Determination of Branched-Chain Amino Acids and Tyrosine in Serum of Patients with Various Hepatic Diseases, and Its Clinical Usefulness

Yutaro Azuma,^1 Maseto Maekawa,^1 Yoshiko Kuwabara,^1 Takeyuki Nakajima,^2 Ken Taniguchi,^3 and Takashi Kanno^1

We developed an automated enzymatic method for determination of the branched-chain amino acids (BCAAs; valine, isoleucine, leucine) and tyrosine in serum, and applied it to the clinical evaluation of patients with various hepatic diseases. Analytically, the test results were acceptably precise and reproducible, and correlated well with results obtained with an amino acid analyzer. Clinically, we found that a decrease in BCAAs, an increase in tyrosine, and the BCAAs/tyrosine ratio in serum paralleled the severity of hepatic parenchymal damage. We conclude that this enzymatic determination of BCAAs and tyrosine is simple and convenient enough for routine clinical laboratory use, and that the ratio of BCAAs/tyrosine obtained may be a good indicator of the severity of hepatic disorders.

Additional Keyphrases: enzymatic methods • monitoring liver disease • plasma/serum differences

Metabolic abnormalities involving free amino acids in plasma, particularly branched-chain amino acids (BCAAs; valine, isoleucine, leucine), aromatic amino acids (AAAs; tyrosine, phenylalanine), and methionine, are frequently observed in patients with hepatic disorders. These amino acids have been determined by ion-exchange chromatography, HPLC, or gas chromatography (1,2). These methods, however, require sophisticated and expensive instrumentation and sample pre-treatment, and analysis times are prolonged because samples must be analyzed sequentially. Recently, an alternative enzymic-spectrophotometric method requiring no extraction or deproteinization of plasma or serum has been described (3–10). In this procedure, leucine dehydrogenase is used in the analysis for BCAAs (3–6) and phenylalanine ammonia-lyase for AAAs (7–10), and results are determined spectrophotometrically or fluorometrically.

Here we report new enzymatic methods developed for both BCAAs and tyrosine. We have applied these methods in an automated clinical chemical analyzer to the routine monitoring of patients with hepatic disorders.

In a clinical application of amino acid analysis, Fischer and Baldessarini (11) reported that the molar ratio of BCAAs/AAAs decreased in parallel with increasing severity of hepatic parenchymal damage. Subsequently, other investigators have reported the connection between this ratio and hepatic disorders, particularly hepatic encephalopathy (12–15). We have determined concentrations of BCAAs and tyrosine in serum and calculated the molar ratio of BCAAs to tyrosine instead of BCAAs to AAAs. Finally, we discuss their utility in monitoring patients with various hepatic disorders.

Materials and Methods

Principle

Figure 1a shows a scheme for determination of BCAAs. BCAAs are oxidatively deaminated in the presence of leucine dehydrogenase (EC 1.4.1.9). The formation of NADH is coupled with the redox systems of 1M-PMS and MTT, and the absorbance of the formazan so produced is measured at 600 nm.

Figure 1b shows a scheme for the determination of tyrosine. Tyrosine is decarboxylated in the presence of tyrosine decarboxylase (EC 4.1.1.25) to yield tyramine. Tyramine oxidase (EC 1.4.3.4) catalyzes the oxidation of tyramine, and 4-hydroxyphenyl acetaldehyde and hydrogen peroxide are produced. The hydrogen peroxide is detected by adding peroxidase (EC 1.11.1.7), 4-amino antipyrine, and TOOS as a chromogen system. Peroxidase acts on hydrogen peroxidase, 4-amino antipyrrine, and TOOS to produce a quinine dye, in the presence of ascorbate oxidase (EC 1.10.3.3). We measure the absorbance of this dye at 546 nm.

Apparatus and Reagents

A Model 835 amino acid analyzer (Hitachi Kokki Co., Ltd., Tokyo, Japan) was used as a conventional method for determining amino acid concentrations. A Shimadzu CL-20 discrete-type clinical chemistry automated analyzer (Shi-

---

1 Department of Laboratory Medicine and 2 Second Department of Internal Medicine, Hamamatsu University, School of Medicine, Hamada-cho 3600, Hamamatsu City, 431-31, Japan.

3 Diagnostic Research Laboratory, Ono Pharmaceutical Co., Ltd., Sakurai 3-1-1, Shimamoto-cho, Mishima-gun, Osaka 618, Japan.

4 Nonstandard abbreviations: BCAAs, branched-chain amino acids; AAAs, aromatic amino acids; 1M-PMS, 1-methoxy-5-methylphenazinium methosulfate; MTT, 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium; and TOOS, N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine.

Received January 17, 1989; accepted April 3, 1989.
madzu Co., Ltd., Kyoto, Japan) was used for the enzymatic methods.

Leucine dehydrogenase from Bacillus species, peroxidase from horseradish, and ascorbate oxidase from Cucumis species were from Toyobo Co., Ltd., Osaka, Japan. Tyramine oxidase from Aspergillus species was from Sigma Chemical Co., St. Louis, MO; pyridoxal 5-phosphate from Tokyo Kasei Co., Tokyo, Japan; Tyrosine decarboxylase from Streptococcus faecalis was from Sigma Chemical Co., St. Louis, MO; pyridoxal 5-phosphate from Tokyo Kasei Co., Tokyo, Japan; 4-amino antipyrine from Nakarai Chemicals Ltd., Kyoto, Japan; TOOS, 1M-PMS, and MTT from Djin Chem. Lab., Kumamoto, Japan; bilirubin conjugate from Purp runy Products Inc., Logan, UT; hemoglobin (Hemocon-N*) from Nippon Shiyui Co., Ltd., Tokyo, Japan; Intra lipid (10%) from Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan; and L-isoleucine and L-tyrosine were from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Other reagents were of analytical grade.

Reagents for Determining BCAAs and Tyrosine

Reagents for determining BCAAs: Each of these reagents was prepared in Tris HCl buffer (50 mmol/L, pH 7.5).

Calibration solution: Isoleucine 500 μmol/L.
Reagent 1: NAD+ 5 mmol/L, 1M-PMS 0.02 mmol/L, and ascorbate oxidase 1 kU/L.
Reagent 2: leucine dehydrogenase 15 kU/L, MTT 0.2 mmol/L.

Reagents for determining tyrosine: Each of these reagents was prepared in phosphate–citrate buffer (pH 6.0).

Calibration solution: tyrosine 200 μmol/L.
Reagent 1: peroxidase 2 kU/L, ascorbate oxidase 2 kU/L, and TOOS 3 mmol/L.
Reagent 2: tyrosine decarboxylase 0.9 kU/L, pyridoxal 5-phosphate 10 μmol/L, tyramine oxidase 1 kU/L, 4-amino antipyrine 0.3 mmol/L.

Procedures

Enzymatic determination of BCAA: Add 400 μL of BCAA reagent 1 to 5 μL of serum or standard solution, and incubate at 37 °C for 5 min. Next, add 100 μL of BCAA reagent 2 to the reaction mixture, incubate at 37 °C for 5 min, and measure the absorbance of formazan at 600 nm (A1). At the same time measure the absorbance of a sample blank, for which the reagent lacks leucine dehydrogenase (A2). Correct the value for BCAAs with the sample blank (A1 – A2).

Enzymatic determination of tyrosine: Add 400 μL of tyrosine reagent 1 to 10 μL of serum or standard solution, and incubate at 37 °C for 5 min. Next, add 100 μL of tyrosine reagent 2 to the reaction mixture, incubate at 37 °C for 5 min, then measure the absorbance of the resulting guinone dye at 546 nm (A1). Correct by subtracting the absorbance (A2) of a sample blank, for which the reagent is used without tyrosine decarboxylase (A1 – A2).

Comparison method: Plasma samples were collected in heparinized tubes, and deproteinized with sulfosalicylic acid (30 g/L final concentration). All samples were analyzed with a Hitachi 835 amino acid analyzer, used according to the manufacturer's instructions.

Subjects

We studied 76 patients with several hepatic disorders (seven with acute hepatitis, 17 with active chronic hepatitis, 21 with compensated liver cirrhosis, five with decompensated liver cirrhosis, and 26 with hepatocellular carci-

Results

Analytical Variables

Linearity: We examined linearity by assaying serial dilutions of the standard solution. The linearity range for isoleucine determinations extends to 3000 μmol/L and that for tyrosine to 1000 μmol/L, without predilution of the specimens. These ranges allowed us to measure the concentrations of these amino acids in serum.

Precision: Within-run (15 assays) and between-run (20 assays) precisions were determined with two different serum pools. Table 1 shows the within- and between-run precision of the method, each <2.4% for both BCAAs and tyrosine.

Analytical recoveries: Different concentrations of isoleucine and tyrosine were added to the serum pool (volume ratio: 1/9) and then measured, as was the serum with no added substance. Analytical recoveries of BCAAs ranged from 91.3% to 99.9%; those of tyrosine ranged from 100.6% to 106.7% (Table 2).

Interference: Conjugated bilirubin, hemoglobin, and In- tratrilipid (a fat emulsion) were added to the serum pool, and their potential interference was measured by comparison with the pool with no added substance. Additions to the serum were averaged, and the percentages of decrease or increase in apparent BCAAs or tyrosine were calculated. As Table 3 shows, there was no interference from conjugated bilirubin or Intralipid in concentrations as high as 100 mg/L and 2.5 mg/L, respectively. However, the tyrosine

<table>
<thead>
<tr>
<th>Table 1. Precision of Enzymatic Determinations of BCAAs and Tyrosine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Conc, μmol/L</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>BCAs</strong></td>
</tr>
<tr>
<td>Sample 1</td>
</tr>
<tr>
<td>Sample 2</td>
</tr>
<tr>
<td><strong>Tyrosine</strong></td>
</tr>
<tr>
<td>Sample 1</td>
</tr>
<tr>
<td>Sample 2</td>
</tr>
<tr>
<td>* Mean ± SD (and CV, %).</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 2. Analytical Recovery of BCAAs and Tyrosine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Conc, μmol/L</strong></td>
</tr>
<tr>
<td><strong>Pooled serum</strong></td>
</tr>
<tr>
<td><strong>BCAs</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Tyrosine</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
measurement was affected in parallel with increasing concentrations of hemoglobin.

Comparison of plasma and serum as samples: Usually, fresh plasma samples are used for amino acid analysis, because serum samples suffer interference from amino acids released from erythrocytes and other cells during blood coagulation or the proteolytic conversion of some peptides during coagulation. However, we used serum samples to apply the present method to the clinical chemistry analyzer.

We compared values for BCAAs and tyrosine obtained with serum samples and plasma samples (Figure 2). A paired t-test showed some significant differences (P < 0.001) in the values obtained for plasma and serum. However, good proportional correlations were obtained, both for plasma and serum. We considered that the results reveal no critical problem, and serum samples could be used for these measurements.

Comparison with amino acid analysis: Results by the present method were compared with those by the conventional amino acid analysis (Hitachi 835). The results are shown in Figure 3. Results of the paired t-test showed good agreement for tyrosine, but a significant difference (P < 0.001) for BCAAs. However, good proportional correlations were obtained for the BCAA values by the two methods. We conclude that there are no critical problems, and that results determined by our method agree with those obtained by amino acid analysis.

Comparison with BCAAs/AAAs and BCAAs/Tyrosine

Fischer et al. (11-13) reported that the ratio of BCAAs to AAAs correlated well with the grade of hepatic encephalopathy, and they attempted to treat these patients with infusions of amino acid solutions with high BCAAs and low AAAs for hepatic encephalopathy (20, 21).

In the present study, our method measures only tyrosine, instead of both tyrosine and phenylalanine. Therefore, we first compared the ratio of BCAAs to AAAs as measured with the amino acid analyzer with the ratio of BCAAs to tyrosine as measured by our method. The coefficient of correlation between the two ratios was 0.808. We concluded that the ratio of BCAAs to tyrosine, obtained with our method, can be used as an indicator of liver damage instead of the ratio of BCAAs to AAAs.

Reference intervals

The distribution patterns of BCAAs in the normal controls proved to be normal, and the distribution patterns of tyrosine and the ratio of BCAAs/tyrosine proved to be logarithmically normal. The reference intervals (mean ± 2 standard deviations) for BCAAs, tyrosine, and the ratio of BCAAs to tyrosine were: 460.5-646.2 μmol/L, 65.7-91.3 μmol/L, and 5.71-9.42, respectively.

Clinical Application

To assess the clinical application of our method, we measured BCAAs and tyrosine and determined the ratio of BCAAs to tyrosine in the serum of patients with various hepatic diseases (Figure 4). BCAA concentrations in serum decreased in parallel with the increasing severity of liver parenchymal damage. In contrast, serum tyrosine concentrations increased in parallel with the severity of liver disease.
damage. As a result of these changes the BCAAs/tyrosine ratio decreased. Therefore we consider the ratio to be a better discriminator of liver parenchymal damage.

We carried out a multivariate correlation analysis on BCAAs, tyrosine, the ratio of BCAAs to tyrosine, and other liver-function tests (Table 4). The increase in tyrosine was inversely correlated with—and the decreases in BCAAs and the BCAAs/tyrosine ratio was positively correlated with—prothrombin time, cholinesterase, total protein, albumin, and total cholesterol.

**Discussion**

A method suited for routine monitoring of patients with hepatic disorders has not been previously reported, because of the complexity of existing methods. Here, we have described an enzymatic method involving use of an automated analyzer and have applied it to the monitoring of various hepatic disorders.

In the analytical study, the linearity of the standard curve for this assay for BCAAs extends to 3000 μmol/L, and that for tyrosine to 1000 μmol/L, without predilution of the specimens. Analytical recovery ranged from 91.3% to 99.9% for BCAAs, from 100.6% to 106.7% for tyrosine. In this assay, conjugated bilirubin and Intralipid caused slight interference, but neither should be a problem for routine monitoring. However, hemoglobin interferes. It can act as a peroxidase in redox reactions. As shown in the scheme (Figure 1b), we used peroxidase to produce a quinone dye for the determination of tyrosine. In this assay, the reagent for the sample blank only lacked tyrosine decarboxylase, so this assay was significantly affected by the peroxidase reaction, which explains the influence of hemoglobin. This is a problem that is yet to be solved.

In the comparison study we observed a good correlation between results of the enzymatic method and those from the amino acid analyzer. It also appears that this method is clinically useful for determining BCAAs and tyrosine in serum.

Phenylalanine hydroxylase and tyrosine aminotransferase are specific for liver cells, and the liver is the principal organ for metabolism of AAs. The concentrations of these amino acids in serum depend on liver-cell functions. Thus, the increased tyrosine seen in serum of patients with liver disease may occur because the liver cannot metabolize tyrosine and it consequently accumulates in the serum. However, BCAAs are mainly metabolized in skeletal muscle. They are transaminated and subsequently oxidized to succinyl CoA. Thus, BCAA concentrations in serum are largely controlled by muscle metabolism.

As reported from the study of infusion of BCAAs (22), BCAAs are associated with glucose metabolism and, in particular, insulin. Uptake of BCAAs into these tissues is promoted by insulin. The liver extracts insulin from the circulation, and the relative hyperinsulinaemia in hepatic failure is reflected by decreases in its extracting ability (17). BCAA concentrations in serum are considerably affected by the serum insulin concentration, which is related to liver functions (16). In accord with this, we observed a good correlation between amino acid concentrations and results of liver-function tests (prothrombin time, cholinesterase, total protein, albumin, total cholesterol) that are indicators of liver parenchymal damage.

Fischer et al. (12) reported that the ratio of BCAAs to AAs was significantly reduced in animals and patients with hepatic encephalopathy. Soeters and Fischer (16) then hypothesized that hepatic encephalopathy might in fact result from changes in the plasma concentrations of amino acids making up this ratio. However, some reports have shown that this ratio is decreased in patients with liver disease, as a result of liver damage rather than because of encephalopathy (14, 15, 19). In addition, some other reports (23, 24) show the usefulness of the BCAAs/AAs ratio, not only for diagnosis of hepatic encephalopathy but for other liver diseases such as liver cirrhosis and acute hepatic necrosis. In this study, we have shown that decreases in the serum ratio of BCAAs/tyrosine in serum could substitute clinically for decreases in the ratio of BCAAs/AAs.

In conclusion, the BCAAs/tyrosine ratio can be determined rapidly and simply by the present enzymatic method, and it can be a very useful indicator of hepatic parenchymal damage in laboratory diagnoses.

**References**


---

**Table 4. Correlation (r) with Results of Liver-Function Tests**

<table>
<thead>
<tr>
<th>Item</th>
<th>No. patients</th>
<th>BCAAs</th>
<th>Tyrosine</th>
<th>BCAAs/tyrosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prothrombin time</td>
<td>65</td>
<td>0.482*</td>
<td>-0.702**</td>
<td>0.743**</td>
</tr>
<tr>
<td>Cholinesterase</td>
<td>74</td>
<td>0.327*</td>
<td>-0.516**</td>
<td>0.617**</td>
</tr>
<tr>
<td>Albumin</td>
<td>69</td>
<td>0.308*</td>
<td>-0.448**</td>
<td>0.538**</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>61</td>
<td>0.244</td>
<td>-0.486**</td>
<td>0.428**</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>75</td>
<td>-0.175</td>
<td>0.328**</td>
<td>-0.335*</td>
</tr>
<tr>
<td>Total protein</td>
<td>68</td>
<td>0.135</td>
<td>-0.145</td>
<td>0.286*</td>
</tr>
<tr>
<td>Total bilirubin</td>
<td>74</td>
<td>-0.110</td>
<td>0.220</td>
<td>-0.220*</td>
</tr>
<tr>
<td>Alanine aminotransferase</td>
<td>76</td>
<td>0.347**</td>
<td>0.016</td>
<td>0.124</td>
</tr>
</tbody>
</table>

* Correlations significant at *P <0.05, **P <0.01.


