Significance of L-Alloisoleucine in Plasma for Diagnosis of Maple Syrup Urine Disease

Peter Schadewaldt, Annette Bodner-Leidecker, Hans-Werner Hammen, and Udo Wendel

Background: The significance of plasma L-alloisoleucine, which is derived from L-isoleucine in vivo, for diagnosis of maple syrup urine disease (MSUD) was examined.

Methods: Branched-chain L-amino acids were measured by automatic amino acid analysis.

Results: Alloisoleucine reference values in plasma were established in healthy adults (1.9 ± 0.6 μmol/L; n = 35), children 3–11 years (1.6 ± 0.4 μmol/L; n = 17), and infants <3 years (1.3 ± 0.5 μmol/L; n = 37). The effect of dietary isoleucine was assessed in oral loading tests. In controls receiving 38 μmol (n = 6; low dose) and 1527 μmol (n = 3; high dose) of L-isoleucine per kilogram of body weight, peak increases of plasma isoleucine were 78 ± 24 and 1763 ± 133 μmol/L, respectively; the peak increase of alloisoleucine, however, was negligible for low-dose (<0.3 μmol/L) and minor for high-dose (5.5 ± 2.1 μmol/L) load. In patients with diabetes mellitus, ketotic hypoglycemia, phenylketonuria, and obligate heterozygous parents of MSUD patients, alloisoleucine was not significantly different from healthy subjects. Therefore, a plasma concentration of 5 μmol/L was used as a cutoff value. In patients with classical MSUD (n = 7), alloisoleucine was beyond the cutoff value in 2451 of 2453 unselected samples. In patients with variant MSUD (n = 9), alloisoleucine was >5 μmol/L in all samples taken for establishment of diagnosis and in 94% of the samples taken for treatment control (n = 624). With the other branched-chain amino acids, the frequency of diagnostically significant increases was <45%.

Conclusions: The present findings indicate that plasma L-alloisoleucine above the cutoff value of 5 μmol/L is the most specific and most sensitive diagnostic marker for all forms of MSUD.

In maple syrup urine disease (MSUD; McKusik 248600), the degradation of the essential branched-chain L-amino acids leucine, valine, and isoleucine and their derived 2-oxoacids is impaired because of an inherited deficiency in branched-chain 2-oxoacid dehydrogenase complex (EC 1.2.4.4) activity. The accumulation of branched-chain compounds in blood and other body fluids can exert neurotoxic effects by as yet unclear mechanisms. There are two clinical types of the disease: a severe (classical) form associated with very low branched-chain 2-oxoacid dehydrogenase complex activity (<2% of control), and variant forms with variable residual activity of the enzyme complex (2–40% of control) [see Ref. (1) for a comprehensive review].

In the classical form, severe neurological symptoms and a maple syrup-like odor appear during the first week of life. Demonstration of grossly increased concentrations of branched-chain amino acids, especially leucine, in plasma firmly establishes the diagnosis.

In patients with the variant form of MSUD, the onset of metabolic derangements associated with ketoacidosis and cerebral symptoms is generally delayed. In these patients, overt clinical symptoms may be absent for months, years, or even decades. Some patients are admitted for medical examination because of psychomotor retardation and are diagnosed incidentally without having a history of ketoacidotic episodes. In other patients, intermittent episodes may arise in infancy and childhood during catabolic states, which are often triggered, in an apparently unpredictable manner, by intercurrent illnesses (1–4). We, for example, recently experienced diagnosis of variant MSUD in two 4- and 5-year-old German patients who were experiencing severe metabolic crises. One of these patients remained undetected although traceably subjected to neonatal screening.

1 Diabetes Forschungsinstitut and 2 Kinderklinik, Heinrich-Heine-Universität, D-40225 Düsseldorf, Germany.

Preliminary results were presented at the Society for the Study of Inborn Errors of Metabolism 36th Annual Symposium, September 1–4, 1998, York, UK.

*Address correspondence to this author at: Diabetes-Forschungsinstitut, Klinische Biochemie, Auf’m Hennekamp 65, D-40225 Düsseldorf, Germany. Fax 49-211-3382-603; e-mail schadewa@uni-duesseldorf.de.

Received May 19, 1999; accepted July 22, 1999.
Detection of MSUD variants can be difficult. In the absence of evident clinical symptoms, the patients often exhibit near normal or moderately increased plasma concentrations of leucine, valine, and isoleucine, which are similar to the concentrations observed in secondary amino acid disturbances such as ketotic hypoglycemia, diabetes mellitus, starvation, and other catabolic states (5–10). Early diagnosis of MSUD is essential, however, to maintain patients under metabolic control during intercurrent episodes to prevent permanent brain damage. Thus, a suitable indicator specific for MSUD is needed that permits early identification of variant MSUD.

We therefore examined the (patho)physiological significance of alloisoleucine plasma concentrations for the differential diagnosis of MSUD. This nonprotein amino acid is formed from isoleucine in vivo. It is consistently present in human plasma and can be reliably determined along with the other branched-chain amino acids (11). In the present study, we established alloisoleucine reference ranges and investigated the effect of dietary isoleucine on plasma l-alloisoleucine. Based on these results, a cutoff value was defined. This value was then used to estimate the sensitivity and specificity of increased alloisoleucine plasma concentrations for the diagnosis of MSUD.

Materials and Methods

SUBJECTS
Adult control subjects [23 males, 12 females; mean age (± SD) 28 ± 9 years] had a routine physical examination, received no medication, and had no acute or chronic illness. Infants (27 males, 23 females; 1.1 ± 1.0 years) and children (9 males, 8 females; 5.6 ± 2.2 years) were without metabolic defects. Patients with diabetes mellitus (33 males, 36 females; 54 ± 14 years) and phenylketonuria (6 males, 9 females; 13 ± 8 years) were from the inpatient clinic of the Diabetes Forschungsinstut and the outpatient clinic of the University’s children’s hospital, respectively. Plasma samples from patients with ketotic hypoglycemia were kindly provided by Dr. O.A.F. Bodamer (Baylor College of Medicine, Department of Molecular and Human Genetics, Houston, TX). The patients with the classical form of MSUD (4 males, 3 females; 13 ± 4 years) were characterized by neonatal onset of the disease, very low protein tolerance, and residual l-[1-14C]leucine oxidation in cultured fibroblasts of <1% of control [see Ref. (12)]. The obligate heterozygotes under study (5 males, 5 females; 38 ± 9 years) were parents of patients with established classical MSUD. The clinical characteristics of patients with variant forms of MSUD are compiled in Table 2. In all the patients, blood was collected on occasion of routine clinical examination or for metabolic monitoring.

ANALYTICAL PROCEDURES
Branched-chain amino acid concentrations in plasma were measured on an automatic amino acid analyzer (LC 5000, LC 6000; Biotronik), using ninhydrin detection and a short program as detailed previously (11). The limit of detection for the branched-chain amino acids was <0.05 nmol (equivalent to plasma concentrations of <0.2 μmol/L). The range for reliable quantification of alloisoleucine was 0.1–10 nmol (equivalent to 0.5–50 μmol/L in plasma). Over this range, the molar response (area per nmol) varied <5%. Typically, the CV was well below 10%, e.g., for alloisoleucine in plasma at 1 (250) μmol/L, the CV within (n = 10) and between runs (n = 9) was 7 (2) % and 8 (2) %, respectively. When the concentration exceeded 50 μmol/L in plasma, the sample was diluted appropriately before analysis. Thus, in specimens from non-MSUD subjects, concentrations of alloisoleucine and the other branched-chain amino acids were generally measured in separate analytical runs. Analytical data of external MSUD patients were provided by the respective attending metabolic centers, which applied an equivalent methodology for the determination of plasma amino acids. Residual activity of branched-chain 2-oxoacid dehydrogenase complex in our laboratory was assessed in cultured fibroblasts using l-[1-14C]leucine as described previously (12).

LOADING TESTS
After an overnight fast, six healthy subjects (five males, one female; 31 ± 5 years) received 5 mg of l-isoleucine per kilogram of body weight (dissolved in 50 mL of diluted citric acid solution; low-dose loading) orally. Three volunteers (two males, one female; 29 ± 9 years) underwent a high-dose loading and ingested 200 mg of l-isoleucine per kilogram of body weight thoroughly mixed with 150 mL of yogurt. The l-isoleucine (from Bachem) was essentially free from l-alloisoleucine (<0.03%). Venous blood was collected into EDTA tubes just before (basal values) and after ingestion of the loading dose according to the time schedules depicted in Fig. 1. Plasma was obtained by centrifugation and analyzed for branched-chain amino acids as described above. Written informed consent was obtained from the participants. The experimental protocol had been approved by the Ethikkommission of the Heinrich-Heine-Universität Düsseldorf.

CALCULATIONS
Unless otherwise noted, the results are presented as means ± SEM with the number of determinations in parentheses. Correlations were checked by simple linear regression analysis (least-squares method). For examination of differences, the Mann–Whitney U-test (two-tailed) was applied. Sensitivity estimates were based on the relationship between the number of plasma samples with alloisoleucine concentrations beyond the cutoff value and the total number of plasma analyses.
Results

REFERENCE VALUES

To establish alloisoleucine reference values, plasma branched-chain amino acids were measured in healthy subjects, children (3–10 years), and infants (<3 years). The mean leucine, valine and isoleucine concentrations were essentially comparable between children and adults (Table 1) and plasma alloisoleucine was 1.6 ± 0.1 μmol/L (n = 17) and 1.9 ± 0.1 μmol/L (n = 35), respectively. In the infants under study (n = 50), the mean alloisoleucine concentra-

Table 1. Branched-chain amino acid concentrations in plasma.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Leu</th>
<th>Val</th>
<th>Ile</th>
<th>Allo a</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adults</td>
<td>129 ± 4 (129; 78–165)</td>
<td>232 ± 7 (233; 145–304)</td>
<td>66 ± 2 (67; 39–91)</td>
<td>1.9 ± 0.1 (1.8; 0.7–3.4)</td>
<td>35</td>
</tr>
<tr>
<td>Children, 3–11 years</td>
<td>169 ± 10 (172; 82–240)</td>
<td>264 ± 15 (251; 166–407)</td>
<td>77 ± 5 (73; 41–124)</td>
<td>1.6 ± 0.1 (1.5; 0.7–2.5)</td>
<td>17</td>
</tr>
<tr>
<td>Infants, &lt;3 years</td>
<td>178 ± 8 (188; 63–299)</td>
<td>253 ± 11 (243; 130–432)</td>
<td>84 ± 4 (86; 34–147)</td>
<td>1.4 ± 0.1 (1.4; 0.5–2.6)</td>
<td>50</td>
</tr>
<tr>
<td>Non-MSUD patients with</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>198 ± 5 (196; 116–318)</td>
<td>316 ± 9 (306; 194–517)</td>
<td>89 ± 2 (92; 56–180)</td>
<td>2.2 ± 0.1 (1.8; 0.8–4.6)</td>
<td>69</td>
</tr>
<tr>
<td>Phenylketonuria</td>
<td>155 ± 20 (131; 44–330)</td>
<td>282 ± 25 (245; 160–464)</td>
<td>79 ± 10 (65; 40–180)</td>
<td>1.6 ± 0.1 (1.5; 0.5–2.6)</td>
<td>15</td>
</tr>
<tr>
<td>Ketotic hypoglycemia</td>
<td>109 ± 7 (101; 78–161)</td>
<td>169 ± 9 (165; 119–211)</td>
<td>56 ± 3 (53; 47–72)</td>
<td>&lt;2.5</td>
<td>10 c</td>
</tr>
<tr>
<td>MSUD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterozygous parents</td>
<td>175 ± 22 (150; 114–310)</td>
<td>261 ± 26 (237; 192–455)</td>
<td>79 ± 11 (63; 53–170)</td>
<td>2.0 ± 0.3 (1.8; 1.1–3.7)</td>
<td>10</td>
</tr>
<tr>
<td>Classical MSUD</td>
<td>409 ± 7 (344; 2–3794)</td>
<td>245 ± 3 (222; 17–1308)</td>
<td>182 ± 2 (168; &lt;1–1290)</td>
<td>127 ± 1 (115; &lt;1–626)</td>
<td>2453 d</td>
</tr>
</tbody>
</table>

a Median and range in parentheses.

b Allo, alloisoleucine.

c Samples from five different patients.

d Samples from seven different patients.

Fig. 1. Effect of oral L-isoleucine loads on the plasma concentrations of isoleucine and alloisoleucine.

After an overnight fast, healthy volunteers received 36 μmol of L-isoleucine (Low dose; n = 6) and 1527 μmol of L-isoleucine (High dose; n = 3) per kilogram of body weight, respectively. Before the load, isoleucine (alloisoleucine) plasma concentrations were 64 ± 6 μmol/L (2.3 ± 0.3 μmol/L) and 64 ± 4 μmol/L (1.8 ± 0.1 μmol/L), respectively. For convenience, plasma concentrations are presented on a logarithmic scale. Results are presented as means ± SE (bars).
Alloisoleucine plasma concentrations were also evaluated in several metabolic defects. In patients with diabetes mellitus exhibiting significantly increased mean leucine (53%), valine (36%), and isoleucine (35%) plasma concentrations \((P < 0.001\) vs adult controls), alloisoleucine concentrations were comparable to control concentrations (see Table 1). In patients with phenylketonuria and ketogenic hypoglycemia, the plasma concentrations of all branched-chain amino acids, including alloisoleucine, were comparable to control concentrations. As checked by regression analysis, the plasma concentrations of alloisoleucine and its metabolic precursor, isoleucine, were statistically not correlated in all non-MSUD study groups, including adults, children, and infants [e.g., linear regression for controls: \(y = 0.002 (\pm 0.002)x + 1.39 (\pm 0.20)\); coefficient of determination = 0.008; \(n = 102\)].

**MSUD Patients**

In the non-MSUD study groups, plasma alloisoleucine was always below 5 \(\mu\text{mol/L}\). The loading experiments also suggest that in controls and non-MSUD patients receiving branched-chain amino acids in typical amounts in their diet, plasma alloisoleucine concentrations should not exceed that value. Therefore, this value was taken as a reasonable cutoff value for the discrimination of MSUD and non-MSUD subjects.

Of note is that alloisoleucine concentrations were below the cutoff in obligate heterozygous parents of patients with the classical form of MSUD (Table 1). In classical MSUD patients, alloisoleucine concentrations >5 \(\mu\text{mol/L}\) were found in 99.9% of a representative number of unselected plasma samples \((n = 2453)\) from seven patients; Table 1). There was a statistically highly significant linear relationship between isoleucine \((x)\) and alloisoleucine \((y)\) concentrations \([y = 0.40 (\pm 0.01)x + 54.6 (\pm 2.0); P\)

### Table 2. Characteristic data of patients with variant form of MSUD.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age at diagnosis</th>
<th>BCOA-DH activity</th>
<th>Protein intake</th>
<th>Plasma concentration, (\mu\text{mol/L})</th>
<th>Increased Allo’ (no. of samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D.G. (f) (female, born 1974)</td>
<td>3 weeks (d) (01/08–09/98)</td>
<td>2</td>
<td>(0.5 – 0.6^c)</td>
<td>Leu: 254 ± 10 (260; 61-496)</td>
<td>135/135</td>
</tr>
<tr>
<td>Y.M. (f) (female, born 1977)</td>
<td>2 months (d) (12/1977–04/89)</td>
<td>4</td>
<td>(0.5 – 0.6^c)</td>
<td>Ile: 145 ± 5 (137; 31-290)</td>
<td>236/236</td>
</tr>
<tr>
<td>S.C. (f) (female, born 1986)</td>
<td>21 months (d) (02/1986–08/86)</td>
<td>7</td>
<td>1.2 – 1.4</td>
<td>Allo: 79 ± 3 (76; 8-132)</td>
<td>71/71</td>
</tr>
<tr>
<td>H.H. (male, born 1985)</td>
<td>7.5 years (d) (02/1989–09/98)</td>
<td>9</td>
<td>0.8 – 1.2</td>
<td>Leu: 421 ± 14 (345; 328-473)</td>
<td>12/12</td>
</tr>
<tr>
<td>S.T. (f) (female, born 1977)</td>
<td>3 weeks (d) (08/77–07/88)</td>
<td>11</td>
<td>1.0 – 1.5</td>
<td>Ile: 242 ± 9 (237; 178-327)</td>
<td>24/24</td>
</tr>
<tr>
<td>T.R. (f) (male, born 1964)</td>
<td>3.2 years (d) (10/1970–03/98)</td>
<td>15</td>
<td>0.6 – 1.0</td>
<td>Allo: 107 ± 6 (105; 69-165)</td>
<td>25/25</td>
</tr>
<tr>
<td>S.K. (f) (male, born 1979)</td>
<td>3 weeks (d) (07/79–11/86)</td>
<td>17</td>
<td>1.5 – 2.0</td>
<td>Leu: 304 ± 30 (339; 99-467)</td>
<td>13/13</td>
</tr>
<tr>
<td>D.N. (f) (male, born 1978)</td>
<td>4 weeks (d) (11/78–09/88)</td>
<td>25</td>
<td>1.5</td>
<td>Ile: 152 ± 12 (150; 77-239)</td>
<td>22/22</td>
</tr>
<tr>
<td>L.F. (female, born 1990)</td>
<td>3 weeks (d) (07/90–12/98)</td>
<td>NA</td>
<td>1.0 – 1.5</td>
<td>Allo: 147 ± 5 (141; 19-29)</td>
<td>28/28</td>
</tr>
</tbody>
</table>

\(f\) Observation period (in month/year) in parentheses.  
\(d\) BCOA-DH, branched-chain 2-oxoacid dehydrogenase; Allo, alloisoleucine; NA, not available.  
\(e\) Dietary recommendations, in g · kg\(^{-1}\) · day.  
\(f\) Data from periods of good to moderate metabolic control (see Results), median and range in parentheses; valine data not shown.  
\(g\) For additional patient data see \(f\) Wendel et al. (18) and \(f\) Boisse et al. (22).  
\(h\) Detection on occasion of or because of: \(i\) metabolic crisis; \(j\) maple syrup odor; \(k\) neonatal screening.  
\(\) Supplemented with branched-chain amino acid-free amino acid mixture.
increased mean plasma concentrations of the other branched-chain amino acids. The lower relative muscle mass in infants compared with older subjects might provide an explanation of this apparent age dependency. Most likely, alloisoleucine is produced as a by-product of isoleucine transamination (14), and the branched-chain amino acid aminotransferase activity in humans is localized mainly in muscle tissue (15).

The results in diabetic patients show that moderately increased branched-chain amino acid concentrations do not in themselves lead to increased alloisoleucine concentrations. Likewise, transient branched-chain aminoacidemia that occurs, for example, in ketogenic hypoglycemia and starvation appears not to be associated with increased alloisoleucine concentrations (5–10). Similarly, the transient approximately twofold increase of plasma isoleucine in the present low-dose isoleucine loading studies exerted no significant effect on plasma alloisoleucine. In the high-dose loads, the isoleucine equivalents administered corresponded to ~4 g of protein/kg of body weight. This amount grossly exceeded the uptake in a typical meal. The effect on increases in alloisoleucine, however, was only slight despite the ~30-fold peak increase in plasma isoleucine. Taken together, these findings indicate that increased plasma concentrations and the routine dietary supply of isoleucine have negligible influence on the alloisoleucine plasma concentrations in non-MSUD subjects. With exception of the high-dose isoleucine loads, we never observed plasma alloisoleucine concentrations >5 μmol/L in the non-MSUD study groups. Therefore, this concentration was taken as a tentative cutoff value for a retrospective analysis in MSUD patients.

The general knowledge that the presence of alloisoleucine is characteristic of MSUD (1, 16, 17) has apparently never been substantiated in quantitative terms. According to the present data, alloisoleucine concentrations below the cutoff value are extremely rare in classical MSUD and occur only when the patients are on a too strict dietary regimen. Generally, increased alloisoleucine persisted even when the isoleucine concentrations were extremely low (<1 μmol/L). The latter was not unexpected in MSUD because it has been shown that plasma alloisoleucine increases within hours after an isoleucine challenge but decreases with sluggish plasma kinetics within days or even weeks (18–20). In the 2 samples (of 2453) in which plasma alloisoleucine was <5 μmol/L, the patient was on a stringently restricted diet, and isoleucine was practically absent from the plasma. Most likely, there was a somewhat prolonged isoleucine deficiency in this patient, which finally led to the disappearance of plasma alloisoleucine.

In our patients showing a rather representative spectrum of variant MSUD, alloisoleucine was below the cutoff value in several plasma samples although isoleucine was generally beyond 30 μmol/L. The overall incidence of increased alloisoleucine decreased with the se-
verity of the disease, in agreement with previous findings showing a graded enhancement of plasma alloisoleucine clearance in MSUD variants (20). In the majority of samples, however, alloisoleucine was >5 μmol/L. In the absence of grossly increased branched-chain amino acid concentrations and any clinical symptoms, increased alloisoleucine was found in at least 78% of the samples taken from an individual patient. Even when the branched-chain amino acid concentrations were normal, the incidence of alloisoleucine beyond the cutoff value was never below 70% (patient L.F.; data not shown).

Regarding the significance of increased plasma branched-chain amino acid concentrations for the diagnosis of MSUD, leucine, valine, and isoleucine should exceed 400, 600, and 250 μmol/L, respectively, to allow reliable differentiation of MSUD-induced increases from secondary disturbances of branched-chain amino acid metabolism that occur, e.g., in ketotic hypoglycemia (5, 6). In our classical MSUD patients, the overall percentages of samples exhibiting plasma concentrations above these threshold values were 43% with leucine, 2% with valine, and 20% with isoleucine compared with >99% of the samples showing increased alloisoleucine. In variants, the overall percentages of increased plasma concentrations were 28% with leucine, 3% with valine, and 14% with isoleucine, compared with 94% with alloisoleucine. Simultaneous increases of leucine, valine, and isoleucine over these threshold values were found in only 2% of the specimens from classical MSUD patients and in 3% of the samples obtained from the variants.

Taken together, the present findings indicate that plasma alloisoleucine is the most sensitive and most specific general diagnostic marker for classical as well as variant forms of MSUD. Alloisoleucine analysis in plasma should allow a differential diagnosis of MSUD even in episodes of mild clinical symptoms and before the development of severe metabolic crises. Urinary analysis cannot be recommended for this purpose. Because of the generally low and rather variable fractional renal clearance of branched-chain compounds, analysis in urine is far less sensitive than in plasma specimens (21).

When based on an alloisoleucine cutoff value of 5 μmol/L, the sensitivity estimates for detection of variant and classical MSUD in the absence of clinical symptoms were >90% and >99%, respectively. The sensitivity in the presence of symptoms and the overall specificity can be expected to be almost 100%.

Supported in part by Grant We 614/9-2 from the Deutsche Forschungsgemeinschaft. We gratefully acknowledge the generous support of colleagues who provided data on their MSUD patients: Dr. D. Leupold (Ulm, Germany), Dr. B. Plecko (Graz, Austria), and Prof. J-M. Saudubray (Paris, France). Plasma samples from patients with ketotic hypoglycemia were kindly provided by Dr. O.A.F. Bodamer (Houston, TX). This communication contains parts of the thesis of A. Bodner-Leidecker.

References


