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Simultaneous enantioselective analysis of chiral urinary metabolites in patients with *Zellweger* syndrome

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Abstract

Enantio-MDGC–MS analysis with heptakis-(2,3-di-*O*-methyl-6-*O*-*tert*.-butyl-dimethylsilyl)-β-cyclodextrin as the chiral main column is a powerful tool for the separation of chiral compounds. This paper reports on the simultaneous stereodifferentiation of 2-hydroxyisocaproic acid (HICA), 3-methyladipic acid (3-MA), 2-hydroxyglutaric acid (2-HG), 3-(4-hydroxyphenyl)-lactic acid (HPLA), 2-hydroxysebacic acid (2-HS) and 3-hydroxysebacic acid (3-HS) in a single chromatographic run. These chiral urinary metabolites are useful in the diagnosis of peroxisomal diseases such as *Zellweger* syndrome (ZS). In this investigation, urine samples from nine patients with ZS were analysed in order to reveal the enantiomeric ratio of these chiral metabolites. The stereodifferentiation of the analysed chiral compounds may provide important information on their biochemical origin.
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Keywords: Enantioselective analysis; *Zellweger* syndrome; Chiral metabolites

1. Introduction

In urine of patients with peroxisomal diseases such as *Zellweger* syndrome (ZS) and neonatal adrenoleukodystrophy (NALD), characteristic amounts of even- and odd-numbered dicarboxylic acids (DA), 2-hydroxy-fatty acids (HICA, 2-HG and 2-HS), 3-hydroxy-dicarboxylic acids (3-HS) and HPLA are excreted [1]. The excretion of an excess of DA in patients with a peroxisomal disease is thought to result from impaired β-oxidation in mitochondria and/or peroxisomes [1]. In patients with ZS, in addition to a dicarboxylic aciduria, there is an increased excretion of 2-HS and HPLA [2,3].

To our knowledge, there is no information on the enantiomeric distribution of the chiral metabolites HICA, 2-HG, 3-MA, HPLA, 2- and 3-HS in patients with ZS. The aim of this study was to present a method for the simultaneous enantioselective analysis of these chiral carboxylic acids in urine samples. Additionally, the elucidation of the absolute configuration of 2- and 3-HS was determined. During this investigation, various urine samples from ZS patients and healthy controls were analysed. The enantio-MDGC–MS system has proved to be the method of choice in analysing chiral metabolites from complex

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matrices such as urine [4–6] and may provide some further insight into the enantiomeric ratios and the origin of these metabolites.

2. Experimental

2.1. Patients

Urine samples were obtained from nine patients with classical ZS. Four urine samples from healthy controls without signs of a peroxisomal disease were obtained. All samples were stored at −20 °C until analysis.

2.2. Chemicals and reagents

(*R*,*S*)-2-Hydroxyisocaproic acid, (*R*,*S*)-2-hydroxyglutaric acid zinc salt, (*R*) and (*S*)-5-oxotetrahydrofuran-2-carboxylic acid and NAD^+ were obtained from Sigma (St. Louis, MO, USA); (*R*,*S*)-3-methyladipic acid, (*R*)-3-methyladipic acid, suberoyl chloride and (*R*,*S*)-3-(4-hydroxyphenyl)-lactic acid hydrate from Aldrich (Milwaukee, WI, USA). (*R*,*S*)-2-Hydroxysebacic acid dimethylester and (*R*)-3-hydroxysebacic acid dimethylester were synthesised as described in Section 2.4. Racemic 3-hydroxysebacic acid was obtained from Prof. E. Brunet (Universidad Autónoma de Madrid, Spain).

Methanol, *n*-hexane, sodium hydrogencarbonate, sodium chloride and sodium sulfate were from Riedel-de Haën (Seelze, Germany). Acetyl chloride, thionyl chloride, dichloromethane, diethyl ether, pyridine, potassium hydroxide, (*S*)-α-methoxy-α-trifluoromethyl-phenylacetyl chloride (*S*-MTPA-Cl), 2,2-dimethyl-1,3-dioxane-4,6-dione (Meldrum's acid) and glycerol dehydrogenase (EC 1.1.1.6) from *Geotrichum candidum* were obtained from Fluka (Buchs, Switzerland). *tert*.-Butylmethyl ether (MTBE), sebacoyl chloride, iodine, bromine, toluene-4-sulfonic acid hydrate, cyclohexane, disodium hydrogenphosphate, potassium carbonate and silica (0.040–0.063 mm, 0.063–0.200 mm) were from Merck (Darmstadt, Germany). Formic acid, sulfuric acid, hydrochloric acid and 2-propanol were from Grüssing (Filsum, Germany). Ethyl acetate was from Roth (Karlsruhe, Germany). (In the following context methyl esters are abbreviated with ''Me'' respectively.)

The column of the GC–MS system and the chiral stationary phase of the MDGC main column was ''custom''-made as previously described [7,8].

2.3. Instrumentation

2.3.1. GC–MS

A Fisons Instrument GC 8000 gas chromatograph was connected to a Fisons Instruments MD800 Quadrupol-MS (EI, 70 eV). The mass range was set at 40–250 amu. The column was fused-silica, 30 m×0.25 mm i.d., coated with a 0.5-μm film of SE-52. The carrier gas was He at 25 kPa, the injector was split–splitless at 230 °C; split 1:20. The interface temperature was 250 °C and the ion-source temperature 200 °C. The initial column temperature was 80 °C increased by 4 °C to 260 °C.

2.3.2. HPLC

The equipment consisted of a SepTechTM pump (1–140 ml) with a cyclic system (cyclomat; Merck, Darmstadt), a 250 mm×50 mm i.d. column packed with LiChrospher 60 (12 μm, Merck) and a Knauer variable wavelength monitor with a Sonntek Vari-PrepCell VP1100. The column was eluted with *n*-hexane/diethylether (65:35, v/v) at a flow-rate of 70 ml/min. Detection was set at 210 nm.

2.3.3. NMR-data

^{1}H-NMR: Bruker ARX 300 spectrometer (Bruker, Karlsruhe, Germany); and ^{13}C-NMR: Bruker AC 200 E (Bruker, Karlsruhe, Germany).

2.3.4. Enantio-MDGC–MS

The enantio-MDGC–MS system consisted of a Siemens SiChromat 2 double oven system with two independent temperature controls. The injections were in the split–splitless mode at 250 °C. The precolumn was equipped with a flame ionisation detector (FID, 270 °C), whereas the main column was connected with an ITD-transfer line (250 °C) and an open split interface (250 °C) to an ITD 800 mass spectrometer (Finnigan MAT, Bremen, Germany), detection in electron ionisation mode. Sweep flow (helium) was 1 ml/min, ion trap manifold 230 °C and electron energy 70 eV.

The pre and main column were connected with a live-switching coupling piece (live-T-piece). The precolumn was a 30 m×0.25 mm i.d. SOLGEL-Wax (SGE, Austin, TX), coated with a 0.25-μm film. A retention gap was installed prior to the precolumn (1 m×0.25 mm i.d. deactivated high temperature fused-silica capillary column).

A fused-silica capillary column (30 m×0.25 mm i.d.), coated with a 0.25-μm film of the chiral stationary phase heptakis (2,3-di-*O*-methyl-6-*O*-*tert*.-butyl-dimethylsilyl)-β-cyclodextrin, dissolved in SE 52 was used as the main column.

Conditions: For the precolumn the carrier gas was helium, pressure 210 kPa; split 1:30; the initial temperature was 80 °C, ramp 4 °C/min to 270 °C and held for 1 h. For the main column the carrier gas was helium, pressure 145 kPa; initial temperature 60 °C raised at 1.5 °C/min to 200 °C, held for 30 min

Analytes (methyl ester)	Cut-times precolumn (min)
(*R*,*S*) 2-Hydroxyisocaproic acid	13.1–13.8
(*R*,*S*) 3-Methyladipic acid	22.7–23.3
(*R*,*S*) 2-Hydroxyglutaric acid/TOF	30.5–31.3
(*R*,*S*) 2-Hydroxysebacic acid	44.5–45.2
(*R*,*S*) 3-Hydroxysebacic acid	46.3–47.0
(*R*,*S*) HPLA	68.7–69.6

2.4. Reactions

2.4.1. Synthesis of (R,S)-2-hydroxysebacic acid diMe

A 5-ml volume of thionyl chloride was mixed with 10 ml of sebacoyl chloride and heated at 55 °C [9]. After the addition of some iodine crystals, 2 ml of bromine was added slowly. The reaction mixture was stirred and heated at 55 °C for 4 h. The excess thionyl chloride was removed. Twenty millilitres of 50% formic acid was added to the residue slowly and heated at 75 °C for 2 h. After concentrating, the residue was suspended in a sodium hydrogencarbonate solution and a small amount of sulfuric acid was added slowly. The suspension was filtered and dried. Hydrolysis was carried out with 2.0 g of the reaction product, which was dissolved in 80 ml 1 *M* K_2CO_3 solution and heated under reflux for 1 h. After cooling to room temperature the reaction mixture was acidified with 1 N sulfuric acid, extracted with diethyl ether and the organic layer dried over Na_2SO_4, filtered and concentrated.

A 1.0-g amount of the reaction product was dissolved in a mixture of dry dichloromethane (100 ml) and methanol (20 ml). After the addition of 0.1 g toluene-4-sulfonic acid hydrate, the reaction mixture was stirred at room temperature overnight [10]. The solution was neutralised with saturated $NaHCO_3$ solution and extracted with MTBE, washed with a saturated solution of NaCl and dried over sodium sulfate. An aliquot (200 mg) was applied to a silica gel column (20 g) and eluted with hexane/diethyl ether (1:1, v/v) to yield pure 2-hydroxysebacic acid diMe, which was identified by GC–MS.

(MS: *m*/*z* 247(1), 187(27), 155(100), 109(59), 95(25), 90(18), 87(25), 74(21), 67(42), 66(50), 59(50), 55(82), 54(88), 43(57), 41(65)).

2.4.2. Preparation of (R)-MTPA-derivatives of (R,S)-2-hydroxysebacic acid diMe

Dry pyridine (1 ml), (*S*)-MTPA-Cl (50 μl, 0.27 mmol) and 2-hydroxysebacic acid diMe (70 mg; 0.27 mmol) were injected into a dry test tube and kept under constant stirring at ambient temperature overnight [11,12]. The resulting (*R*)-MTPA-esters were isolated by diluting the reaction mixture with diethyl ether and washing with 1 N HCl, dest. H_2O, saturated $NaHCO_3$ solution and saturated NaCl solution. The organic layer was dried over sodium sulfate and directly analysed by GC. The diastereomeric (*R*)-MTPA-esters were isolated by HPLC and their purity monitored by GC.

2.4.3. ^{1}H-NMR-investigation of pure (R)-MTPA-diastereomers of 2-hydroxysebacic acid diMe

The pure diastereomers were concentrated and dissolved in $CDCl_3$ with TMS as standard.

^{1}H-NMR (300 MHz; $CDCl_3$/TMS): (*R*),(*R*)-MTPA-diastereomer: δ ppm 7.64 (m, 2H); 7.40–7.43 (m, 3H); 5.14–5.18 (t, 1H, J=6.5 Hz); 3.79 (s, 3H); 3.65–3.66 (s, 6H); 2.26–2.30 (t, 2H, J=7.5 Hz); 1.84–1.86 (m, 2H); 1.11–1.30 (m, 10H).

(*R*),(*S*)-MTPA-diastereomer: δ ppm 7.57–7.58 (m, 2H); 7.41–7.43 (m, 3H); 5.15–5.19 (t, 1H, J=6.3 Hz); 3.75 (s, 3H); 3.67 (s, 3H); 3.56 (s, 3H); 2.27–

2.32 (t, 2H, J=7.5 Hz); 1.87–1.94 (m, 2H); 1.11–1.29 (m, 10H).

2.4.4. MTPA-ester cleavage and chirality evaluation

Aliquots (10 mg) of the isolated MTPA-ester were dissolved in 2 ml methanol and 200 μl of a solution of KOH in methanol (5%) were added. After stirring at room temperature overnight the reaction mixture was acidified with 1 N HCl and extracted three times with diethyl ether. The combined organic extracts were washed with saturated $NaHCO_3$ solution, saturated NaCl solution and dried over sodium sulfate. The organic phase was analysed directly by enantio-MDGC.

2.4.5. Enantioselective synthesis of D-3-hydroxysebacic acid diMe

2.4.5.1. Preparation of 3-oxosebacic acid diMe. Meldrum's acid (2.9 g, 20 mmol) was suspended in 25 ml of dry dichloromethane. To this solution, 3.2 ml (40 mmol) of pyridine was added slowly at 0 °C [13]. The reaction mixture was treated at 0 °C with 4 ml (22 mmol) of suberoyl chloride, diluted in 25 ml dichloromethane, for approximately 2 h under nitrogen. It was stirred for 1 h at 0 °C and additionally for 2 h at room temperature. The reaction mixture was washed twice with diluted HCl. The organic layer was washed with saturated NaCl solution and dried over sodium sulfate. The crude reaction product was isolated by evaporation at reduced pressure. The residue was dissolved in 50 ml methanol and refluxed for 3 h. The raw product was purified by flash-chromatography on silica gel [14] with cyclohexane/ethylacetate (2:1, v/v) as the eluent. 3-Oxosebacic acid diMe was identified by ^{13}C-NMR.

^{13}C-NMR (100 MHz; $CDCl_3$/TMS): δ ppm 23.2; 24.7; 28.7; 28.8; 33.9; 42.9; 49.0; 51.4; 52.3; 167.6; 174.0; 202.6.

2.4.5.2. Stereoselective reduction of 3-oxosebacic acid diMe [15]. In a micro reaction vessel, 4 mg (0.016 mmol) of 3-oxosebacic acid diMe was suspended in 150 μl 0.1 *M* Na_3PO_4 buffer (pH 7.0). To this solution, 0.15 U glycerol dehydrogenase from *Geotrichum candidum* (EC 1.1.1.6), 0.16 mmol of 2-propanol and 0.19 μmol NAD^+ were added. The reaction mixture was stirred for 2 days at room temperature.

The suspension was extracted twice with 500 μl of MTBE. The organic layer was washed with saturated NaCl solution and dried over sodium sulfate. The generated D-3-hydroxysebacic acid diMe was identified by enantio-MDGC–MS (Fig. 3).

2.5. Sample preparation

Urine samples (1 ml) were neutralised and lyophilised in a 5-ml GC vial. The dry residue was taken up in 1 ml acetyl chloride/methanol (1:9, v/v), transferred to a 5-ml reaction vessel and heated for 10 min at 110 °C [16]. After cooling to room temperature, it was neutralised with sodium hydrogencarbonate solution and extracted twice with 1 ml *tert*.-butylmethyl ether. The organic layer was dried over anhydrous sodium sulfate and evaporated to ~50 μl [17].

3. Results and discussion

The presented enantio-MDGC–MS system using an achiral precolumn and modified cyclodextrins as the main column easily and successfully afforded the separation of the chiral metabolites HICA, 2-HG, 3-MA, HPLA, 2-HS and 3-HS (as methyl esters) in a single chromatographic run. On the polar precolumn, the investigated compounds were separated from many other urinary metabolites and transferred to the chiral column with mass selective detection to ensure their unambiguous identification (Fig. 1). This approach improves our understanding of enzymatic activities and metabolic pathways, particularly those involved in inborn errors of metabolism.

For the analysis of the carboxylic acids, they were derivatised to their corresponding methyl esters. The derivatisation method with acetyl chloride/methanol has been shown to proceed smoothly without any racemisation [6,16]. As a minor product, tetrahydrofuran-5-oxo-2-carboxylic acid (TOF)—the corresponding γ-lactone product of 2-HG—occurs on storage or during derivatisation (Fig. 1).

The elution order of the chiral carboxylic acids analysed in this report was established using either commercially available and enantiopure or enriched

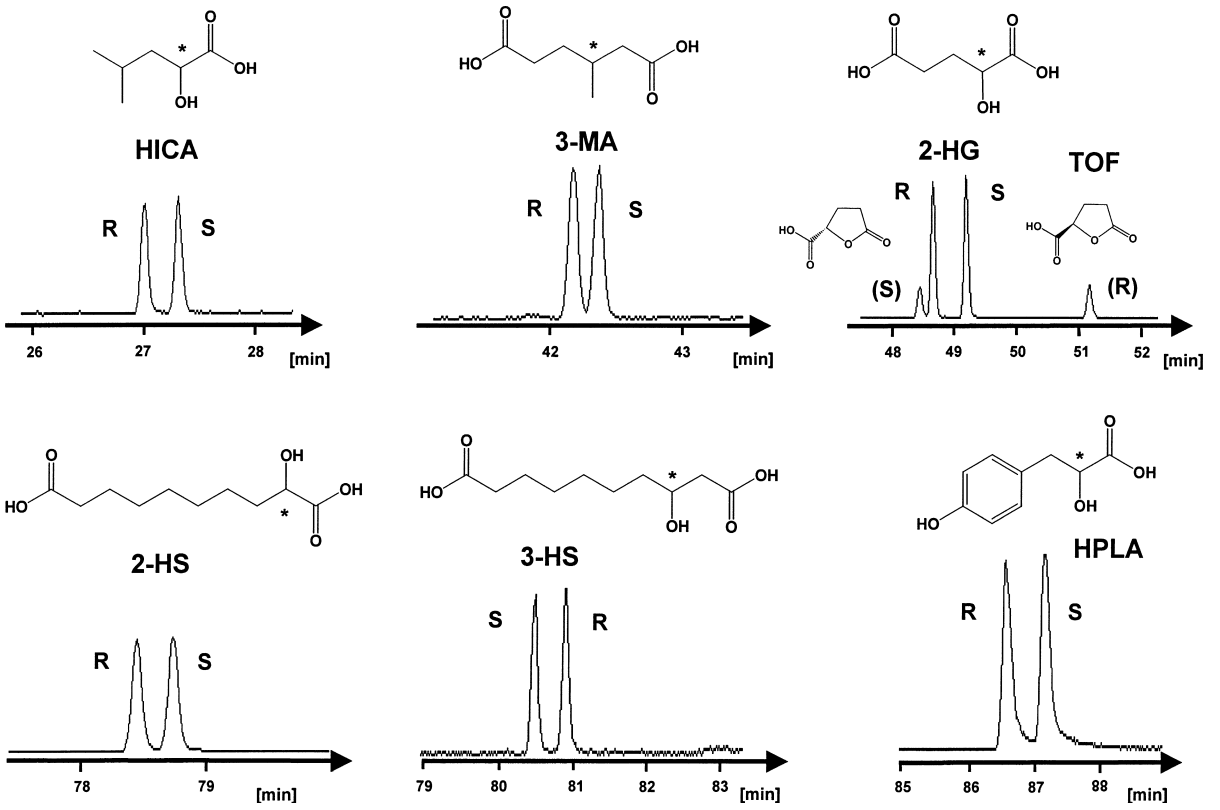

Fig. 1. Enantio-MDGC–MS analysis of chiral metabolites: main column separation of reference compounds (HICA, 3-MA, 2-HG/TOF, 2-HS, 3-HS, HPLA) into enantiomers. All reference compounds were analysed as methyl esters and dimethylesters, respectively.

standards of definite absolute configuration (Fig. 1), synthesised in our laboratory.

3.1. Absolute configuration and enantioselective analysis of 2- and 3-hydroxysebacic acid diMe

3.1.1. Absolute configuration of 2-hydroxysebacic acid diMe

The diastereomeric esters were separated by HPLC and characterised by ^{1}H-NMR. Elucidation of the absolute configuration of 2-hydroxysebacic acid diMe was deduced from the ^{1}H-NMR spectroscopic behaviour of the diastereomeric (*R*)-MTPA-ester according to the Mosher model [11,12]. This method is well established and is based on chemical shift differences for protons with equivalent constitution. The different chemical shifts for the diastereotopic groups result from the upfield shift caused by the anisotropic magnetic field around the phenyl ring of the acid moiety. The singlet signal of the 1-COOMe group of the 2-hydroxysebacic acid moiety is the ^{1}H-NMR spectral indicator of the diastereomeric esters. The absolute configurations of the 2-hydroxysebacic acid diMe enantiomers were concluded from the ^{1}H-NMR data in connection with the model considerations as outlined in Fig. 2.

After mild alkaline ester cleavage with retention of the absolute configuration [18], the pure enantiomers of 2-hydroxysebacic acid diMe were directly analysed by enantio-MDGC and the resulting elution order was proved to be (*R*) (I) and (*S*) (II) (Fig. 1).

3.1.2. Absolute configuration of 3-hydroxysebacic acid diMe

The absolute configuration of 3-hydroxysebacic acid diMe was determined by enantioselective reduction of synthesised 3-oxosebacic acid diMe.

The enantioselective reduction was carried out

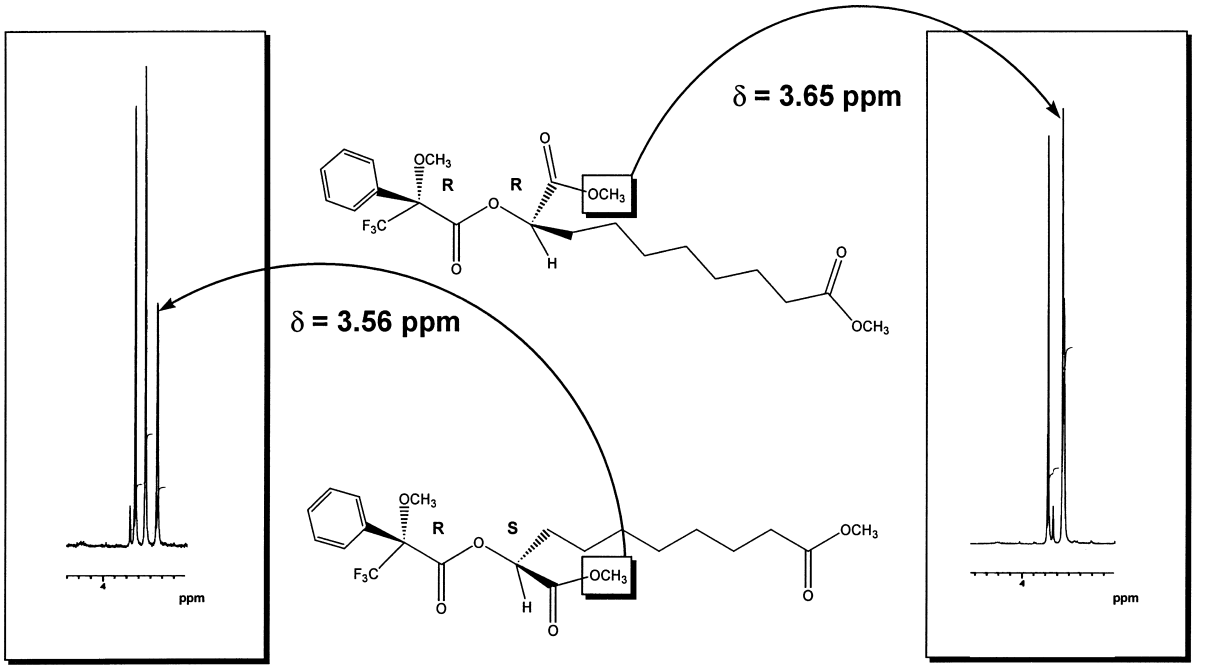

Fig. 2. ^{1}H-NMR-spectra of the investigated (*R*)-MTPA-diastereomers of 2-hydroxysebacic acid diMe.

with glycerol dehydrogenase (EC 1.1.1.6) from *Geotrichum candidum*, which reduces β-ketoesters with high enantioselectivity to the corresponding D-hydroxyester [15]. Following the Cahn–Ingold–Prelog convention, D-3-hydroxysebacic acid diMe can be assigned to the 3*R*-configuration, respectively. The product of the enzymatic reduction was analysed by enantio-MDGC–MS (Fig. 3).

The elution order was assigned to be (*S*) (I) and (*R*) (II), using the synthesised enantiopure reference compound in comparison with the racemic mixture of 3-hydroxysebacic acid diMe (Fig. 3).

3.2. Analysis of chiral carboxylic acids in urine samples

The results of the enantiomeric ratios of the investigated urine samples are summarised in Table 1.

In all urine samples of the ZS patients, the excretion of HPLA was increased. This has also been found in patients with tyrosinaemia and/or liver failure. Endogenously formed HPLA consists of only the (*S*)-enantiomer [19]. In the investigated urine samples, HPLA occurred predominantly in the (*S*)-form (>99%).

The metabolite HICA was observed in three of nine ZS cases, the (*R*)-enantiomer being predominant compared to the (*S*)-form. HICA is a metabolite of the amino acid L-leucine and the (*R*)-enantiomer can be formed only as product of certain bacteria [19].

In the present investigation, the urine samples of five ZS patients demonstrated elevated (*S*)-2-HG. 2-Hydroxyglutaric aciduria exists in two distinct clinical phenotypes [4] and an enantioselective analysis can easily distinguish between the two different phenotypes. An elevation of (*S*)-2-HG is present in urine of patients with characteristic neurological and cerebellar abnormalities [20]. For those patients with (*R*)-2-hydroxyglutaric aciduria, only developmental delay has been observed. Only in case 4 was an increased (*R*)-enantiomer excretion detected, for which we as yet have no explanation.

3-MA might be a metabolite of the ω-oxidation of phytanic acid, which is a constituent of the normal diet. The precursor phytanic acid occurs in two diastereomeric forms: (3*S*, 7*R*, 11*R*) and (3*R*, 7*R*, 11*R*). In animals both diastereomers occur, whereas

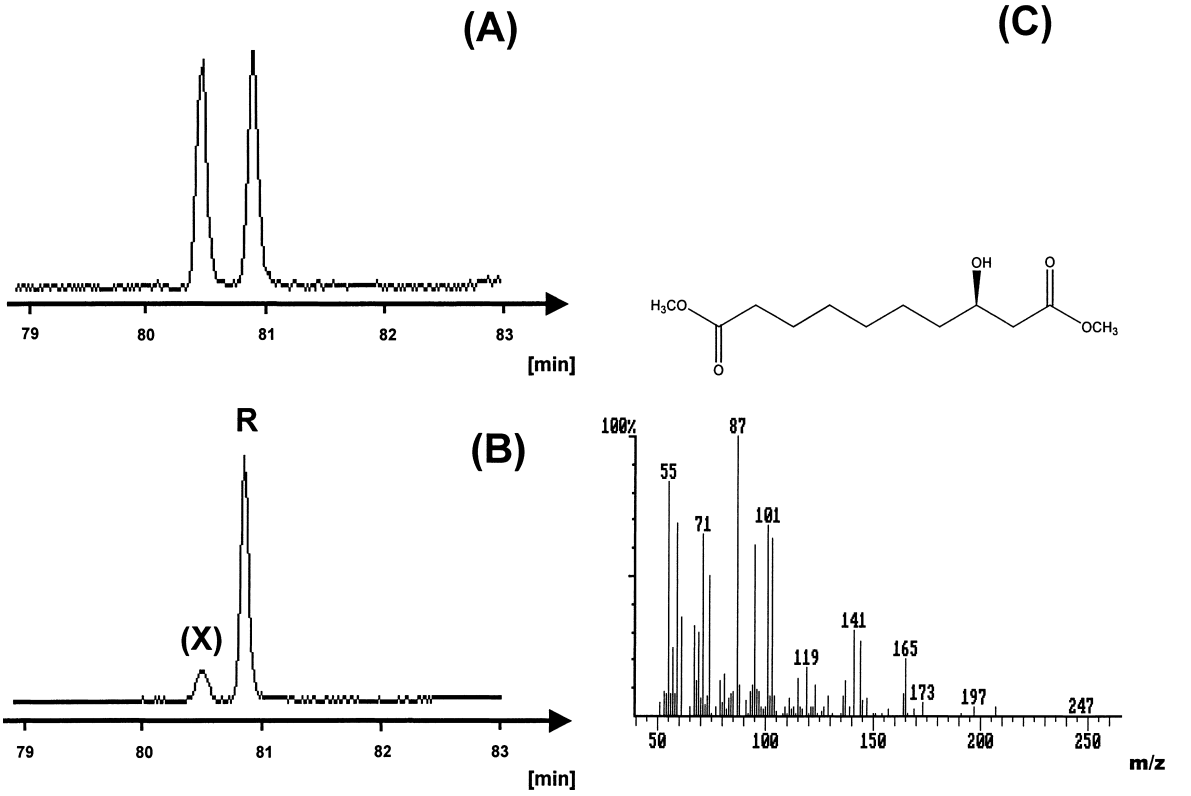

Fig. 3. Enantio-MDGC–MS analysis of the stereoselective reduction of 3-oxo-sebacic acid diMe with glycerol dehydrogenase (EC 1.1.1.6) from *Geotrichum candidum*. (A) Main column separation of the reference compound: racemic 3-hydroxysebacic acid diMe. (B) Main column chromatogram of the stereoselective reduction of 3-oxo-sebacic acid diMe in favour of (*R*)-3-hydroxysebacic acid diMe; the peak (X) is not identical with (*S*)-3-hydroxysebacic acid diMe, as proved by a different mass spectrum (base peak $m/z = 147$). (C) Mass spectrum of 3-hydroxysebacic acid diMe.

in terrestrial mammals the (3*R*, 7*R*, 11*R*) isomer predominates [21]. 3-MA was found in the urine of some ZS patients and in small amounts in the control urines. In all these samples the amount of the (*R*)-enantiomer was increased (66–84%).

In all urine samples of the ZS patients, an increased excretion of 2-HS was present and for the first time it was shown that the amount of the (*R*)-enantiomer (80–92%) predominates over the (*S*)-enantiomer (Fig. 4B).

2-HS may be a result of the ω-oxidation and subsequent β-oxidation of 2-hydroxy-long-chain fatty acids (LCFA) and 2-hydroxy-very-long-chain fatty acids (VLCFA) [1,3]. 2-Hydroxy-VLCFA (C_{22}–C_{26}) are characteristic components of brain cerebrosides and sulfatides, the major components of myelin lipids. The absolute configuration of naturally occurring VLCFA (i.e. cerebronic acid) was determined to be the (*R*) form [22].

The excretion of 3-HS in human urine is described in cases with increased mobilisation of fatty acids and inhibited fatty acid β-oxidation such as long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency, dicarboxylic aciduria and under fasting conditions [23]. In general, 3-HS is not a biochemical marker for defects in fatty acid oxidation. Infants with a normal fatty acid oxidation may produce 3-hydroxy-dicarboxylic acids when they are fed with a MCT-containing formula. The metabolic origin of 3-HS is proposed to result from the ω-oxidation of 3-hydroxy-monocarboxylic acids in liver mitochondria [23]. In the metabolic pathway of the fatty acid β-oxidation, the formation of the 3-hydroxyacyl-CoA-compound is enantioselective in favour of the

Table 1
Enantio-MDGC–MS analysis: enantiomeric ratios in urine samples

Sample no.	Status	HICA		3-MA		2-HG		2-HS		3-HS		HPLA	
		R	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*S*	*R*	*R*	*S*
1	ZS	n.d.	n.d.	n.d.	n.d.	18	81	81	19	76	24	<1	>99
2	ZS	n.d.	n.d.	n.d.	n.d.	56	44	89	11	73	27	<1	>99
3	ZS	n.d.	n.d.	n.d.	n.d.	55	45	89	11	75	25	<1	>99
4	ZS	69	31	n.d.	n.d.	87	13	80	20	65	35	<1	>99
5	ZS	n.d.	n.d.	n.d.	n.d.	26	74	90	10	78	22	<1	>99
6	ZS	>99[a]	<1	84	16	35	65	80	20	62	38	<1	>99
7	ZS	n.d.	n.d.	83	17	13	87	84	16	83	17	2	98
8	ZS	>99	<1	66[a]	34	50	50	92	8	84	16	2	98
9	ZS	n.d.	n.d.	n.d.	n.d.	19	81	80	20	82	18	<1	>99
10	Control	n.d.	n.d.	74[a]	26	49	51	n.d	n.d.	n.d	n.d.	n.d	n.d.
11	Control	n.d.	n.d.	n.d.	n.d.	57	43	n.d.	n.d.	n.d	n.d.	n.d	n.d.
12	Control	n.d.	n.d.	76[a]	24	46	54	n.d.	n.d.	n.d	n.d.	n.d	n.d.
13	Control	n.d.	n.d.	80[a]	20	48	52	n.d.	n.d.	n.d	n.d.	n.d	n.d.

n.d., not detectable; ZS, patients with *Zellweger* syndrome.
[a] Trace.

(*S*)-configuration. Furthermore, 3-hydroxyacyl-CoA dehydrogenase is selective for the oxidation of (*S*)-3-hydroxyacyl-CoAs. The formation of both enantiomers could result from more than one enzyme or even more than one metabolic pathway being involved in the formation of 3-hydroxyacyl-CoAs.

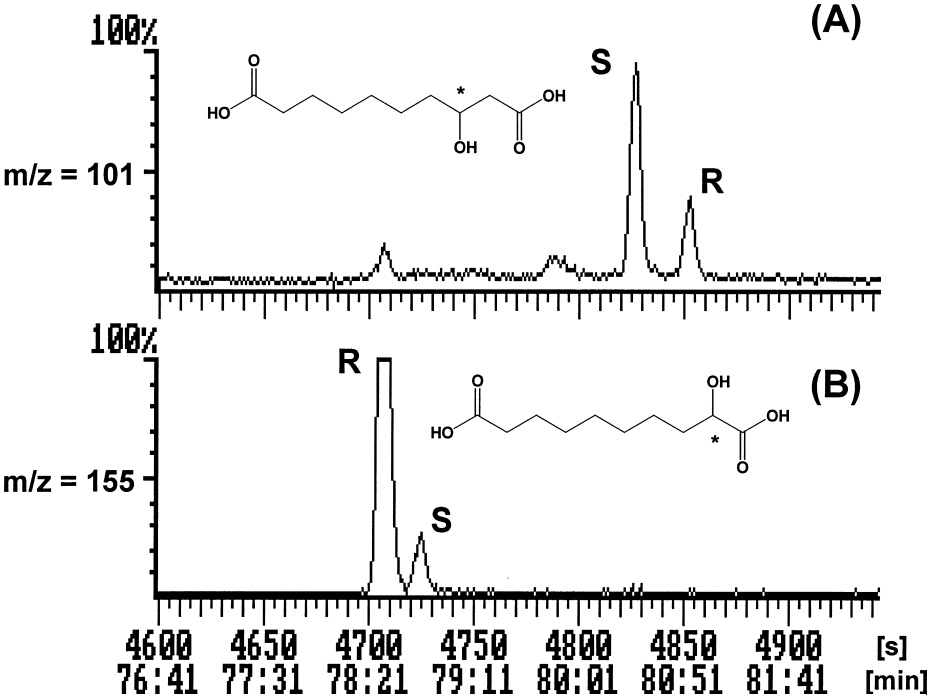

Fig. 4. Enantio-MDGC–MS analysis. (A) Main column separation of 3-HS-enantiomers—analysed as dimethyl esters from a ZS patient urine (case no. 3). (B) Main column separation of 2-HS-enantiomers—analysed as dimethyl esters from a ZS patient urine (case no. 3).

From previous studies in isolated mitochondria, the configuration of the precursor 3-hydroxy-fatty acids was shown to be an almost racemic mixture with a slightly increased D(*R*)-isomer (58±3%) [24]. The racemisation of the 3-hydroxy-fatty acids was explained as a rapid dehydration–rehydration step.

For the determination of the enantiomeric distribution of the urinary metabolite 3-HS, we could not confirm the results of the previous study [24]. The present study clearly demonstrates that the (*S*)-enantiomer of 3-HS (62–84%) is predominant in all investigated urine samples of the ZS patients (Fig. 4A). These finding could be explained since an impaired β-oxidation would lead to an accumulation of the (*S*)-enantiomer of 3-hydroxyacyl-CoAs. To our knowledge, the biological relevance for the formation of the (*R*)-enantiomer remains unknown.

4. Conclusions

The enantio-MDGC–MS system enables the simultaneous stereodifferentiation of chiral urinary metabolites, even in trace amounts. This method allows a sensitive, selective and unambiguous identification of intermediary compounds. The results of the investigated enantiomeric distributions of HICA, 2-HG, 3-MA, HPLA, 2-HS and 3-HS allow conclusions to be drawn concerning the biosynthesis of these urinary metabolites in patients with peroxisomal diseases such as *Zellweger* syndrome. This approach is a promising perspective in diagnostic certainty and may help to formulate specific dietary treatments. The enantio-MDGC–MS is a versatile analysis method for the diagnosis of inherited metabolic diseases. Further clinical investigations are in progress.

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