New insights in dihydropyrimidine dehydrogenase deficiency: a pivotal role for β-aminoisobutyric acid?

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DPD (dihydropyrimidine dehydrogenase) constitutes the first step of the pyrimidine degradation pathway, in which the pyrimidine bases uracil and thymine are catabolized to β-alanine and the *R*-enantiomer of β-AIB (β-aminoisobutyric acid) respectively. The *S*-enantiomer of β-AIB is predominantly derived from the catabolism of valine. It has been suggested that an altered homoeostasis of β-alanine underlies some of the clinical abnormalities encountered in patients with a DPD deficiency. In the present study, we demonstrated that only a slightly decreased concentration of β-alanine was present in the urine and plasma, whereas normal levels of β-alanine were present in the cerebrospinal fluid of patients with a DPD deficiency. Therefore the metabolism of β-alanine-containing peptides, such as carnosine, may be an important factor involved in the homoeostasis of β-alanine in patients with DPD deficiency. The mean concentration of β-AIB was approx. 2–3-fold lower in cerebrospinal fluid and urine of patients with a DPD deficiency, when compared with controls. In contrast, strongly decreased levels (10-fold) of β-AIB were present in the plasma of DPD patients. Our results demonstrate that, under pathological conditions, the catabolism of valine can result in the production of significant amounts of β-AIB. Furthermore, the observation that the *R*-enantiomer of β-AIB is abundantly present in the urine of DPD patients suggests that significant cross-over exists between the thymine and valine catabolic pathways.

Key words: β-alanine, γ-aminobutyric acid, β-aminoisobutyrate, dihydropyrimidine dehydrogenase, enantiomer, valine.

INTRODUCTION

In human beings, the pathway for the catabolism of uracil and thymine consists of three consecutive steps and is primarily confined to the liver (Scheme 1). DPD (dihydropyrimidine dehydrogenase) is the initial and rate-limiting enzyme in the catabolism of the pyrimidine bases and it catalyses the reduction of uracil and thymine to 5,6-dihydrouracil and 5,6-dihydrothymine respectively. The second step is catalysed by dihydropyrimidinase and consists of a reversible hydrolysis of dihydrouracil and dihydrothymine to *N*-carbamoyl-β-alanine and *N*-carbamoyl-β-AIB (where β-AIB stands for β-aminoisobutyric acid) respectively. Finally, *N*-carbamoyl-β-alanine or *N*-carbamoyl-β-AIB is converted into β-alanine or β-AIB, ammonia and CO_2 by β-ureidopropionase.

DPD is also responsible for the breakdown of the widely used antineoplastic agent 5-fluorouracil, thereby limiting the efficacy of the therapy. In this light, a pharmacogenetic disorder has been described concerning cancer patients with a complete or partial deficiency of DPD who suffered from severe toxicity, including death, following the administration of 5-fluorouracil [1,2]. In children, a deficiency of DPD is often accompanied by a neurological disorder, although a considerable variation in the clinical presentation among these patients has been reported [3].

It is generally thought that the pyrimidine degradation pathway is the main route for the synthesis of β-alanine in human beings [4]. β-Alanine is a structural analogue of GABA (γ-aminobutyric acid) and glycine, which are the major inhibitory neurotransmitters in the central nervous system. It has been suggested that β-alanine itself might function as a neurotransmitter since it is an agonist of both the glycine and $GABA_A$ receptors [5,6]. Moreover, the presence of a high-affinity uptake system in glial and neuronal cells, which regulates the concentration of β-alanine in the brain, supports a role for β-alanine in the modulation of the neuronal response [7,8]. A deficiency in one of the enzymes of the pyrimidine degradation pathway might, therefore, be accompanied by decreased levels of β-alanine and contribute to some of the clinical abnormalities encountered in patients with a DPD deficiency [3].

β-AIB is a non-protein amino acid originating from the catabolism of thymine and valine (Scheme 2). The concentration of β-AIB is normally low in urine as β-AIB is further catabolized by β-aminoisobutyrate aminotransferases to methylmalonic acid semialdehyde and propionyl-CoA. β-AIB occurs in two isomeric forms and both enantiomers of β-AIB can be detected in human urine and plasma [9–12]. In plasma, the *S*-enantiomer is the predominant type due to active renal reabsorption [11]. In contrast, urine almost exclusively contains the *R*-enantiomer of β-AIB, which is eliminated both by filtration and tubular secretion [9–12]. Persistently increased levels of β-AIB have been observed in individuals with a deficiency of *R*(−)-β-aminoisobutyrate-pyruvate aminotransferase. In addition, transient high levels of β-AIB have been observed under a variety of pathological conditions such as lead poisoning, starvation, total body irradiation and in a number of malignancies [13].

It has been demonstrated that the *R*-enantiomer of β-AIB originates from the catabolism of thymine, whereas the *S*-enantiomer is a degradation product of valine [14–16]. Loading tests with thymine and valine showed that the vast majority of β-AIB in urine is derived from the catabolism of thymine and that only a very small amount (< 10 %) of β-AIB is produced from the degradation of valine [9,17]. Hence, an impaired pyrimidine

Abbreviations used: β-AIB, β-aminoisobutyric acid; CSF, cerebrospinal fluid; DPD, dihydropyrimidine dehydrogenase; GABA, γ-aminobutyric acid; OPA, *o*-phthaldialdehyde.

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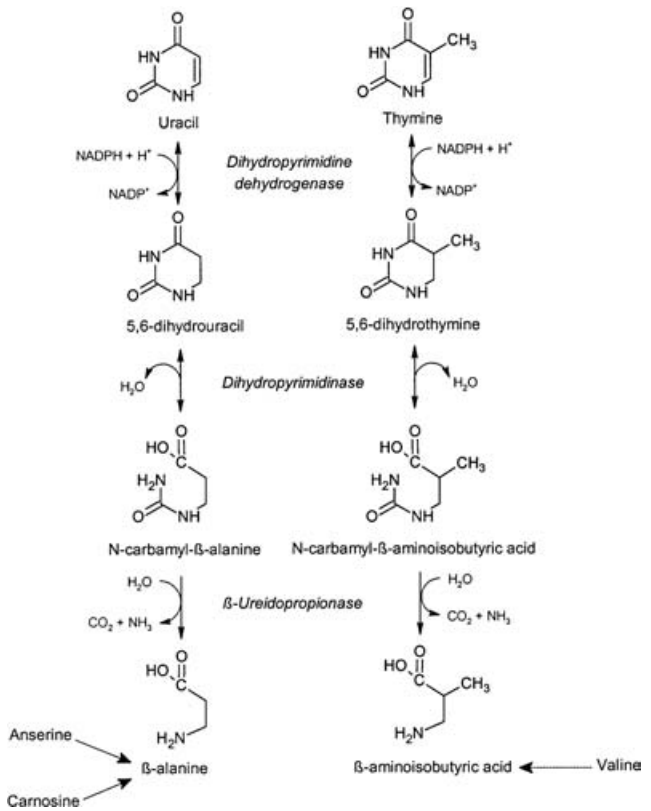
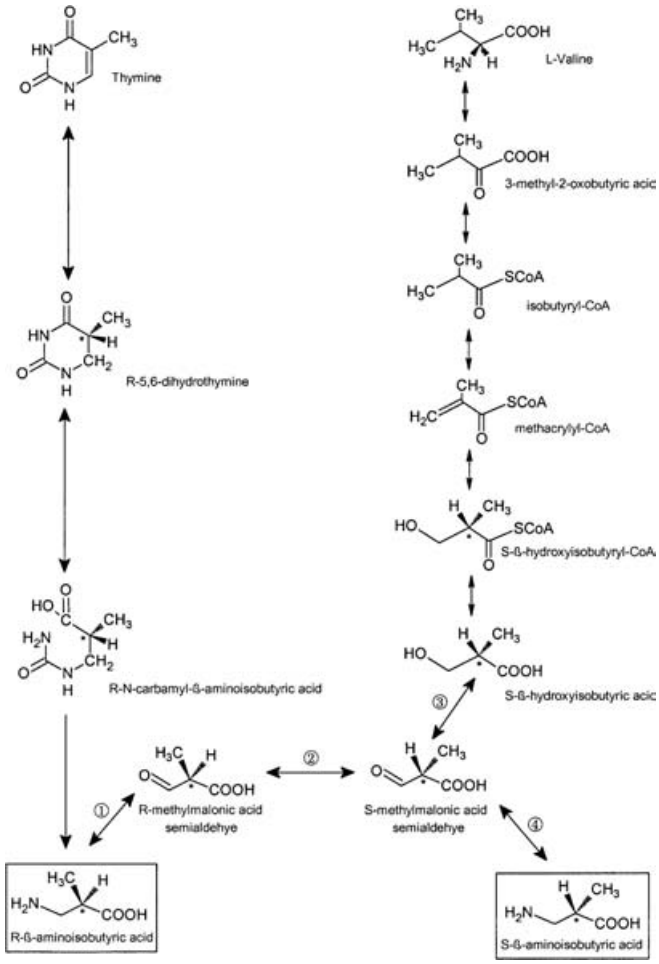


Scheme 1 Catabolic pathway of the pyrimidine bases uracil and thymine

β-Alanine can also be synthesized through the hydrolysis of anserine and carnosine. Valine is a distant precursor of β-AIB.



Scheme 2 Metabolic pathways for the synthesis of *R*- and *S*-β-AIB

The chiral atoms are indicated with an asterisk (*). ①, $R(-)$-β-aminoisobutyrate-pyruvate aminotransferase; ②, non-enzymic/racemase?; ③, β-hydroxyisobutyrate dehydrogenase; ④, $S(+)$-β-aminoisobutyrate–α-ketoglutarate transaminase.

degradation pathway should have profound effects on the levels of β-AIB in general and, in particular, on the levels of the *R*-enantiomer of β-AIB.

Recently, the analysis of CSF (cerebrospinal fluid) from a single DPD patient indicated the presence of normal levels of β-alanine [18]. However, until now, there was no systematic analysis of the concentrations of β-alanine and β-AIB in patients with DPD deficiency. Therefore in the present study, we have investigated the impact of a DPD deficiency on the homoeostasis of β-alanine and both enantiomers of β-AIB.

MATERIALS AND METHODS

Patient and control populations

The control population consists of patients admitted to our hospital with clinical and biochemical findings not indicative of inborn errors in the purine and pyrimidine metabolism. Samples were obtained according to the 'Code for proper use of human tissue' as formulated by the Dutch Federation of Medical Scientific Societies. The characteristics of the patients with a DPD deficiency and the controls are summarized in Table 1.

Table 1 Characteristics of patients and controls

	DPD patients	Controls
Urine		
Age (years)		
Median	4	5
Range	0.1–42	0.1–55
Sex		
Male	14	23
Female	22	21
Plasma		
Age (years)		
Median	7	12
Range	0.1–42	0.1–78
Sex		
Male	11	31
Female	15	21
Cerebrospinal fluid		
Age (years)		
Median	4	7
Range	0.1–15	0.1–75
Sex		
Male	8	14
Female	5	19

Sample preparation for determination of β-alanine and β-AIB

Urine, plasma and CSF samples (200 μl) were deproteinized by thoroughly mixing with 20 μl of 12 %, 35 % and aq. 5 % (w/v) sulphosalicylic acid respectively and storing the samples at 4 °C for 30 min. After centrifugation (11 000 ***g***, 10 min), 100 μl of

plasma and CSF and 20 μl of urine supernatants were collected and mixed with 100 and 380 μl of 0.3 M lithium citrate (pH 3.0) respectively. The OPA (*o*-phthaldialdehyde) derivatization reagent was prepared by dissolving 26 mg of OPA in 0.5 ml of methanol followed by the addition of 5 ml of 0.6 M potassium borate and 30 μl of 14.2 M 2-mercaptoethanol. Derivatization of the amino acids occurred before injection into the HPLC apparatus and was performed automatically in a Gilson 231 XL autosampler (Gilson Medical Electronics, Villiers Le Bel, France) by mixing 20 μl of OPA derivatizing reagent with 40 μl of sample. After an incubation period of 30 s, 20 μl of the reaction mixture was injected into the HPLC apparatus.

HPLC configuration

Separation of the derivatized amino acids was performed on an OPA-HR LC-8 analytical column (5 μm particle size, 150 mm × 4.6 mm inner diameter; Alltech, Deerfield, IL, U.S.A.) with a guard column (type Pelliguard, 40 μm particle size, 20 mm × 4.6 mm inner diameter; Supelco, Bellafonte, PA, U.S.A.) and an Alltima C-18 analytical column (5 μm particle size, 250 mm × 4.6 mm inner diameter; Alltech). Switching between both analytical columns occurred via a 6-position automated Vici switching valve. Both columns were thermostatically controlled at 21 °C using a Hubner minichiller cooling water bath.

HPLC analysis of β-alanine and β-AIB

The content of β-alanine in urine, plasma and CSF was determined with a dual-column reversed-phase HPLC, as described before [19]. Briefly, solvent A used for chromatography on column I (LC-8) consisted of 70 mM sodium acetate (pH 5.75) and 10 % (v/v) methanol. Solvent B consisted of 90 % methanol and 2.0 % (v/v) propan-2-ol. Elution was performed by applying a linear gradient from 90 % solvent A to 60 % solvent A in 15 min at a flow rate of 1 ml/min. β-Alanine eluting from column I between 15 and 18 min was introduced, by means of column switching, to column II (LC-18) and elution was performed isocratically with a buffer containing 100 mM sodium acetate and 50 % methanol (pH 5.0) at a flow rate of 1 ml/min.

For the determination of β-AIB, elution was performed by applying a linear gradient from 90 % solvent A to 70 % solvent A in 14 min at a flow rate of 1.0 ml/min and subsequently kept at 70 % solvent A for 9 min. β-AIB eluting from column I between 19 and 23 min was introduced to column II (LC-18) and elution was performed isocratically with a buffer containing 100 mM sodium acetate (pH 6.1) and 47 % methanol at a flow rate of 1 ml/min. Fluorescence of both amino acids was measured at λ_{ex} (excitation wavelength) = 330 nm and λ_{em} (emission wavelength) = 450 nm. Quantification of the amounts of β-alanine and β-AIB was performed by comparison with external standards.

The detection limits of β-alanine and β-AIB, defined as three times the baseline noise, were approx. 0.7 and 1.5 pmol respectively. The intra- and inter-assay variation for both procedures to determine the concentration of β-alanine and β-AIB was <10 %.

Detection of *R*- and *S*-β-AIB in urine

β-AIB was isolated from 10 ml of urine on a column of 5 ml of Dowex 50WX8. After washing the column with an excess of water, the amino acids were eluted with a 0.2 M ammonia solution. The eluate was dried in vacuum and the residue was dissolved in 1 ml of 0.2 M HCl. Subsequently, a 0.5 ml fraction was applied to a 6 ml SPE™ C-18 disposable extraction column (Bakerbond, Deventer, The Netherlands) and eluted with 1 ml of water. The eluate was collected, vacuum-dried and dissolved in 0.2 ml of 0.2 M HCl.

N-trifluoroacetylated isopropyl esters of β-AIB were prepared using the TFA-IPA derivatization kit (Alltech). *R*- and *S*-enantiomers of β-AIB were separated by GC on a 50 mm × 0.25 mm, 0.16 μm film, Chirasil-Val fused silica capillary (Alltech). The oven temperature was set at 65 °C for 2 min and subsequently increased to 130 °C (rate, 1 °C/min) and 220 °C (rate, 30 °C/min). The oven temperature was subsequently kept at 220 °C for 15 min. Temperatures of the injection port and flame-ionization detector were set at 240 and 300 °C respectively. *R*- and *S*-β-AIB peaks in urine samples from controls and patients with a DPD deficiency were identified and confirmed by spiking the samples with standards of purified *R*- and *S*-β-AIB.

Isolation of *R*- and *S*-enantiomers of β-AIB

R- and *S*-enantiomers of β-AIB were separated on a Crownpak CR(+) column (150 mm × 4 mm; Daicel Chemical Industries, Osaka, Japan). Elution was performed isocratically at 3 °C with 0.11 M $HClO_4$ (pH 1), at a flow rate of 0.4 ml/min with on-line UV detection at 210 nm. The peak fractions of both enantiomers were collected and neutralized with K_2CO_3. The purity of the isolated *R*- and *S*-enantiomers of β-AIB was confirmed by GC–flame-ionization detector analysis, as described above.

The identity of the *R*-enantiomer was confirmed by preparing *R*-β-AIB enzymically according to the following procedure. Urine sample of a patient with *N*-carbamoyl-β-amino aciduria was fractionated on a Phenomenex Aqua C-18 column (250 mm × 4.6 mm, 5 μm particle size) using 50 mM acetic acid adjusted to pH 4.0 with 13 M NH_4OH, at a flow rate of 1 ml/min. *R*-β-AIB was prepared by the enzymic hydrolysis of a fraction containing *N*-carbamoyl-β-AIB with recombinant human β-ureidopropionase, essentially as described before [20].

Statistical analysis

Normal distribution and variances of the data were analysed using the Kolmogorov–Smirnov test and Levene's test for equality of variances respectively. Differences between the mean concentrations of β-alanine and β-AIB between patients and controls were analysed with the two-sample *t* test. For a non-normal distribution or unequal variances, the log-transformed data were used or the original data were tested with the non-parametric Mann–Whitney test. They were analysed with the Statistical Package for Social Sciences (SPSS, Chicago, IL, U.S.A.).

RESULTS

β-AIB and β-alanine levels

To investigate whether the impaired degradation of uracil and thymine in patients with a DPD deficiency would result in altered levels of β-alanine and β-AIB, we measured these β-amino acids in body fluids of controls and patients with a complete deficiency of DPD (Figure 1). In patients with a complete deficiency of DPD, the range and mean concentration of β-AIB were moderately decreased in urine and CSF and profoundly decreased in plasma, when compared with controls. For a child suffering from an anaplastic large cell lymphoma and a DPD deficiency [21], a normal concentration of β-AIB was detected in the urine (25 μmol/mmol of creatinine) at diagnosis. During treatment,

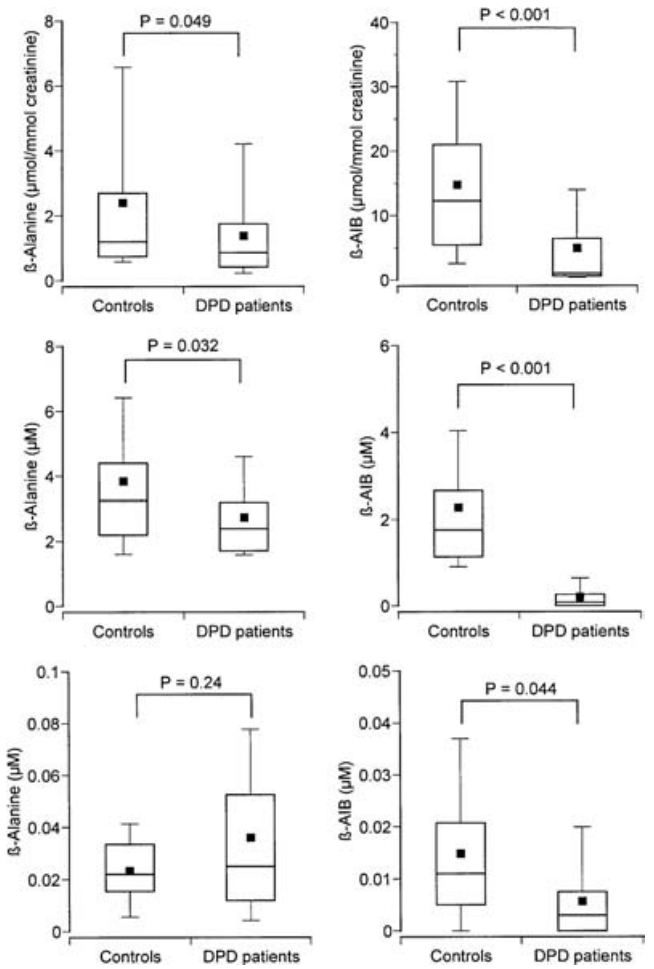


Figure 1 Box plots of the β-alanine and β-AIB concentrations in controls and DPD patients

The top, bottom and line through the middle of a box correspond to the 75th, 25th and 50th percentile respectively. The whiskers on the bottom extend from the 10th and top 90th percentile. ■, Mean concentration. Distributions of β-alanine and β-AIB are indicated for urine (top panel), plasma (middle panel) and CSF (bottom panel).

the concentration of β-AIB increased up to 106 μmol/mmol of creatinine, which was above the upper limit of a control population (Table 2). In this patient with an increased tissue degradation, excretion of β-AIB was less than that usually observed in patients with haematological malignancies [13], which can be explained by the DPD deficiency. Surprisingly, the concentration of β-alanine was only slightly lower in urine and plasma, whereas the concentration of β-alanine was normal in CSF from DPD patients when compared with controls (Table 2).

Detection of *R*- and *S*-β-AIB in urine

To investigate the absolute configuration of β-AIB in patients with a DPD deficiency, N-trifluoroacetylated isopropyl esters of *R*- and *S*-β-AIB were prepared and separated using GC (Figure 2). A typical gas chromatogram, showing that urine from controls contains almost exclusively the *R*-enantiomer of β-AIB, is shown in Figure 2(B). The mean relative level of *R*-β-AIB in urine from five controls was 93 ± 3 %. Surprisingly, the analysis of urine samples from DPD patients showed significant amounts of *R*-β-AIB as well, with a mean relative level of *R*-β-AIB of 58 ± 36 % ($n = 6$). Figure 2(C) shows that in a DPD patient with an anaplastic large-cell lymphoma, β-AIB was almost exclusively present in the *R*-configuration.

Table 2 Levels of β-alanine and β-AIB in controls and DPD patients

n.d., not detectable; *n*, number of patients investigated.

	DPD patients	Controls	*P*
Urine			
β-Alanine (μmol/mmol of creatinine)			
Means $\pm$ S.D.	1.4 ± 1.4 ($n = 31$)	2.4 ± 3.0 ($n = 44$)	0.049
Range	0.2–5.3	0.1–13.2	
β-AIB (μmol/mmol of creatinine)			
Means $\pm$ S.D.	5.0 ± 9.4 ($n = 36$)	14.8 ± 11.7 ($n = 39$)	< 0.001
Range	0.1–49	0.6–50	
Plasma			
β-Alanine (μM)			
Means $\pm$ S.D.	2.7 ± 1.3 ($n = 26$)	3.8 ± 2.9 ($n = 52$)	0.032
Range	0.9–6.2	1.3–20	
β-AIB (μM)			
Means $\pm$ S.D.	0.2 ± 0.3 ($n = 22$)	2.3 ± 1.9 ($n = 52$)	0.001
Range	n.d. (< 0.05)–1.1	0.5–10.5	
Cerebrospinal fluid			
β-Alanine (μM)			
Means $\pm$ S.D.	0.036 ± 0.034 ($n = 12$)	0.024 ± 0.013 ($n = 32$)	0.24
Range	n.d. (< 0.005)–0.124	n.d. (< 0.005)–0.051	
β-AIB (μM)			
Means $\pm$ S.D.	0.006 ± 0.009 ($n = 8$)	0.015 ± 0.014 ($n = 35$)	0.044
Range	n.d. (< 0.005)–0.025	n.d. (< 0.005)–0.057	

DISCUSSION

In patients with a DPD deficiency a wide spectrum of clinical abnormalities, ranging from very mild to quite severe, can be encountered [3]. Since the synthesis of β-alanine from uracil is impaired in patients with a DPD deficiency, the presence of almost normal levels of this amino acid in urine, plasma and CSF of DPD patients indicates the existence of alternative pathways for the synthesis of β-alanine. In this respect, the metabolism of β-alanine-containing peptides such as carnosine (β-alanyl-histidine) and anserine (β-alanyl-1-methylhistidine) might be an important factor involved in the homoeostasis of β-alanine.

The majority of β-alanine in the human body occurs as the dipeptide carnosine, which is present in high concentrations (2–20 mM) in the skeletal muscles of many vertebrates, including human beings [22]. In addition, carnosine is present in the brain, particularly in the olfactory bulb. In human beings, carnosine is hydrolysed by two different enzymes: tissue (cytosolic) carnosinase and serum carnosinase [4]. Tissue carnosinase or prolinase is present in most human tissues, especially in the kidney, liver and brain. Owing to its low affinity towards carnosine and the low concentrations of carnosine in the human brain, it is unlikely that it will significantly contribute to the hydrolysis of carnosine *in vivo*. However, serum carnosinase is also present in the human brain and subsequently secreted by these cells into the CSF [23].

It is important to stress that human brain cells are not able to synthesize β-alanine via the catabolism of uracil since DPD is the sole enzyme present in the pyrimidine degradation pathway [24]. Previously, it has been demonstrated that β-alanine can cross the blood–brain barrier and that the uptake is mediated by an

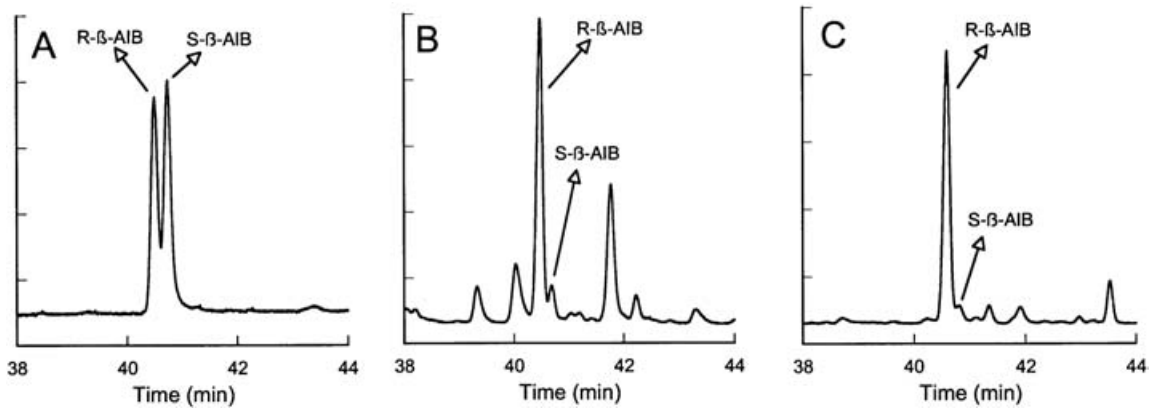


Figure 2 Gas chromatographic separation of N-trifluoroacetylated isopropyl esters of *R*- and *S*-β-AIB

(**A**) Chromatographic profile of a prepared β-AIB standard (*R*/*S* = 1:1). The separation of both *R*- and *S*-enantiomers of β-AIB in urine samples from a control and a DPD patient are shown in (**B**, **C**) respectively.

active transporter that is common to β-amino acids [25]. Thus the uptake of the amount of β-alanine produced from carnosine and anserine by serum carnosinase may be an important pathway for the brain to sustain a sufficient level of β-alanine. It is interesting to note that an opposing mechanism of action has been proposed for carnosine and β-alanine. Whereas carnosine induces vascular relaxation, β-alanine actually produces vasoconstriction [26].

A conspicuous finding was the presence of significant amounts of β-AIB in the urine of patients with a DPD deficiency. It has been shown that thymine is normally the main precursor of β-AIB and that only a very small amount of β-AIB is produced from valine [9,16,17]. The inability of DPD patients to synthesize β-AIB via the degradation of thymine demonstrates that, under pathological conditions, the catabolism of valine can result in the production of significant amounts of β-AIB. Furthermore, the observation that the *R*-enantiomer of β-AIB is abundantly present in the urine of DPD patients suggests that significant cross-over exists between the thymine and valine catabolic pathways.

The interrelationship between thymine and valine catabolism was originally proposed by van Gennip et al. [12], who observed a linear relationship between the *R*- and *S*-enantiomers of β-AIB in human urine. The cross-over between both pathways probably occurs at the level of the *R*- and *S*-methylmalonic acid semialdehyde (Scheme 2). Racemization of the *R*- and *S*-methylmalonic acid semialdehyde can occur spontaneously, due to the instability of the compound [27]. Furthermore, the presence of a racemase has also been proposed [12,16]. Interestingly, metabolic labelling studies in human beings have indicated that a flow from *S*- to *R*-methylmalonic acid semialdehyde exists [16]. Furthermore, in patients suffering from ketoacidosis, the large amount of β-AIB present in urine, which is most probably derived from the degradation of valine, proved to be predominantly the *R*-enantiomer of β-AIB [28]. These observations would be in line with the fact that a significant amount of *R*-β-AIB could be detected in the urine of DPD patients with an impaired degradation of thymine.

Reasoning along these lines, the extremely low β-AIB levels in the plasma and only moderately decreased levels of β-AIB in urine from patients with a DPD deficiency might reflect the increased channelling of catabolic products of valine into *R*-methylmalonic acid semialdehyde and subsequently to that of *R*-β-AIB, followed by the active secretion of the *R*-enantiomer by the kidney. In normal individuals with an intact pyrimidine degradation pathway, *R*-methylmalonic acid semialdehyde can be synthesized directly from the catabolism of thymine (Scheme 2). Hence, there might be less cross-over between the valine and thymine pathway, allowing the conversion of *S*-methylmalonic acid semialdehyde into *S*-β-AIB and the subsequent accumulation of *S*-β-AIB in plasma [11].

Apart from being a precursor for the synthesis of succinoyl CoA, the functional role of β-AIB is not known. The administration of large amounts of β-AIB to mice suffering from acute kidney failure induced twitching, cramps and death in some cases [29]. Furthermore, Armstrong et al. [9] observed pronounced pharmacological effects in men, who have been treated with large oral doses of *R*-β-AIB. They consisted of a marked prickling and burning sensation of the skin, beginning with the face and extremities and finally affecting the whole body surface. A conceivable possibility might be that β-AIB penetrated into nerve tissue and either mimicked or interfered with the destruction of GABA [9]. In addition, it has been shown that β-AIB is a partial agonist of the glycine receptor [6,30]. Thus the decreased concentration of β-AIB in CSF of patients with a DPD deficiency might, therefore, have a profound effect on the degree of activation of the glycine receptor and the efficacy of GABA. In this respect, it is interesting to note that the clinical condition of a DPD patient suffering from arthrogryposis multiplex congenita improved considerably when he was treated with both β-alanine and β-AIB [31].

We thank Dr M. Duran and F. Ward for a critical reading of this paper.

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Received 24 September 2003/23 December 2003; accepted 5 January 2004
Published as BJ Immediate Publication 5 January 2004, DOI 10.1042/BJ20031463