ELSEVIER

Journal of Chromatography B, 739 (2000) 313–324

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Enantioselective analysis of ketone bodies in patients with β-ketothiolase deficiency, medium-chain acyl coenzyme A dehydrogenase deficiency and ketonemic vomiting

Martin Heil[a], Frank Podebrad[a], Elisa Prado[a], Thomas Beck[a], Armin Mosandl[a,*], Adrian C. Sewell[b], Hans Böhles[b], Willy Lehnert[c]

[a]*Institut für Lebensmittelchemie, Biozentrum J.W. Goethe-Universität, Marie-Curie-Strasse 9, D-60439 Frankfurt am Main, Germany*
[b]*Kinderklinik der J.W. Goethe-Universität, Frankfurt am Main, Germany*
[c]*Kinderklinik der A.L. Universität, Freiburg, Germany*

Received 12 July 1999; received in revised form 3 December 1999; accepted 10 January 2000

Abstract

Enantioselective multidimensional gas chromatography–mass spectrometry (enantio-MDGC–MS) is a valuable tool for the differentiation of enantiomers from complex matrices when present in trace amounts. The separation of chiral compounds provides further information on the diagnosis of diseases, and on normal and abnormal biochemical pathways. The formation of the normal urinary metabolite 3-hydroxy-2-methylbutanoic acid (HMBA), excreted in abnormally high amounts in β-ketothiolase deficiency, is not absolutely clarified. Metabolic pathways involving this metabolite are isoleucine catabolism, as well as presumably β-oxidation of fatty acids and ketogenesis. The latter two pathways are distinguishable in their enantioselectivity. Enantioselective analysis gives further information on interfering metabolic pathways and the selectivity of the enzyme(s) forming HMBA. Different ratios of the stereoisomers of HMBA in control urine samples and patients with β-ketothiolase deficiency were detected. Analogous to HMBA urinary 3-hydroxybutanoic acid (HBA) was investigated in several diseases. The formation of HBA and HMBA is expected to result from the same or similar metabolic pathways. Differences in the enantiomeric ratio of HMBA may originate from the enantioselectivity of different enzyme systems.

Keywords: Enantiomer separation; Ketone bodies; 3-Hydroxy-2-methylbutanoic acid; 3-Hydroxybutanoic acid; β-Ketothiolase

1. Introduction

3-Hydroxy-2-methylbutanoic acid (HMBA) is (esterified with CoA) a normal metabolite of isoleucine catabolism preceeded in the pathway by tiglyl-CoA and followed by 2-methyl-3-oxobutanoyl-CoA (MOBA-CoA) (Fig. 1) [1].

Patients with β-ketothiolase deficiency (mitochondrial acetoacetyl-CoA thiolase deficiency), an autosomal recessive inherited disease, lack the enzyme β-ketothiolase (mitochondrial acetoacetyl-CoA thiolase) which cleaves MOBA-CoA to acetyl-CoA and propanoyl-CoA resulting in a significant disturbance of isoleucine catabolism. This defect leads to a characteristic pattern of urinary organic acids with an increased excretion of HMBA (Fig. 2) [1–3].

*Corresponding author.

0378-4347/00/$ – see front matter
PII: S0378-4347(00)00022-0

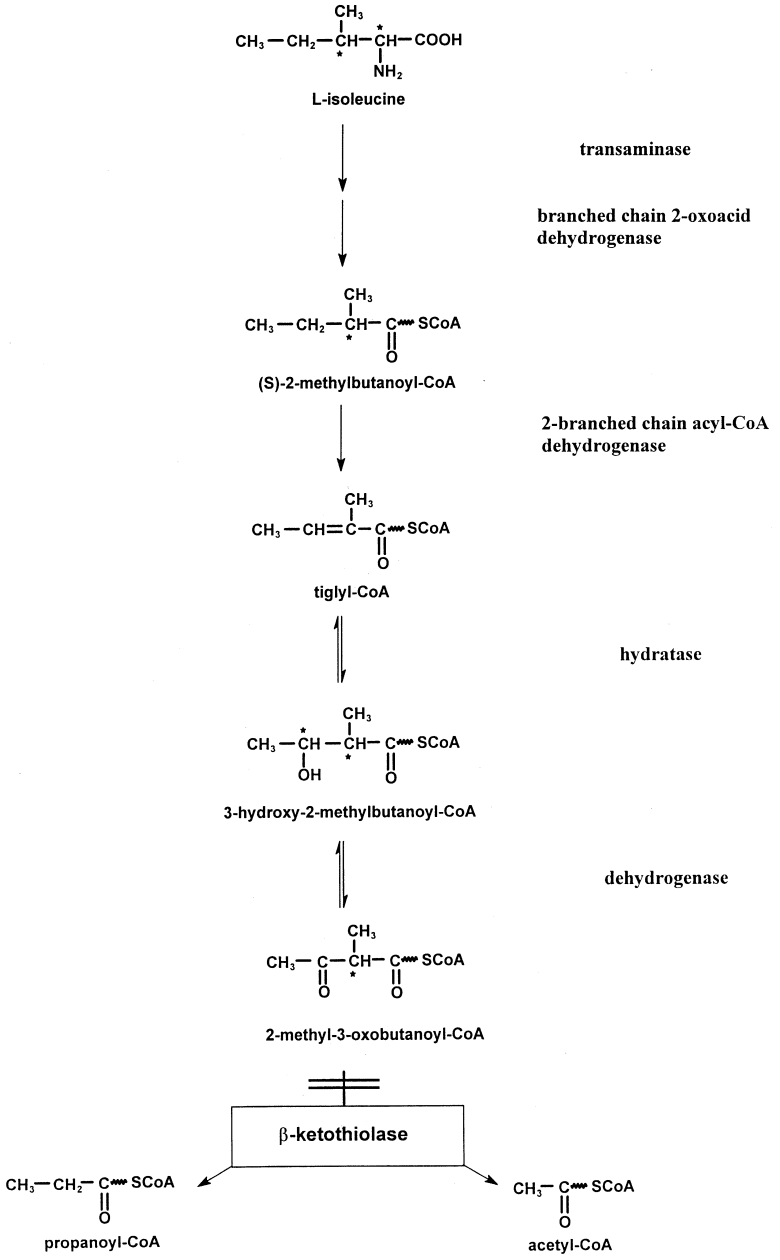


Fig. 1. Catabolism of L-isoleucine.

HMBA has two chiral centers and exists in four stereoisomeric forms. Due to its structural properties, HMBA is an interesting chiral synthon in the synthesis of natural compounds as antibiotics [4], pheromones [5,6], bioactive saponins [7] or their analogues as well as a reference substance in describing and testing enantioselective synthesis [8,9]. The absolute configuration has been elucidated [10,11],

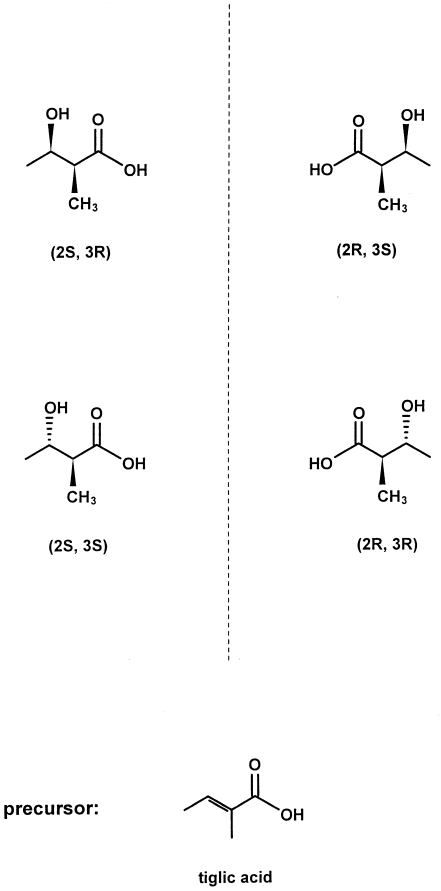


Fig. 2. HMBA stereoisomers and tiglic acid, the precursor of HMBA in L-isoleucine catabolism.

but the methods were inappropriate and unsuitable for trace analysis. In this paper a method for the enantioselective analysis of HMBA in urine of patients with β-ketothiolase deficiency and of controls is presented.

To our knowledge the elucidation of the absolute configuration of HMBA in human urine has not been carried out until now.

A second aspect is the investigation of 3-hydroxybutanoic acid (HBA), because the underlying metabolic pathways are likely to be the same or at least very similar to HMBA. In the β-oxidation of fatty acids the (*S*)-enantiomer is formed enantioselectively whereas ketogenesis leads to formation of the (*R*)-enantiomer [12,13]. Enantioselective analysis of HBA may provide some insight into the extent of these two metabolic pathways and on the origin of HBA. Regarding this aspect, several samples from patients with β-ketothiolase deficiency, ketonemic vomiting, a symptom of unknown origin with vast amounts of excreted ketone bodies, and medium chain acyl-CoA dehydrogenase (MCAD) deficiency, a defect in the degradation of fatty acids which in some cases coincides with excretion of ketone bodies of unknown origin, were investigated.

We present a method for the enantioselective analysis of HMBA and HBA. The ratio of enantiomers may shed light on the ways of formation of these metabolites in affected children and control samples.

2. Experimental

2.1. Patients

Urine samples were obtained in the course of selective screening for inborn errors of metabolism. Where possible the diagnosis was confirmed by respective enzyme assays in cultured skin fibroblasts or by mutation analysis.

The following patients were investigated:

Four patients with β-ketothiolase deficiency. The clinical details of patient S. have been reported [14]. Patient C., a 7-year-old Turkish boy, had a severe hypoglycaemic episode with increased ketonuria. He had no prior attacks. Branched chain amino acids in plasma were increased and urinary organic acid analysis demonstrated the characteristic pattern of β-ketothiolase deficiency (3-hydroxybutanoate, 3-hydroxy-2-methylbutanoate and tiglylglycine). Since then he was set on a protein-restricted diet and has had no further episodes of metabolic decompensation.

In the case of patient Nos. 3 and 4 no clinical data were available.

Thirteen patients with medium chain acyl-CoA dehydrogenase deficiency in whom 3-hydroxybutanoate excretion was also prominent.

Two patients with ketonemic vomiting who had no other symptoms of metabolic disease.

A group of five children without evidence of metabolic disease served as controls.

2.2. Chemicals and reagents

Racemic 3-hydroxybutanoic acid was from Sigma (St. Louis, MO, USA), 2-methyl-3-oxobutanoic acid ethyl ester and sodium (*S*)-(+)-3-hydroxybutanoic acid from Aldrich (Milwaukee, WI, USA), *tert.*-butyl methyl ether (MTBE), ethanol, toluene-4-sulfonic acid monohydrate and diatomaceous earth were from Merck (Darmstadt, Germany). Sodium borohydride and acetyl chloride were obtained from Fluka (Buchs, Switzerland). Sucrose was from Roth (Karlsruhe,Germany). Dichloromethane, sodium hydroxide, sodium hydrogencarbonate and sodium sulfate were purchased from Riedel-de Haën (Seelze, Germany). Hydrochloric acid, sulfuric acid and sodium chloride were from Grüssing (Filsum, Germany), methanol from Mallinckrodt–Baker (Deventer, The Netherlands) and diethyl ether from Krämer & Martin (St. Augustin, Germany). Baker's yeast was obtained from two different local supermarkets and yeast nutrients in tablet-form from Arauner (Kitzingen, Germany).

All GC columns were prepared as previously described [15,16].

2.3. Instrumentation

2.3.1. Enantioselective multidimensional gas chromatography–mass spectrometry

The chromatographic system comprised a Siemens SiChromat 2 double-oven system with two separate temperature controls. Injections were split–splitless at 250°C.

The pre-column was a 30 m×0.25 mm I.D. high-temperature fused-silica capillary column coated with a 0.38 μm film of PS 268. Conditions: carrier gas hydrogen at 1.4 bar; split 27 ml/min; initial temperature 40°C; ramp 1°C/min to 120°C; ramp 5°C/min to 250°C.

Prior to the pre-column a protection column (2 m×0.25 mm I.D. deactivated high-temperature fused-silica capillary column) was installed. A fused-silica capillary (30 m×0.32 mm I.D.) coated with a 0.64 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: carrier gas hydrogen at 0.65 bar; initial temperature 40°C raised at 0.5°C/min to 70°C, then raised with 2°C/min to 200°C (for HMBA-, HBA- and MOBA-methyl esters).

A flame ionisation detector (270°C) was used as the pre-column detector, 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), with detection in the ion impact mode. Sweep flow (helium) was 1 ml/min, ion trap manifold was 230°C and electron energy was 70 eV.

For the separation of HMBA- and MOBA-ethyl esters the temperature program of the main column was modified: initial temperature 40°C held for 5 min; raised at 2°C/min to 200°C. (Methyl ester and ethyl ester are in the following abbreviated with ''Me'' or ''Et'', respectively.)

2.4. Reactions

2.4.1. Reduction of MOBA-Et to HMBA-Et using $NaBH_4$

Into an ice-cooled flask containing 0.02 g NaOH, 200 μl water, 1 ml absolute ethanol and 1.8 ml MOBA-Et 500 mg of $NaBH_4$ in 25 ml absolute ethanol was added dropwise and stirred for 2 h.

Subsequently, the solution was acidified with 2 *M* HCl and twice extracted with MTBE. The organic phase was washed with saturated $NaHCO_3$ solution and saturated sodium chloride solution. The MTBE phase was dried over sodium sulfate and analyzed by GC.

Since the starting material could not be detected, the reaction appeared to be complete. All four peaks of HMBA-Et were detected in comparable amounts.

2.4.2. Hydrolysis of HMBA-Et to HMBA

A 500-μl volume of HMBA-Et was added to 10 ml of 15% NaOH and stirred overnight. After smooth acidification with hydrogen chloride the solution was extracted with MTBE. The MTBE phase was purified with a saturated solution of sodium chloride and dried over Na_2SO_4 before evaporating the solvent.

2.4.3. Esterification of HMBA to HMBA-Me

A 5-mg amount of product of reaction (see Section 2.4.2) was mixed with 1 ml of methanolic HCl solution [17], generated from acetyl chloride and

methanol in the ratio 1:9, and heated for 10 min at 110°C. After cooling to room temperature the solution was extracted with MTBE. The MTBE phase was neutralized with saturated $NaHCO_3$ solution, washed with a saturated solution of sodium chloride and dried over Na_2SO_4. Finally the organic phase was concentrated by evaporation in an apparatus consisting of an pear-shaped distillation flask with a Vigreux column. The resulting solution was analyzed by GC.

2.4.4. Transesterification of HMBA-Et to HMBA-Me

The transesterification was carried out analogously to Section 2.4.3. The degree of conversion was determined using GC and yielded about 30%.

2.4.5. Stereoselective reduction of MOBA-Et to HMBA-Et using baker's yeast [6]

A 1-g amount of MOBA-Et, 30 g baker's yeast and 5 g sucrose were suspended in 130 ml water. In order to enhance the performance of the yeast a nutritive salt tablet for yeast containing dibasic ammonium phosphate and ammonium sulfate was added. The suspension was stirred during fermentation for 2 days at room temperature. Approximately every 12 h an additional amount of 5 g sucrose was added.

The suspension was filtered by suction using diatomaceous earth as a filter aid. The filtrate was extracted three times with ethyl acetate. The combined extracts were washed twice with a saturated solution of $NaHCO_3$ and once with water. The organic phase was dried over anhydrous sodium sulfate and analyzed by GC.

2.4.6. Esterification of HBA to HBA-Me

The commercially available salt was esterified as described in Section 2.4.3.

2.4.7. Hydrolysis of MOBA-Et [18]

A 25-ml volume of 1 *M* NaOH solution was added under stirring to 3 ml MOBA-Et at room temperature. The reaction flask was stirred for 20 h and extracted twice with diethyl ether. The water containing phase was acidified with 10 ml of 20% H_2SO_4, saturated with NaCl and extracted twice with diethyl ether. The organic phase was washed with saturated NaCl solution and dried over sodium sulfate. The solvent was removed using a rotary evaporator at 0°C.

2.4.8. Esterification of MOBA to MOBA-Me

For esterification of the free MOBA 100 μl of the raw product of reaction (see Section 2.4.7) was mixed with 800 μl of dry dichloromethane, 200 μl methanol and a spatula-tipfull of toluene-4-sulfonic acid monohydrate [19]. After vigorous shaking the solution was kept at ambient temperature overnight. Water and MTBE were added, the organic phase dried over sodium sulfate and subjected to GC analysis.

2.5. Sample preparation

All urine samples were stored at −20°C prior to analysis.

The investigated samples had a neutral pH. A 1-ml volume of each sample was lyophilized and the dry residue derivatized to its corresponding methyl ester according to reaction (see Section 2.4.3). The obtained solution containing methanolic HCl was not extracted, but evaporated as described in Section 2.4.3 in order to reduce the volume to about 100 μl under smooth conditions and evaporation of methanol and HCl under the addition of MTBE. The concentrated solution was neutralized with solid $NaHCO_3$ and analyzed directly by enantio-MDGC–MS.

For derivatization of MOBA only method (see Section 2.4.8) was used. Other methods resulted in thermal decarboxylation.

3. Results and discussion

Combining enantioselective synthesis with baker's yeast and enantioselective analysis using enantio-MDGC–MS, the absolute configuration of HMBA and the sequence of elution of all four stereoisomers was elucidated. Urine samples of patients with β-ketothiolase deficiency and from healthy children were investigated and compared. Some distinct differences in the ratio of all four stereoisomers between these two groups could be observed. Furthermore, enantioselective analysis allows some conclu-

sions on the underlying enzymatic activities and metabolic pathways to be drawn. In this context it should be mentioned that it is not definitely clear which enzymes are responsible for the formation and degradation of HMBA [1]. It may be that degradation of L-isoleucine has its own enzymes, but alternatively it seems to be reasonable that enzymes of other pathways like the β-oxidation of fatty acids or perhaps ketogenesis are involved, especially in the case of β-ketothiolase deficiency. The hydration step from tiglic acid-CoA to HMBA-CoA should be crucial, because in this reaction the starting material is achiral and two chiral centers are introduced.

Using enantio-MDGC–MS conclusions on the selectivity and the transition state of this enzymatic reaction are expected to be drawn.

3.1. Absolute configuration and chromatographic behavior of HMBA stereoisomers

The absolute configuration of HMBA stereoisomers was determined in the following manner:

Firstly MOBA-Et was reduced in a non enantioselective reaction by sodium borohydride yielding all four stereoisomers of HMBA-Et in comparable amounts. The stereoisomers were saponified, re-esterified to their corresponding methyl esters and separated by enantio-MDGC–MS. All four stereoisomers were clearly separated and their retention times defined (Fig. 3A). The methyl esters had to be synthesized for comparison and identification of sample compounds.

In a second step, MOBA-Et was reduced stereoselectively by baker's yeast yielding the (2*R*,3*S*) and (2*S*,3*S*) stereoisomers of the corresponding HMBA-Et as reported in the literature [6,20,21]. The obtained stereoisomer ratio of (2*R*,3*S*):(2*S*,3*S*) 87:13 was in excellent agreement with the literature data [6,20,21]. The ethyl esters were transesterified as described in Section 2.4.4 to the corresponding HMBA-Me that were stereoanalyzed by enantio-MDGC–MS (Fig. 3B) showing peaks 1 and 4 of the HMBA-Me stereoisomers in the same ratio as the ethyl esters and were assigned with (2*R*,3*S*) and (2*S*,3*S*) diastereomers, respectively.

The employed enantio-MDGC system was equipped with a pre-column, coated with a achiral

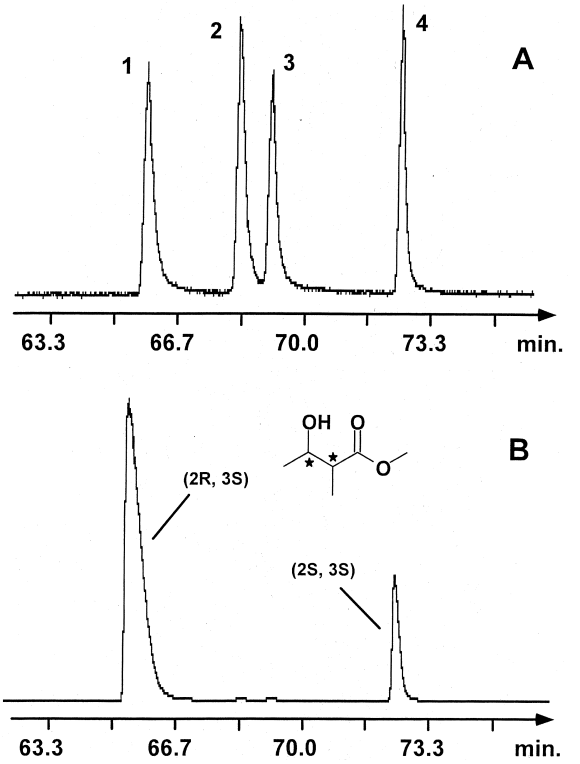


Fig. 3. Enantio-MDGC–MS main column chromatograms. (A) Analysis of all four stereoisomers of HMBA-Me. (B) Diastereomeric HMBA-Me from stereoselective reduction of MOBA ester by baker's yeast.

stationary phase, suitable for the separation of HMBA diastereomers, whereas the main column additionally resolved the HMBA enantiomers. As the stereoisomers of HMBA may occur as enantiomeric pairs of diastereomers one diastereomer was transferred to the main-column and separated into its enantiomers (Fig. 3A). Reduction by baker's yeast yields (3*S*)-configured diastereomers [peak 1 (2*R*,3*S*), peak 4 (2*S*,3*S*)] consequently the second and the third peaks are conclusively assigned as (2*S*,3*R*) and (2*R*,3*R*) stereoisomers, respectively (Table 1).

3.1.1. Samples of healthy children

In most samples all four stereoisomers were detected. The (2*S*,3*S*) stereoisomer dominated (>90%), whereas the (2*R*,3*S*) stereoisomer was detectable in only minor amounts (about 1%). The

Table 1
Retention times of HMBA-, MOBA- and HBA-esters

Compound	Cut time pre-column (min)	Retention time main-column (min)
Methyl ester		
(2*R*,3*S*)-HMBA	15.7–17.3	65.9
(2*S*,3*R*)-HMBA	15.7–17.3	68.3
(2*R*,3*R*)-HMBA	14.7–15.5	69.2
(2*S*,3*S*)-HMBA	14.7–15.5	72.5
(*S*)-HBA	9.65–10.40	47.6
(*R*)-HBA	9.65–10.40	49.2
MOBA	15.55–15.9	52.0 and 53.3
Ethyl ester		
(2*R*,3*S*)-HMBA	22.75–24.00	37.5
(2*S*,3*R*)-HMBA	22.75–24.00	38.0
(2*R*,3*R*)-HMBA	21.50–22.50	38.3
(2*S*,3*S*)-HMBA	21.50–22.50	39.2
MOBA	22.3–22.7	34.7 and 34.9

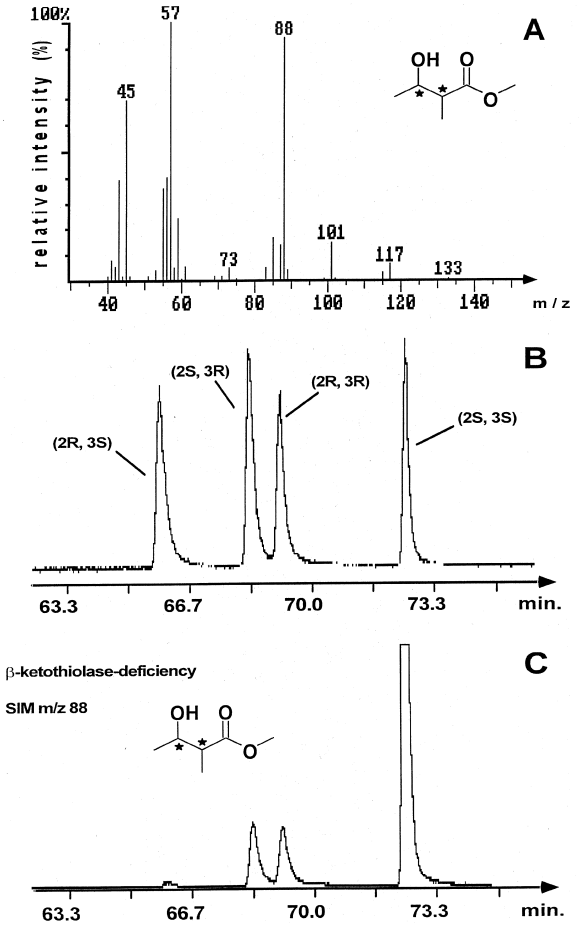


Fig. 4. Enantio-MDGC–MS main column chromatograms. (A) Mass spectrum of HMBA-Me. (B) All four stereoisomers of HMBA-Me, assignment of absolute configuration. (C) HMBA-Me stereoisomers from patients, affected by β-ketothiolase deficiency.

(3*R*)-configured stereoisomers appeared in the range between 1 and 5%. Except for the (2*S*,3*S*) stereoisomer, the other stereoisomers were only detectable in rather small amounts when single-ion monitoring was used. In two control urine samples, the (2*R*,3*S*) stereoisomer could not be determined (data not shown).

3.1.2. β-Ketothiolase patients

Similar to healthy children, the (2*S*,3*S*) stereoisomer was the dominating compound in affected children, but also both (3*R*)-configured stereoisomers were clearly elevated (ca. 10%). This finding might be explained by the reduction of MOBA to HMBA, i.e., the reversed reaction induced by the deficiency of ketothiolase or ketogenesis (Fig. 4 and Table 2).

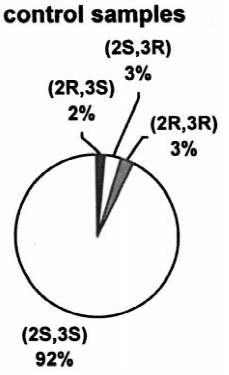
3.2. Conclusions on the hydration of tiglic acid

During catabolism of isoleucine tiglic acid is hydrated to HMBA. This step is catalyzed by an enzyme that is not yet clearly identified [1]. Considering that the hydration creates two new chiral centers, some conclusions might be drawn from the enzyme's selectivity. Four clearly discernible transition states are reasonable for hydration. Therefore it is likely that the enzyme catalyzed hydration of tiglyl-CoA leads to a single stereoisomer of HMBA-CoA in one and the same enzyme. In human urine samples all stereoisomers were detectable (Fig. 4), but in extremely different amounts. Therefore, it is concluded that more than one enzyme or even more than one metabolic pathway is involved in the formation of HMBA.

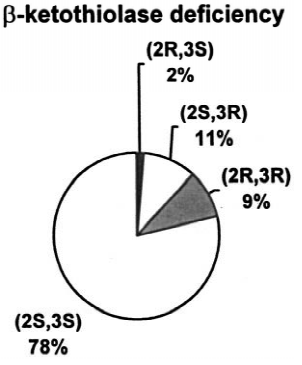
In view of the predominance of the (2*S*,3*S*) stereoisomer, especially in control samples, a *cis*-addition (at C-2 from Re and at C-3 from Si) has to

Table 2
HMBA-ratios in control and patients urine

	HMBA-ratio in control urine (%)			
	Sample 1	Sample 2	Sample 3	Average
(2*R*,3*S*)	1.2	2.8	1.2	1.7
(2*S*,3*R*)	4.7	1	3.9	3.2
(2*R*,3*R*)	4.9	1.1	2.1	2.7
(2*S*,3*S*)	89.2	95	92.8	92.3



	HMBA-ratio in urine of patients with β-ketothiolase deficiency (%)				
	Sample 1	Sample 2	Sample 3	Sample 4	Average
(2*R*,3*S*)	1.1	1.9	1.6	1.1	1.5
(2*S*,3*R*)	11.7	9.6	10.1	5.6	10.5
(2*R*,3*R*)	11.1	11.8	5.2	6	9.4
(2*S*,3*S*)	76	76.7	83	87.3	78.6



be taken into account for the hydration of tiglic acid during isoleucine catabolism (Fig. 5).

It is improbable that one and the same enzyme is able to catalyze a hydration reaction in different transition states. The reverse reaction in enzyme catalyzed reactions must have the same stereoselectivity as the forward reaction. But perhaps the dehydrogenase responsible for the oxidation of HMBA-CoA to MOBA-CoA possesses low stereoselectivity.

Regarding the altered enantiomeric and diastereomeric ratios in affected children it is rather probable that different metabolic pathways are involved. Even the fact that all four stereoisomers are detectable in control urine samples points to several pathways acting together in L-isoleucine catabolism.

3.3. Comparison with β-oxidation of fatty acids and ketogenesis

In the course of fatty acid β-oxidation and isoleucine catabolism similar reactions are known [12] (Fig. 6).

The 2,3-enoyl-CoA moiety of fatty acid breakdown is hydrated to a (3*S*)-hydroxy acid-CoA compound. In this case only one stereogenic center is generated and to our knowledge there are no literature data available concerning the *cis*- or *trans*-

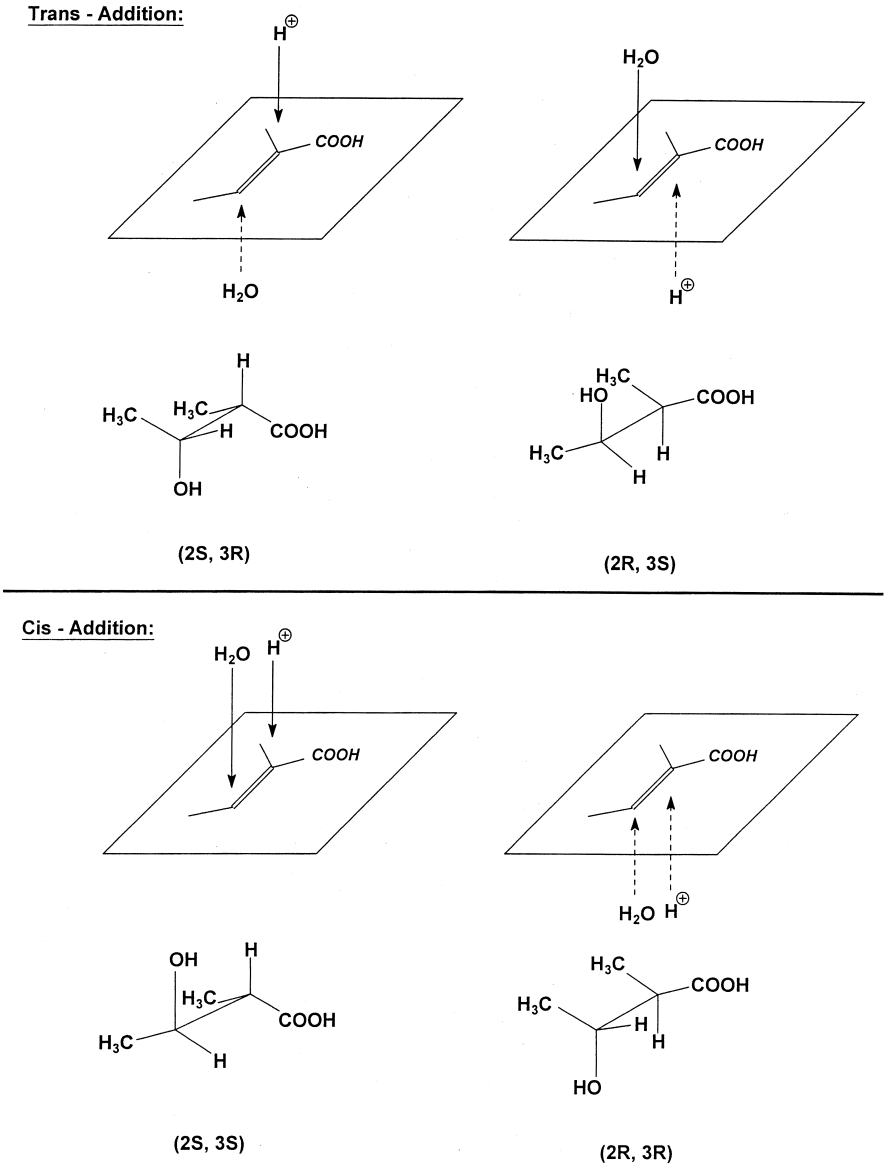


Fig. 5. Mechanistic pathways for the hydration of tiglic acid and corresponding HMBA stereoisomers.

mechanism of hydration. Nevertheless, the corresponding enzyme seems to be selective for the (3S)-configuration. Therefore, it is reasonable, that this enzyme may also be responsible for the hydration of tiglic acid to HMBA [1,22,23]. However, the formation of ketone bodies [1,13] as an alternative biosynthetic pathway has to be taken into account. Acetoacetyl-CoA is selectively reduced to (3*R*)-hydroxybutanoyl-CoA.

This reaction is analogous to the reverse reaction of the oxidation of HMBA-CoA to MOBA-CoA during isoleucine catabolism. The rate of this parallel reaction is expected to be elevated in β-ketothiolase deficiency. The finding of increased levels of (3*R*)-

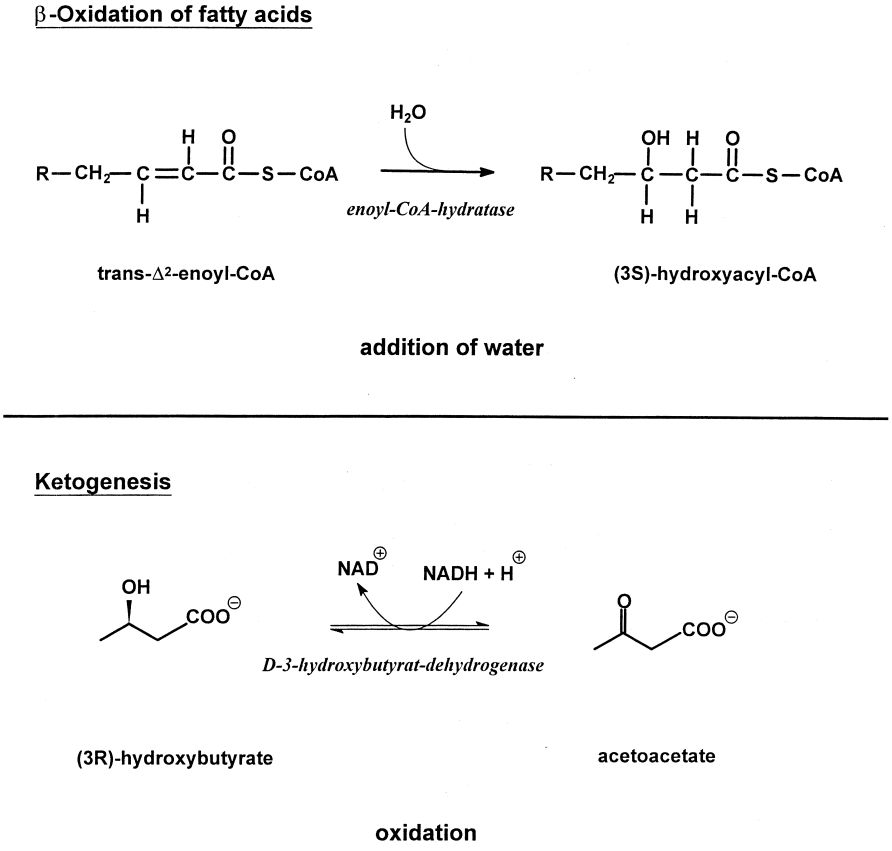


Fig. 6. Formation of 3-hydroxybutanoate during β-oxidation of fatty acids and ketogenesis.

stereoisomers of HMBA in affected patients fits this biosynthetic pathway. The responsible enzyme is presumably not selective for the C-2, but only for the C-3 position. The ratio of HMBA stereoisomers may give information on the extent of the course of these metabolic pathways.

It should be noted that MOBA racemizes rapidly (MOBA-Et half-life is about 1.5 h [20]). In one sample from a patient with β-ketothiolase deficiency the enantiomeric ratio of MOBA was tested using enantio-MDGC–MS after derivatizing to its methyl ester catalyzed by toluene-4-sulfonic acid and was found to be racemic as expected. Thus, the starting material for the reverse reaction in L-isoleucine catabolism is racemic and it is reasonable to assume that the responsible enzyme is exclusively selective for the C-3 position, whereas the stereogenic C-2 position of HMBA is racemic.

3.4. 3-Hydroxybutyric acid in patients with medium-chain acyl-CoA dehydrogenase deficiency

MCAD deficiency is a disorder of fatty acid degradation. Normally ketone bodies occur when fatty acids are preferentially utilized as a source of energy, but this is not the case in MCAD patients. Under some circumstances MCAD-deficient patients excrete unusual high amounts of ketone bodies of unknown origin.

As mentioned above, HBA may result from fatty acid degradation or ketogenesis and may be distinguished by the absolute configuration at C-3. In the view of an existing defect in fatty acid metabolism, it is quite possible that the HBA results from fatty acid degradation and is (S)-configured. Furthermore, de novo fatty acid syntheses or bacterial origin are reasonable sources of HBA excretion.

The present investigations clearly demonstrate that the (3*R*)-enantiomer dominates. The (3*S*)-enantiomer only occurs in minor amounts (about 3–5%). It seems that as less ketone bodies are excreted, a higher proportion of the (*S*)-enantiomer is present, although a quantitation was not performed.

In MCAD patients with urinary ketone bodies the excreted HBA seems to originate from usual ketogenesis (Table 3).

One patient (No. 5) showed a disproportionately high amount of the (*S*)-enantiomer. There is no explanation for this as yet. This patient had classical MCAD deficiency and had the common A985-G mutation. The diagnosis was confirmed by a phenyl propanoate loading test which gave an increased excretion of 3-phenyl propanoylglycine. Another source of HBA was perhaps more likely in this case.

In the control urine samples, HBA was not detected. These samples were most likely collected under non fasting conditions.

In comparison to the results for MCAD deficiency, samples of patients with ketonemic vomiting and β-ketothiolase deficiency were investigated with regard to the enantiomeric ratio of 3-hydroxybutanoic acid excreted during ketoacidosis. The *S*:*R* ratio in ketonemic vomiting was ca. 1:99, compared to 5:95 in β-ketothiolase deficiency.

Table 3
Enantiomeric ratio of 3-hydroxybutanoic acid in several diseases

Patients		3-Hydroxybutyric acid	
Defect	Sample	(*S*)	(*R*)
MCAD	1	2.3	97.7
	2	4.5	95.5
	3	3.0	97.0
	4	3.3	96.7
	5	11.6	88.4
	6	1.8	98.2
	7	3.4	96.6
	8	1.7	98.3
	9	1.9	98.1
	10	1.4	98.6
	11	4.1	95.9
	12	2.2	97.8
	13	5.9	94.1
Ketonemic vomiting	K.	<1	>99
	A.	<1	>99
β-Ketothiolase deficiency	S.	4.2	95.8
	C.	5.7	94.3

Under the conditions investigated the (3*R*)-enantiomer was clearly dominant, but the amounts of the (3*S*)-enantiomer were somewhat different. The reason for this remains unknown. It seems likely that there are several enzymes leading to HBA, but they may be different in selectivity and activity. In some diseases the activities may be altered.

4. Conclusions

The use of enantio-MDGC–MS enables the enantioselective analysis of trace amounts of chiral compounds in complex matrices. The enantioselective analysis of metabolites in human urine may give important clues for diagnosis, for monitoring treatment and for a general understanding of metabolic pathways and possible side reactions.

Enantioselective analysis of HMBA revealed that its formation is interfered by more than one metabolic pathway. Besides the normal catabolism of L-isoleucine, where perhaps enzymes of the β-oxidation of fatty acids are involved, very probably enzymes of ketogenesis are active, especially when MOBA cannot be degraded as in the case of β-ketothiolase deficiency. Perhaps further in vivo studies using stable isotopes may shed more light on the pathways of ketone body formation.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (DFG).

References

[1] L. Sweetman, J.C. Williams, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), 7th Ed., The Metabolic and Molecular Bases of Inherited Disease, Vol. I, McGraw-Hill, New York, 1995, pp. 1387–1422.
[2] R.S. Daum, P.H. Lamm, O.A. Mamer, C.R. Scriver, Lancet ii (1971) 1289.
[3] B. Middleton, K. Bartlett, A. Romanos, J. Gomez Vazquez, C. Conde, R.A. Cannon, M. Lipson, L. Sweetman, W.L. Nyhan, Eur. J. Pediatr. 144 (1986) 586.

[4] M.A. Sutter, D. Seebach, Liebigs Ann. Chem. (1983) 939.
[5] A. Tai, T. Sugimura, T. Kikukawa, C. Naito, Y. Nishimoto, N. Morimoto, Biosci. Biotech. Biochem. 56 (1992) 1711.
[6] R.W. Hoffmann, W. Ladner, K. Steinbach, W. Massa, R. Schmidt, G. Snatzke, Chem. Ber. 114 (1981) 2786.
[7] G. Massiot, X.-F. Chen, C. Lavaud, L. Le Men-Olivier, C. Delaude, A. Viari, P. Vigny, J. Duval, Phytochemistry 31 (1992) 3571.
[8] R. Noyori, M. Tokunaga, M. Kitamura, Bull. Chem. Soc. Jpn. 68 (1995) 36.
[9] M. Reggelin, Nachr. Chem. Tech. Lab. 45 (1997) 789–793.
[10] A. Tai, M. Imaida, Bull. Chem. Soc. Jpn. 51 (1978) 1114.
[11] G. Fráter, Helv. Chim. Acta 62 (1979) 2825.
[12] L. Stryer, in: Biochemie, 4th Ed., Spektrum, Heidelberg, Berlin, Oxford, 1996, pp. 640–641.
[13] L. Stryer, in: Biochemie, 4th Ed., Spektrum, Heidelberg, Berlin, Oxford, 1996, pp. 644–645.
[14] A.C. Sewell, J. Herwig, I. Wiegratz, W. Lehnert, H. Niederhoff, X.-Q. Song, N. Kondo, T. Fukao, J. Inherit. Metab. Dis. 21 (1998) 441.
[15] K. Grob, Making and Manipulating Capillary Columns for Gas Chromatography, Hüthig, Heidelberg, 1986.
[16] A. Dietrich, B. Maas, A. Mosandl, J. Microcol. Sep. 6 (1994) 33.
[17] H. Allmendinger, Thesis, Eberhard-Karls-Universität Tübingen, Tübingen, 1986.
[18] M. Tanaka, O. Oota, H. Hiramatsu, K. Fujiwara, Bull. Chem. Soc. Jpn. 61 (1988) 2473.
[19] V. Karl, Thesis, Johann Wolfgang Goethe-Universität, Frankfurt/Main, 1994.
[20] K. Nakamura, T. Miyai, A. Nagar, S. Oka, A. Ohno, Bull. Chem. Soc. Jpn. 62 (1989) 1179.
[21] K. Nakamura, Y. Kawai, T. Miyai, A. Ohno, Tetrahedron Lett. 31 (1990) 3631.
[22] R.S. Daum, C.R. Scriver, O.A. Mamer, E. Delvin, P. Lamm, H. Goldman, Pediat. Res. 7 (1973) 149.
[23] S. Landaas, Clin. Chim. Acta 64 (1975) 143.