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Enantioselective multidimensional gas chromatography–mass spectrometry in the analysis of urinary organic acids

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Abstract

Enantioselective multidimensional gas chromatography–mass spectrometry is a valuable tool for the enantioselective analysis of compounds from complex matrices. Samples are separated initially on a precolumn and selected substances then transferred on-line to a main-column coated with suitable chiral stationary phase for enantioselective analysis with subsequent mass selective detection. In this paper the method is used as an adjunct to urinary organic acid analysis to provide information in patients with suspected inborn errors of metabolism. Lactic acid, α-hydroxyisocaproic acid, 3-phenyllactic acid and 3-(4-hydroxyphenyl)-lactic acid are separated in a single run. In addition, the enantioselective analysis of pyroglutamic acid is presented. D-Enantiomers of amino acids and α-hydroxycarboxylic acids derived from amino acids, point to a bacterial origin whereas the L-enantiomer is predominantly of endogenous origin. Therefore the enantiomeric ratio of chiral metabolites is an important parameter for the understanding of metabolic processes.

Keywords: Enantiomer separation; Organic acids; α-Hydroxycarboxylic acids; Pyroglutamic acid

1. Introduction

The characteristic pattern of urinary organic acids is often an important parameter in the diagnosis of inherited metabolic disease. Analysis is usually accomplished by gas chromatography with mass spectrometric detection. This method is quite sensitive, but the identification of trace metabolites can be hampered by the complex biological matrix.

Using multidimensional gas chromatography–mass spectrometry (MDGC–MS) this problem can be minimised considerably providing a highly sophisticated, selective and sensitive analysis. This system separates the sample on a GC precolumn and then selected compounds can be transferred on-line via a live-T-piece on to a main-column with different selectivity and polarity. Subsequent mass selective detection gives structural information and renders the method also suitable for stable isotope enrichment studies.

For the diagnosis of inborn errors of metabolism it is insufficient to merely analyse urinary organic acids using a column coated with a nonchiral stationary phase. There are diseases known where only certain

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enantiomers are excreted and the identification of absolute configuration will determine the course of therapeutic management. Until now, samples were firstly analysed on a normal (nonchiral) column and then a repeat analysis was performed using a column coated with a chiral stationary phase. Using MDGC–MS (column combination: nonchiral/chiral), enantiomers can be separated directly providing a rapid and unequivocal diagnosis.

We wish to present the distinct advantages of this methodology illustrated by samples from patients with inborn errors of metabolism.

2. Experimental

2.1. Patients and samples

Urine samples were available from the following patients.

Case 1. J.K. is a 4-year-old boy with short-bowel syndrome. He was admitted because of increasing tiredness and metabolic acidosis. Microbiological investigations in stool revealed a massive *Lactobacillus* excretion whereon he was treated with isocillin, penicillin and metronidazole. Plasma L-lactate was slightly elevated (3.3 mmol/l) whereas plasma D-lactate was grossly elevated (8.3 mmol/l). Urinary organic acid analysis showed a very high lactate excretion together with phenyllactate.

Case 2. M.M. This 6-year-old Arab girl presented with developmental delay and mental retardation. Urinary organic acid analysis revealed increased 2-hydroxyphenylacetate and 3-phenyllactate suggesting the diagnosis of phenylketonuria.

Case 3. Patient A.G. is a 23-year-old female of Russian descent with classical phenylketonuria who was poorly treated and has immense compliance problems.

Case 4. Patient R.K., a Greek girl, presented at age 1 year with an urinary tract infection. She developed a hepatopathy and a metabolic acidosis. Routine screening revealed a generalised hyperaminoaciduria and glycosuria. Plasma tyrosine was increased. Urinary organic acid analysis demonstrated increased 3-(4-hydroxyphenyl)-lactic acid, 3-(4-hydroxyphenyl)pyruvic acid and succinyl acetone. The diagnosis of tyrosinaemia type I was confirmed by lack of fumarylacetoacetase activity in cultured skin fibroblasts. The patient died at the age of 2 years.

Case 5. D.K., a 3-year-old German boy, was admitted because of increasing lethargy, lack of appetite, constipation and general malaise. He had an enlarged thyroid gland and was hypothyroid. He had been breastfed until the age of 1 year, but thereafter had consumed only phytoproteins. He had a vitamin B12 deficiency, iron-associated anaemia and a slight vitamin D deficiency. Urinary organic acid analysis revealed an increased pyroglutamic acid excretion.

Case 6. Patient I.P. has glutathione synthetase deficiency (enzymatically confirmed).

Control urine samples were obtained from healthy children aged 1–7 years in whom an inborn error of metabolism was not suspected. All urine samples were stored at −80°C prior to analysis.

2.2. Chemicals and reagents

D,L-Lactic acid, D-(+)-3-phenyllactic acid, L-(−)-3-phenyllactic acid, L-pyroglutamic acid, D-glutamic acid, methyl chloroformate, pyridine, acetyl chloride and L-leucine were obtained from Fluka (Buchs, Switzerland). L-Lactic acid was from Roth (Karlsruhe, Germany). D,L-α-Hydroxyisocaproic acid was from Sigma (St. Louis, MO, USA), D,L-3-(4-hydroxyphenyl)-lactic acid hydrate and L-glutamine from Aldrich (Milwaukee, WI, USA), L-(−)-tyrosine, sodium nitrite, buffer solution pH 4.00, buffer solution pH 7.00, *tert*.-butylmethyl ether, methanol and L-glutamic acid were from Merck (Darmstadt, Germany). Methylene chloride was purchased from Riedel-de-Haen (Seelze, Germany).

L-3-(4-Hydroxyphenyl)-lactic acid [1] and L-α-hydroxyisocaproic acid [2] were synthesised from the corresponding amino acids by diazotation under retention of absolute configuration.

D-Pyroglutamic acid was synthesised from D-glutamic acid by heating at pH 3.2 and 100°C under reflux [3] with subsequent water evaporation and extraction with methanol.

2.3. Sample preparation

2.3.1. Derivatisation with acetyl chloride–methanol [4].

Urine samples (1 ml) were brought to pH 6–8 and lyophilised in a 5-ml GC vial. The dry residue was

extracted with 1 ml acetyl chloride–methanol (1:9, v/v), transferred to a gas-tight 5-ml vial and heated for 10 min at 100°C. After cooling to room temperature, 1 ml 10% disodium hydrogenphosphate solution was added and the whole extracted twice with 400 μl *tert.*-butylmethyl ether. The organic layer was dried over anhydrous sodium sulphate and evaporated to ~50 μl [5]. The sample was then ready for enantio-MDGC–MS analysis.

2.3.2. Derivatisation with methyl chloroformate (MCF) [6,7]

This was used for pyroglutamic acid analysis. In a 5-ml GC vial, 1 ml of phosphate buffer pH 7.0 was added to 1 ml of urine and lyophilised. The dry residue was suspended in 500 μl methylene chloride, 100 μl methanol and 700 μl pyridine. MCF (500 μl) was added dropwise and the suspension shaken carefully. After cooling to room temperature, 1 ml of citrate buffer pH 4.0 and 1 ml of saturated NaCl solution were added and the suspension extracted twice with 500 μl *tert.*-butylmethyl ether. The organic layer was dried over anhydrous sodium sulphate and reduced to ~100 μl [5] prior to enantio-MDGC–MS analysis.

2.4. Instrumentation

The chromatographic system comprised a Siemens SiChromat 2 double oven system with two separate temperature controls (Fig. 1). Injections were split–splitless at 250°C. The GC columns were prepared as previously described [8,9].

The pre-column was a 30 m×0.25 mm I.D. high temperature fused-silica capillary coated with a 0.38 μm film of PS 268. Conditions: carrier gas hydrogen 1.4 bar; split 27 ml/min; initial temperature 70°C for 5 min; ramp 2°C/min to 250° held for 30 min.

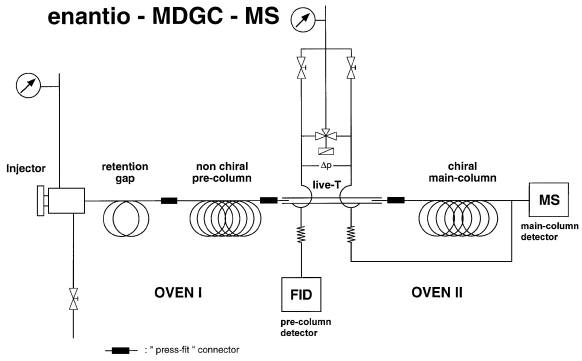

Fig. 1. Enantio-MDGC system.

Prior to the precolumn was a retention gap (2 m×0.25 mm I.D. deactivated high temperature fused-silica capillary column). A fused-silica capillary (30 m×0.32 mm I.D.), coated with a 0.64 μm film of heptakis-(2,3-di-O-methyl-6-O-*tert.*-butyldimethylsilyl)-β-cyclodextrin dissolved in SE 52 was used as the chiral main-column. Conditions: carrier gas hydrogen at 0.65 bar; initial temperature 60°C raised at 1°C/min to 94°C, then raised with 4°C/min to 110°C, then with 1.5°C/min to 180°C, 0.5°C/min to 185°C and finally 4°C/min to 210°C held for 30 min.

A flame ionisation detector (270°C) was used as precolumn detector, whereas the main-column was connected with an ITD-transfer line (250°C) with an open split interface (250°C) to an ITD 800 mass spectrometer (Finnigan MAT, Bremen, Germany) in ion impact mode as detector. Sweep flow (helium) was 1 ml/min, ion trap manifold 230°C, and electron energy 70 eV.

3. Results and discussion

With enantio-MDGC–MS using modified cyclodextrins as chiral stationary phase, the investigated metabolites lactic acid (LA), α-hydroxyisocaproic acid (HICA), 3-phenyllactic acid (PLA) and 3-(4-hydroxyphenyl)-lactic acid (HPLA) (Fig. 2) could be separated into enantiomers as methyl esters in a single chromatographic run (Fig. 3). Pyroglutamic acid (PGA) had to be derivatised separately (see below). The elution order of chiral compounds was established using enantiopure or enriched standards of defined absolute configurations either commercially available or synthesised in our laboratory (Table 1).

Derivatisation with acetyl chloride–methanol proceeded smoothly without racemisation effects as concluded from experiments with enantiopure standards. This method was however, unsuitable for the derivatisation of PGA since under these conditions, glutamine cyclicises to PGA (glutamic acid does not) and the lactam ring of PGA might open. Therefore for PGA, derivatisation with MCF was used. Under these conditions no racemisation of the

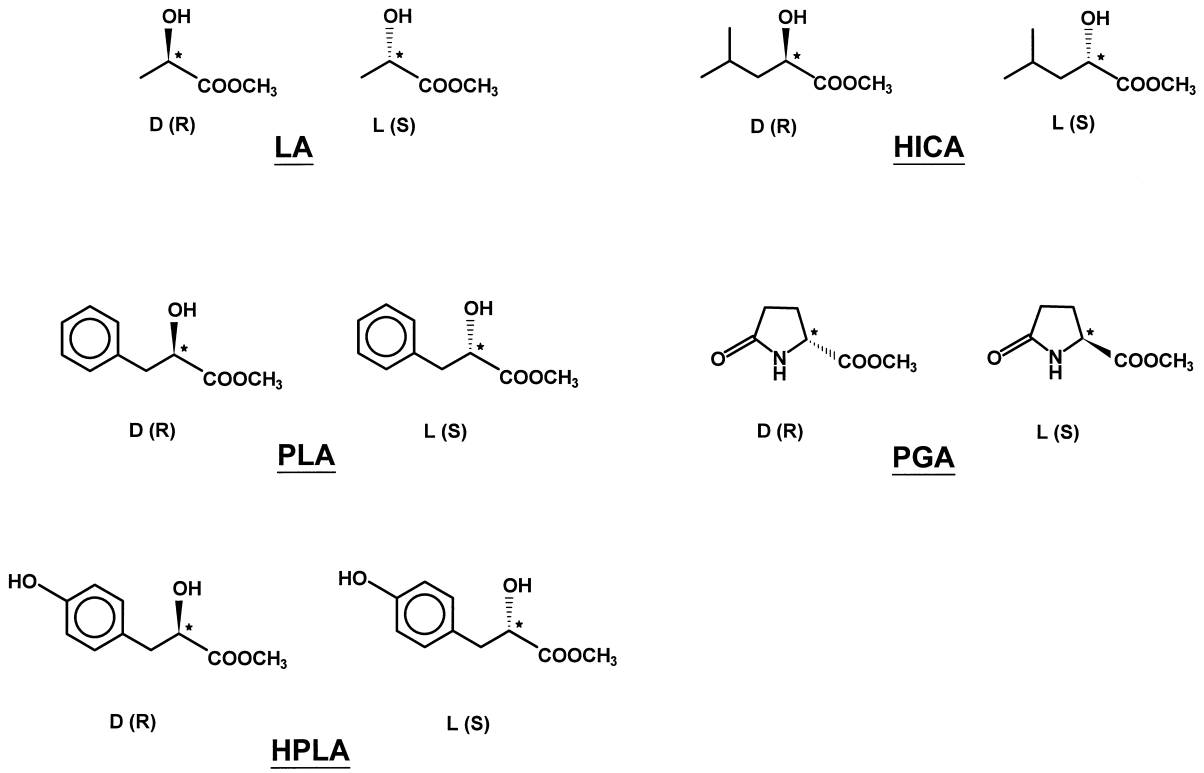

Fig. 2. Methyl ester enantiomers of the examined metabolites lactic acid (LA), α-hydroxyisocaproic acid (HICA), 3-phenyllactic acid (PLA), pyroglutamic acid (PGA) and 3-(4-hydroxyphenyl)-lactic acid (HPLA); relative and absolute configurations are given.

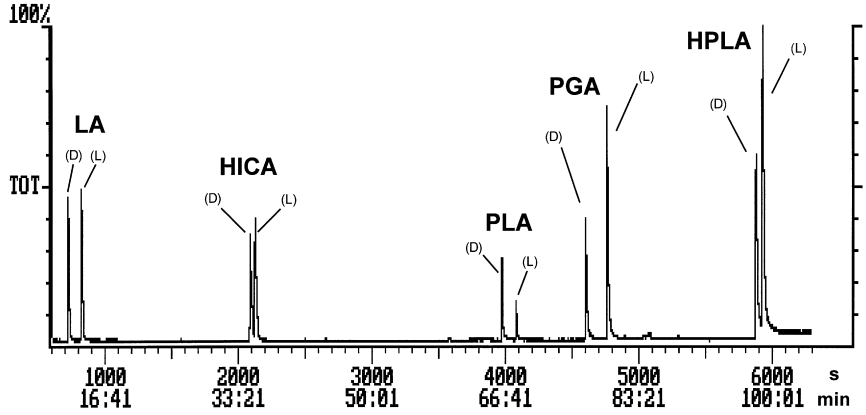

Fig. 3. Enantio-MDGC–MS analysis; main column separation of reference compounds into enantiomers: lactic acid (LA), α-hydroxy-isocaproic acid (HICA), 3-phenyllactic acid (PLA), pyroglutamic acid (PGA), 3-(4-hydroxyphenyl)-lactic acid (HPLA). All reference compounds were derivatised singly and a standard mixture prepared.

Table 1
Elution order of the investigated metabolites

Compound	Abbreviation	Cut-time pre-column (min)	Elution order main-column
Lactic acid	LA	3.28–3.64	D (*R*)/L (*S*)
2-Hydroxyisocaproic acid	HICA	12.25–13.45	D (*R*)/L (*S*)
3-Phenyllactic acid	PLA	39.70–40.95	D (*R*)/L (*S*)
Pyroglutamic acid	PGA	42.60–43.50	D (*R*)/L (*S*)
3-(4-Hydroxyphenyl)-lactic acid	HPLA	61.00–64.00	D (*R*)/L (*S*)

enantiopure standard was detected. It was also tested to what extent glutamic acid or glutamine cyclicise to PGA under these conditions. After derivatisation and processing, small amounts of PGA were detected, but it was assumed this originated from the amino acid standards used and was not a result of analytical handling. PGA could be detected in the glutamic acid standard by TLC [10]. It is also well known that glutamine can cyclicise spontaneously under storage conditions. Derivatisation with MCF was unsuitable for PLA and HPLA since their derivatives failed to separate. For these compounds, derivatisation with MCF seemed to render enantiomeric separation more difficult. In both derivatisation methods, the metabolites are first derivatised and then extracted and not in the reverse order, so that the yield of polar compounds might be improved.

3.1. Analysis of α-hydroxycarboxylic acids

The advantages of enantio-MDGC–MS were assessed by analysing urine samples from patients with confirmed inborn errors of metabolism or with secondary metabolic complications. Case 1 with short-bowel syndrome showed a lactate excretion containing more than 99% of the D-enantiomer (Table 2). Lactic acid is derived from the anaerobic metabolism of glucose and the reduction of pyruvate by lactate dehydrogenase [11]. Since increased urinary lactate levels may be primary or secondary, determination of plasma levels is important. Normally the L-form of lactic acid is present in the body, but certain bacteria produce the D-form. Whereas L-lactic acid produced by bacteria such as *Bifidobacterium*, *Lactobacillus* or *Eubacterium* [12] is further metabolised in the large intestine, the D-enantiomer is very slowly metabolised, enriched and excreted in the urine [13]. Both D- and L-forms of lactic acid can be analysed e.g. enzymatically [14] or separated by MDGC [15]. The risk of bacterial overgrowth in short-bowel syndrome is high with a further risk of D-lactic acidosis, therefore enantioselective analysis is vital for management [14,16].

Further metabolites in short-bowel syndrome are HICA, PLA and HPLA, their D-forms being of bacterial origin. The D-enantiomer of HICA was found to dominate over the L-form in the urine of Case 1 (D:L-ratio, 89:11). The range of D-HICA in short-bowel syndrome has previously determined in the range of 81–100% [17]. This urine sample was also analysed according to [18] confirming the obtained result. Additionally, 2-(*R*,*S*)-hydroxy-3-(*S*)-methylvaleric acid (HMVA), the corresponding α-hydroxy acid of isoleucine, could be detected in

Table 2
Results of the enantio-MDGC–MS analysis of Cases 1–4

Patients	LA		HICA		PLA		PGA		HPLA	
	D (*R*)	L (*S*)	D (*R*)	L (*S*)	D (*R*)	L (*S*)	D (*R*)	L (*S*)	D (*R*)	L (*S*)
Case 1 (Short-bowel syndrome)	>99	<1	89	11	86	14	N.D.	N.D.	49	51
Case 2 (untreated PKU)	68	32	N.D.	N.D.	1	99	35	65	<1	>99
Case 3 (untreated PKU)	45	55	N.D.	N.D.	31	69	56	44	25	75
Case 4 (Tyrosinaemia type I)	<1	>99	N.D.	N.D.	3	97	N.D.	N.D.	2	98

N.D.=not determined (levels too low).

greater amounts than HICA. The 2*R*-diastereomer of HMVA dominated (2*R*,3*S*/2*S*,3*S*-ratio, 99:1). PLA and HPLA, originating from phenylalanine and tyrosine metabolism, have been analysed as diastereomeric esters on nonchiral columns [17,19] and PLA previously on modified cyclodextrins [20]. D-PLA and D-HPLA have been reported to appear in short-bowel syndrome in the range of 60–75% and 43–61%, respectively [17]. In the present study, in Case 1 the urinary ratio of D/L-PLA was 86:14, and that of D/L-HPLA was 49:51 (Table 2), further confirming short-bowel bacterial overgrowth. It could be that food may also contain D-enantiomers. L-LDH (L-lactatedehydrogenase) may produce small amounts of the D-enantiomer [4] and there may be enzymes that act upon or produce D-lactic acid in the human body [4].

PLA and HPLA also occur in liver disease [21] or in errors of phenylalanine and tyrosine degradation. In classical phenylketonuria, the conversion of phenylalanine to tyrosine is inhibited causing phenylalanine to be transaminated to phenylpyruvic acid and partially hydrogenated to PLA [22]. An enrichment of HPLA should not occur. In Case 2, enantio-MDGC–MS analysis revealed an immense excretion of L-PLA (>99%) confirming previous findings [17] but L-HPLA was also found in significant amounts (99% L-form) (Table 2). In Case 3, PLA and HPLA occurred at low levels. The enantiomeric ratio was 2:1 (L/D for PLA) and 3:1 (L/D for HPLA) (Table 2). The D-enantiomers were present in significant amounts. The lower levels are explained by the fact that this patient had been on diet and although compliance was far from acceptable, her urinary metabolites should be far less than in case 2 who had received no treatment at all.

In inherited tyrosinaemia type I due to fumarylacetoacetase deficiency [23], the transamination product of tyrosine, *p*-hydroxyphenylpyruvate, is hydrogenated to HPLA which is elevated [17]. Enantio-MDGC–MS confirmed this finding in Case 4 with 98% of HPLA being the L-enantiomer (Table 2). The far lower amount of PLA in Case 4 was also the L-enantiomer (97%).

3.2. Analysis of pyroglutamic acid

PGA, the cyclisation product of glutamic acid or glutamine, plays an important role in the γ-glutamyl cycle [24]. Analysis of PGA is difficult because both glutamic acid and glutamine may cyclicise on storage or during derivatisation. Cyclisation is pH dependent and is promoted under slightly acidic conditions [3]. Increased heat also promotes cyclisation [25] and low pH induces lactam ring opening. The enantiomeric analysis of PGA has usually been accomplished after hydrolysis to glutamic acid, L-glutamic acid being removed by L-glutamate decarboxylase and the remaining D-enantiomer analysed on an amino acid analyser [10]. Our initial attempts at derivatisation for enantio-MDGC–MS with acetyl chloride–methanol were impractical, since although no cyclicisation of glutamic acid occurred, glutamine cyclicised rapidly. Derivatisation with MCF is ideal since no cyclicisation occurs and the lactam ring remains closed.

Whereas PGA in the γ-glutamyl cycle consists only of the L-enantiomer, in urine of healthy individuals the D-enantiomer is also reported [24]. This appears somewhat contradictory. Enantio-MDGC–MS analysis with selected ion monitoring confirmed the predominance of the D-enantiomer of PGA (60–80%) in the urine of the healthy controls (Table 3) (Fig. 4A and B).

The explanation appears speculative since D-enantiomers may be of bacterial origin or may occur as dietary artifacts in fermented foodstuffs [26–28].

There are at least two known inborn errors of the γ-glutamyl cycle leading to increased PGA excre-

Table 3
Results of the enantio-MDGC–MS analysis of PGA in Cases 5–6 and the control urine samples

Patients	PGA	
	D (*R*)	L (*S*)
Case 5 (acute state)	<1	>99
Case 5 (morning urine)	59	41
Case 5 (night urine)	57	43
Case 6 (Glutathionsynthetase deficiency)	<1	>99
Control urine sample 1	78	22
Control urine sample 2	71	29
Control urine sample 3	78	22
Control urine sample 4	79	21
Control urine sample 5	71	29
Control urine sample 6	71	29
Control urine sample 7	63	37
Control urine sample 8	69	31

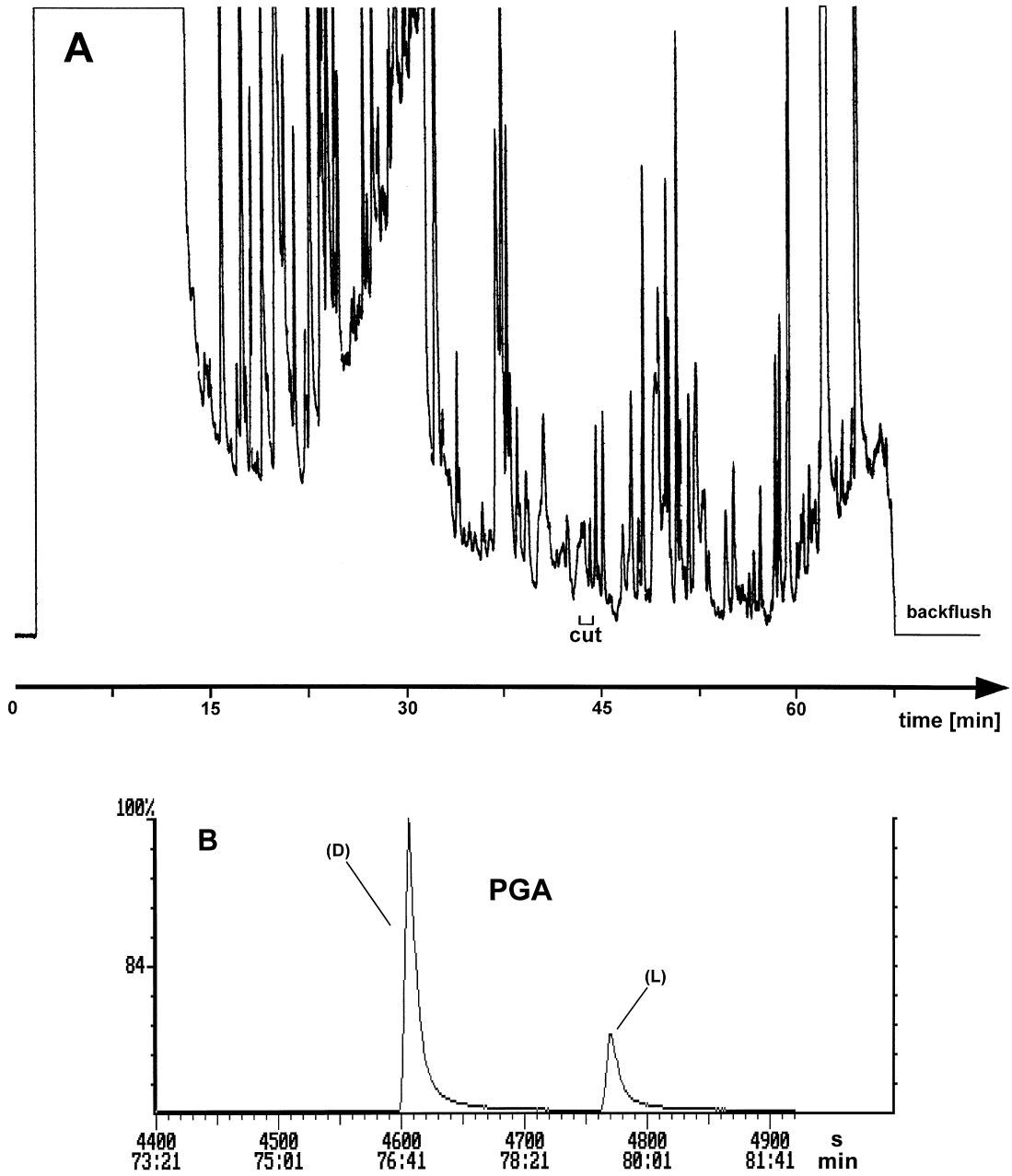

Fig. 4. Enantio-MDGC–MS analysis of urinary acids after derivatization with MCF from control urine sample 4; precolumn chromatogram (A) after splitless injection; the cut time for PGA is marked. (B) Main column chromatogram of PGA-methyl ester.

tion: glutathione synthetase deficiency and 5-oxoprolinase deficiency and the L-enantiomer predominates in both diseases [27]. Our Case 5 revealed a high excretion of PGA which consisted of 99% of the L-enantiomer. This patient exhibited none of the classical symptoms of glutathione synthetase deficiency (severe metabolic acidosis, haemolytic anaemia etc.). Analysis of two further urine samples (morning urine, night urine) revealed a normal enantiomeric ratio (D/L 57–59/41–43). We have no

explanation for the increased L-enantiomer excretion. Recently reports have described increased urinary PGA excretion in normal adults consuming vegetarian or low-protein diets [29] or during recovery from severe childhood malnutrition, in particular related to glycine intake [30]. This child had a most unusual vegetarian diet, but this could not explain the initially high excretion of the L-form. Case 6, a classical case of glutathione synthetase deficiency, showed a massive excretion of L-PGA (>99%), thus confirming previous data [27].

Enantio-MDGC–MS is a rapid, reliable and accurate tool for the enantioselective analysis of urinary metabolites. Removing many components on a non-chiral column followed by enantioselective analysis circumvents the problem of tedious extraction and shortens the analysis time. In the field of inborn errors of metabolism, this technique will be a most valuable addition to the battery of investigations required for the diagnosis of such diseases. Many chiral compounds can be separated in a single gas chromatographic run with a minimum of derivatisation and sample preparation. Our results on a few patients with inborn errors of metabolism demonstrate that a new field has been opened with new questions to be answered. Further investigations are in progress.

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