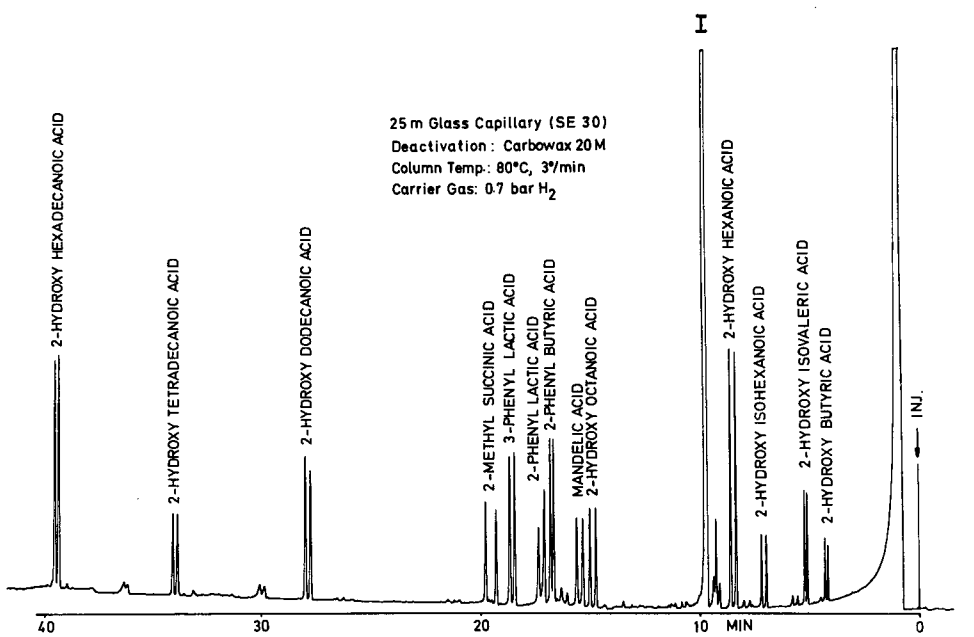

Figure 14.9 Gas chromatogram on a SE-30 WCOT capillary column (25 m × 0.3 mm I.D.) of a series of *O*-trifluoroacetylated (+)-3-methyl-2-butyl esters of DL-2-hydroxycarboxylic acids and (+)-3-methyl-2-butyl esters of DL-2-alkyl(aryl)-carboxylic acids. Internal standard (I): *N*-trifluoroacetyl-L-valine (+)-3-methyl-2-butyl ester. (From Ref. 51.)

* After preparation of the manuscript.

volatile and show slightly larger resolution factors than the O-trimethylsilylated derivatives. Peak assignments were only made for DL-lactic acid, DL-malic acid, and DL-mandelic acid, showing that the L enantiomers have the longer retention times. It has to be noted that the derivatives of DL-3-hydroxybutyric acid could not be separated (see also Section V.C).

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