and CV were 102.5% and 16%, respectively. Intra- and interassay precision was assessed by replicate analysis of specimen aliquots on a single day or on successive days and is shown in Table 1. Good precision was obtained, demonstrating the accuracy of the method for the quantitative determination of VMA.

The stability of a prepared specimen was investigated by repeat injections of multiple aliquots (n = 5) on the same day (day 1) and subsequent days (days 2 and 5) with interim storage at 4 °C. All aliquots gave a value of 1.1 mg/L, which indicates the stability of VMA and d5-VMA on refrigeration for at least 5 days after sample preparation.

For the purpose of method comparison, we compared the MS/MS method against a commercial HPLC assay (Bio-Rad Laboratories) previously used in our laboratory (6, 7). The acquisition time for the HPLC method is relatively long (6 min/specimen) and is prone to several problems: shift of retention time, interference by coeluting compounds (an average of 5% of specimens were rejected after analysis), detector instability, and the necessity of frequent maintenance.

Unused portions of 324 specimens successfully analyzed by the HPLC method were retested using the new method. The correlation between the new (y) and old (x) methods was: $y = 1.05x + 0.06 \text{ mg/L}$, with an average difference of 0.2 mg/L. Bland–Altman and Deming (inset; range, 0–20 mg/L) plots are shown in Fig. 1. More than 99% of compared values differed by less than 2 SD. Results above our adult reference interval (<8 mg/24 h) were assessed on the basis of clinical sensitivity; for example, for a specimen with an average VMA value of 57.5 mg/L, a difference of 7.4 mg/L corresponded to a markedly increased VMA value: 680 and 598 mg/24 h by the new and old methods, respectively. Analysis of proficiency testing specimens carried out during the method comparison gave VMA values in agreement at low concentrations [5.2 and 5.2 mg/L; mean (SD), 5.4 (0.4) mg/L] and increased concentrations [22.1 and 23.7 mg/L; mean (SD), 22.8 (0.7) mg/L].

In summary, we have developed and validated a MS/MS method for the determination of VMA in urine that uses a stable-isotope-labeled internal standard. Our method includes automated solid-phase extraction, an isocratic mobile phase, and quantification against a stable-isotope-labeled internal standard. Sample preparation is fully automated, and a rapid analytical time (3 min/sample) is achieved with little or no need to repeat analyses.

**Table 1. Precision statistics of the LC-MS/MS assay.**

<table>
<thead>
<tr>
<th>No. of aliquots</th>
<th>Detected, mg/L</th>
<th>Intraassay</th>
<th>Interassay</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>2.5 (0.12)</td>
<td>4.7</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>3.8 (0.14)</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>59.7 (3.6)</td>
<td>6.1</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>4.0 (0.1)</td>
<td>1.2</td>
<td>2.5</td>
</tr>
<tr>
<td>6</td>
<td>20.1 (0.5)</td>
<td>1.0</td>
<td>2.5</td>
</tr>
</tbody>
</table>

*a Mean (SD).  
*b VMA calibrator.

With the introduction of modern biochemical techniques, more than 50 enzymes have been developed for diagnos-

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**References**


**Fig. 1.** Bland–Altman plot of the difference vs the mean of paired VMA urine values (n = 324) for the LC-MS/MS method and our previous HPLC method (4–6).

The inset shows the comparison of the two methods (Deming regression) in the 0–20 mg/L range.
tics in liver tests. Among the enzymes, aspartate aminotransferase (AST; EC 2.6.1.1; formerly known as GOT) was introduced in the mid-1950s and has remained the mainstay of enzyme diagnosis for liver disease for more than half a century \( (1) \). AST is widely distributed among human organs as cytosolic and mitochondrial isotypes \( (2) \). Increased activity of this enzyme is considered one of the most sensitive indicators of hepatocellular damage \( (3) \). Previously, several attempts were made to develop an immunoassay procedure for measuring AST mass rather than enzyme activity, but they are currently not used for the diagnosis of liver diseases \( (4–8) \). In this study, we generated monoclonal antibodies (mAbs) to AST and used them to develop a sandwich ELISA to measure the enzyme mass in sera.

Because the amino acid sequences of the human and porcine enzymes are similar, we used porcine AST as an immunogen for the production of mAbs \( (9) \). The porcine enzyme is commercially available as a highly purified form in large quantities. Twenty hybridoma cells to porcine AST were initially screened by ELISA from several fusions, and four clones were selected for further characterization.

To confirm the specificity of the mAbs to the human enzyme, we immunoblotted partially purified human enzyme and total proteins extracted from a human liver with the mAbs. The antibodies specifically recognized a single protein band of 45 kDa, which coincided well with the expected size of AST. To evaluate whether the mAbs recognized a cytosolic or a mitochondrial form of AST in human tissues, we prepared cytosolic and mitochondrial fractions from a human liver and processed them for Western blotting. The mAbs recognized a single protein band in the cytosolic fraction only, and we observed no detectable band in the mitochondrial fraction, indicating that the mAbs were specific for the cytosolic form.

The sandwich ELISA assay was optimized by a standard procedure with porcine AST as a calibrator to achieve assay precision appropriate for the concentrations seen in sera. The limit of determination in serum was 3 \( \mu \)g/L, calculated as the AST concentration whose signal corresponded to the mean + 3 SD for 20 replicates of a zero calibrator. The measurement range was 3–100 \( \mu \)g/L.

The patients enrolled in this study were from the Chuncheon Sacred Heart Hospital (Table 1). Healthy volunteers were defined by analysis of various biochemical tests, including alanine aminotransferase (ALT)/AST, albumin, bilirubin, \( \gamma \)-glutamyltransferase, alkaline phosphatase, and lactate dehydrogenase (LDH), which were performed on a Hitachi 747 (Roche Diagnostics). Hepatitis C (HCV) and B (HBV) tests were serologically confirmed with an AxSYM system (Abbott Laboratories). The mean ages of the various patient groups were similar to those of the control group.

Informed consent was obtained from all patients and healthy volunteers before their participation in the study. Statistical differences between means were calculated using the Student \( t \)-test and ANOVA with Bonferroni adjustment. Pearson correlation coefficients and linear regression with the least-squares method were used to evaluate correlations between the patient and control groups. \( P \) values \(< 0.05 \) were considered statistically significant.

The mean (SD) AST concentration in healthy individuals was 34 (10) \( \mu \)g/L (Table 1, control group), which was lower than the values reported earlier by others: 84 (18) \( \mu \)g/L \( (7) \) and 65–145 \( \mu \)g/L \( (10) \). The 2.5th percentile of the general population was 19 \( \mu \)g/L, and the 97.5th percentile was 58 \( \mu \)g/L. There was no significant difference in AST mass between males or females, which is consistent with the results obtained by other groups \( (10,11) \). However, some studies showed that AST and ALT activities are higher in males than in females and vary with age \((12,13)\).

We next measured AST mass in different groups of liver disease patients. The distribution of AST mass in patient groups is shown in Table 1. The mean (SD) AST mass for liver disease patients was 119 (101) \( \mu \)g/L compared with 34 (10) \( \mu \)g/L for healthy individuals, demonstrating a significant difference between healthy individuals and all liver disease groups \( (P < 0.0001) \). The difference in AST mass between groups, including the control group, was statistically significant \( (P < 0.05) \) except between the chronic hepatitis and liver cirrhosis groups. Patients with acute hepatitis had the highest AST concentration [194 (138) \( \mu \)g/L], followed by those with chronic hepatitis [101 (58) \( \mu \)g/L]. The patients with cirrhosis had the lowest serum AST mass [75 (53) \( \mu \)g/L]. Because there was a bias of gender distribution in some patient groups, e.g., only 5 females of 41 patients with chronic hepatitis and cirrhosis, we reevaluated the data with males only, but we did not find a significant differ-

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Age, years</th>
<th>M/F</th>
<th>ALT, U/L</th>
<th>AST, U/L</th>
<th>cAST,(^{abc}) ( \mu )g/L</th>
<th>Viral infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute hepatitis</td>
<td>47 (16)</td>
<td>11/7</td>
<td>374 (492)</td>
<td>374 (434)</td>
<td>194 (138)</td>
<td>HBV 0</td>
</tr>
<tr>
<td>Chronic hepatitis</td>
<td>39 (14)</td>
<td>14/3</td>
<td>116 (81)</td>
<td>94 (7)</td>
<td>101 (58)</td>
<td>16 1</td>
</tr>
<tr>
<td>Liver cirrhosis</td>
<td>49 (10)</td>
<td>22/2</td>
<td>53 (85)</td>
<td>70 (84)</td>
<td>75 (53)</td>
<td>9 3</td>
</tr>
<tr>
<td>Control</td>
<td>42 (13)</td>
<td>25/18</td>
<td>24 (11)</td>
<td>23 (7)</td>
<td>34 (10)</td>
<td>0 0</td>
</tr>
</tbody>
</table>

\(^a\) Data are expressed as the mean (SD) for continuous variables.

\(^b\) cAST, cytosolic AST.

\(^c\) The \( P \) value for AST mass between all patients with liver disease and the control group was \(< 0.0001 \). The \( P \) value among different groups was statistically significant \((P < 0.05)\) except between the chronic hepatitis and liver cirrhosis groups.
ence between the males-only and total groups. When we compared specific activities, the values were much higher than those reported by other groups (7, 14). One explanation for the high specific activity may be the porcine AST that we used to construct the calibration curve. The antibodies differed in their cross-reactivities between human and porcine AST, which could account for the discrepancy in specific activities.

When we examined the relationship between the two methods by plotting AST activity against AST mass from the control and patient groups, there appeared to be distinct regions with different slopes in the linear regression plot. We observed a poor correlation in the range <50 U/L (Fig. 1A). The correlation was not as good in the range 50–200 U/L (Fig. 1B). The poor correlation at activities <200 U/L, such as those we observed for most of chronic hepatitis and liver cirrhosis patients, may be explained by the loss of AST catalytic activity on release into circulation, whereas the enzyme protein remains immunologically active in the circulation for some time (6, 7). In contrast, we observed a good correlation at activities >200 U/L (Fig. 1C). AST activities for most patients with acute liver disease were in this range.

Attempts to develop an immunochemical procedure for the quantification of serum AST have been published previously (4). Other, more recent reports have described more accurate and sophisticated AST immunoassays (5–8, 14), but immunoassays have not replaced the conventional enzyme activity assays. Previously, authors observed that there is a considerable excess of immunologically active but catalytically inactive AST in the sera of healthy individuals and patients with liver disease (6, 7). There appear to be some similarities and differences between the results of previous studies and our results. One difference is the antibody specificity. We used mAbs that specifically recognized AST, with a single band in the Western blot, but others used polyclonal antibodies, which could have caused cross-reactivity. Suzuki et al. (8) generated mAbs to human mitochondrial AST but failed to detect the mitochondrial AST by their ELISA method. Another possible explanation for the different results is that autoantibodies may combine with

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Fig. 1. Distribution and correlation of AST mass and activity at three different ranges of enzyme activity: <50 U/L (A), 50–200 U/L (B), and >200 U/L (C).

• healthy controls; ×, patients with cirrhosis; □, patients with acute hepatitis; △, patients with chronic hepatitis.
enzymes in the serum and modulate enzyme activity or interfere with the binding of polyclonal antibodies to the protein. The addition of polyclonal antibodies to cytosolic AST produced complete inhibition of the enzyme activity (5).

The proposed immunologic method for measuring AST seems to have potential advantages over the conventional enzyme activity assay. In a single liver cell, two types of AST are present: a mitochondrial and a cytosolic form. The mitochondrial form is released into circulation in cases of more severe liver cell damage (2,15). Thus, the degree of liver cell injury may be estimated by determining the ratio of serum mitochondrial and cytosolic AST. A higher concentration of mitochondrial AST may indicate more severe liver damage. Another issue to be considered is the degradation of enzymes in the circulation, with loss of activity by denaturation or degradation.

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References

Method for Hemoglobin A1c Measurement Based on Peptide Analysis by Electrospray Ionization Mass Spectrometry with Deuterium-labeled Synthetic Peptides as Internal Standards, Toyofumi Nakaniishi, Ken Iguchi, and Akira Shimizu (1, 2)* (1) Department of Clinical Pathology and (2) Clinical Laboratory, Osaka Medical College, 2-7, Daigaku-machi, Takatsuki, Osaka 569-8686, Japan; * address correspondence to this author at: Department of Clinical Pathology, Osaka Medical College, 2-7, Daigaku-machi, Takatsuki, Osaka 569-8686, Japan; fax 81-726-84-6548, e-mail shimizu@poh.osaka-med.ac.jp

Hemoglobin A1c (HbA1c), which is defined as Hb that is irreversibly glycated at the N-terminal valine of the β-chain, is an important index in the monitoring of glucose control in patients with diabetes (1). Considerable discrepancies in the measured values of HbA1c have been observed among methods and among laboratories (2, 3). To standardize HbA1c methods, Kobold et al. (4) proposed a high-level reference method based on liquid chromatography combined with electrospray ionization mass spectrometry (ESI-MS) analysis of the glycated and nonglycated N-terminal hexapeptides of the Hb β-chains, which are released by enzymatic cleavage of the intact Hb molecule by endoproteinase Glu-C. Their method is now the officially approved IFCC reference method (5).

In our laboratory, Hb variants have been measured by ESI-MS (6, 7). Specimens containing Hb variants showed unexpected HbA1c values, especially when analyzed by HPLC. To correctly estimate the degree of Hb glycation in such specimens, we have applied the peptide method using ESI-MS as proposed by Kobold et al. (4), but the ion signals of peptides occasionally fluctuate, depending on the MS conditions in our laboratory.

We devised a method with a stable-isotope-labeled internal standard to obtain more reproducible values in laboratories where mass spectrometers are used for multiple purposes. We also used synthetic nonlabeled peptides to calibrate the measurement, as reported previously (6, 7). For HbA1c standardization, we used the calibrators provided by the IFCC working group. The calibrators were prepared by mixing isolated HbA0 and HbA1c (8).

Four hexapeptides containing the six N-terminal amino acids of the Hb β-chain were chemically synthesized by Peptide Institute Inc. (Osaka, Japan): a nonglycated, unlabeled hexapeptide (Val-His-Leu-Thr-Pro-Glu; HD0, lot no. 749-901201), 1-deoxyfructosyl-hexapeptide (GD0; lot no. 480709), hexapeptide labeled with isopropyl-d7-leucine (HD7; lot no. 740-101295), and 1-deoxyfructosyl-hexapeptide labeled with the same deuterated amino acid (GD7; lot no. 740-102051). The purity of the peptides was ascertained by HPLC as 99.1%, 99.3%, 99.4%, and 99.5%, respectively. We weighed ~3 mg of each peptide and dissolved it in distilled water to adjust the concentration to 90 mmol/L. The peptides were mixed in the desired molar ratios. Endoproteinase Glu-C (lot no. PBIO 160-016), an enzyme that specifically cleaves the carboxyl side of glutamic acid residues, was purchased from PE Biosystems. Other reagents (spectrophotometric grade) were