Ginkgo biloba seeds (GBS) have from ancient times been consumed in China and Japan for medicinal purposes or as food, but toxic symptoms appear if the intake of these seeds is too great. During the food shortage in Japan after the Second World War, there were many cases of toxicosis and even reports of deaths due to consumption of the seeds. Since then, there have been few such cases of poisoning, but recently some cases have occurred, mainly in children.\(^1\)\(^2\)

The principal symptoms of poisoning due to GBS are vomiting and tonic or convulsive convulsions, which can at first easily be misdiagnosed as symptoms of epilepsy. Because the convulsions appear between 1 and 12 h after ingestion of the seeds, and in many cases attacks occur repeatedly.\(^3\)\(^4\) For a long time the agent responsible for the intoxication could not be identified, but it was finally revealed by the work of Wada et al.\(^5\) to be 4-O-methylpyridoxine (MPN). MPN competitively inhibits the action of vitamin B\(_6\), which is a coenzyme in the body’s amino acid metabolism. Among its properties is that by inhibiting glutamate decarboxylase it inhibits the production of \(\gamma\)-aminobutyric acid (GABA), which is an inhibitory neurotransmitter, in the brain; it is thought that it initiates convulsive attacks as a result of a relative increase in the level of glutamate, which is an excitatory neurotransmitter.\(^6\)

The pharmacokinetics of MPN in humans are not yet clearly understood, but we were interested in determining whether it was possible to predict the increase in severity of GBS poisoning from the concentration of MPN in patients’ serum and from the time elapsed since the seeds were consumed. Our reasoning was that, if early prediction of severity were possible, it would also be possible to plan, on a scientific basis, the administration of preparations of the anticonvulsant diazepam and the specific MPN antagonist pyridoxal phosphate to treat recurrent convulsions.\(^3\)

The methods of determining MPN levels in the serum that have so far been reported comprise that of Yagi et al.\(^7\) which is a reverse-phase HPLC technique using fluorescence detection; our own methods of reverse-phase HPLC using UV detection,\(^8\) and GC/MS analysis.\(^9\) However, since all these procedures require solid-phase extraction, it was considered that a faster method of analysis would be useful to improve the treatment approach in the clinical setting.

In the present study, after simply deproteinizing serum MPN, we introduced it directly onto the HPLC column using an ion-pair reagent in the mobile phase, and thus tested the new method for rapid separation and quantitation on an ODS column. In addition, we applied the method in the analysis of the sera of 5 patients who had ingested GBS and experienced convulsions. Further, GBS were collected from the main regions of Japan where they are sold, and we investigated whether the method could be employed for the determination of MPN in GBS.

**MATERIALS AND METHODS**

**Chemicals and Solutions** MPN was synthesized by the method described by Harris.\(^9\) The purity of the synthesized MPN was determined using HPLC\(^6\) to be 99%.

The following vitamin B\(_6\) compounds were purchased: pyridoxine from Sigma Chemical Co. (St. Louis, MO, U.S.A.); and pyridoxal hydrochloride, pyridoxamine dihy-
drochloride hydrate, and 4-pyridoxic acid from Aldrich Chem. Co. (Milwaukee, WI, U.S.A.). IPCC-MS3 was purchased from GL Sciences Inc. (Tokyo, Japan). All other compounds were of analytical reagent grade and were obtained from Wako (Osaka, Japan).

**Biological Specimen Collection** The standard human serum specimens used in the recovery experiments were purchased from Sigma Chemical Co. The serum used for the actual analysis to determine toxicosis in patients was collected from 5 patients who in 2002 had had convulsions as a result of consuming GBS and had been referred to our institution by their family physician for serum MPN determination. The amounts of GBS that the patients had eaten and the symptoms that appeared are listed in Table 1. It should be mentioned that Kuwa et al. have already reported on the condition of patient 4.\(^\text{10}\) The collected sera were stored at −40 °C until analysis.

**Pretreatment of Serum** One hundred microliters of acetone was added to 100 µl of serum containing 1—0.002 µg/ml of MPN, the liquid was mixed thoroughly with a Vortex mixer, and centrifugation was carried out at 2500 × g for 5 min. The resulting supernatant was filtered through a filter with pore size of 0.45 µm (Ekicrodisc®; Gelman Sciences, Tokyo, Japan), and 10 µl of the filtrate was directly introduced onto the HPLC column.

**HPLC Conditions** The HPLC instrument (SCL10AVP; Shimadzu, Kyoto, Japan) was adjusted so that the mobile phase of 0.1 v/v% aqueous IPCC-MS3 solution/acetonitrile at a ratio of 90/10 had a flow rate of 1.0 ml/min. Infused samples were separated on an Inertsil® ODS-3 column (4.6 mm × 150 mm, 5 µm particle size; GL Sciences Inc., Tokyo, Japan) with an Inertsil® ODS-3 guard column at 40 °C. Fluorescence detection (model RF-10AXL, Shimadzu) was carried out at an excitation wavelength of 290 nm and an emission wavelength of 400 nm. Quantitation was performed using the absolute calibration curve method using analysis software (Class VP; Shimadzu). The amount injected onto the HPLC column was 10 µl.

Also, under these HPLC conditions, the separation of pyridoxine, pyridoxal, pyridoxamine, and 4-pyridoxic acid, which have chemical structures similar to that of MPN, were also present in the body and in nature, was confirmed. IPCC-MS3, and the separation from the serum components was achieved with formation of 0.45-µm pore filter (Ekicrodisc®; Gelman Sciences), 10 µl of the filtrate was introduced directly onto the HPLC column.

**Recovery Test** Standard MPN solution 10 µl was added to 0.99 ml of human reference serum to prepare four dilutions of MPN: 1.0, 0.1, 0.01 and 0.002 µg/ml. Pretreatment was carried out using 100 µl of each of these, and the MPN levels were determined on HPLC. The recovery rates were calculated by subtracting the noise value of a “blank” reference serum containing no MPN from these levels and then comparing them with the measured values, after the identical pretreatment procedure had been performed, in 1.0, 0.1, 0.01, and 0.002 µg/ml of standard aqueous solutions (n = 5 for each concentration).

**Determination of Free MPN Content in GBS** An experiment was done to ascertain whether this was a feasible method for determining the MPN content of GBS. In eight major market areas of Japan where GBS are gathered, Asahikawa, Aomori, Niigata, Nagano, Tokyo, Osaka, Okayama, and Fukuoka, we purchased GBS harvested locally and conducted separate analyses of 10 from each region. We used Wada et al.’s method\(^\text{26}\) for pretreatment of the seeds. Specifically, after drying the shelled seeds for 7 d in a dryer at 40 °C, we pulverized them in a mixer. To 10 mg of the resulting powder, 10 ml of distilled water was added, and the free-form MPN was extracted while mixing for 60 min at 40 °C. Next, in a preliminary study, we confirmed that no MPN was detectable in the residue after the extraction process. After the extracted solution had been passed through a 0.45-µm pore filter (Ekicrodisc®; Gelman Sciences), 10 µl of the filtrate was introduced directly onto the HPLC column to measure the amount of MPN. Examination of the differences of the mean MPN content per gram of GBS from each region was carried out by analysis of variance (ANOVA) using multiple comparisons, and Scheffe’s procedure was used in ANOVA. The level of significance was set at 5%.

**RESULTS AND DISCUSSION**

**HPLC Conditions** Figure 1 is a chromatogram showing the separation of a mixed standard solution of MPN, pyridoxine, pyridoxal, pyridoxamine, and 4-pyridoxic acid (0.01 µg/ml each) using an Inertsil® ODS-3 column. In this way, a clear differentiation was demonstrated from and between the members of the vitamin B\(_6\) group of substances, of which the structures resemble that of MPN, IPCC-MS3, which was used in the mobile phase, has a molecular weight of 214.04. It is a fluorinated organic acid with a C3 carbon chain and with a boiling point of 120 °C, and it is used as an ion-pair reagent for cationic compounds.\(^\text{11}\) In a preliminary experiment, we attempted to use sodium 1-dodecylsulfonate as the ion-pair reagent, but the reproducibility of the retention time of MPN was poor, with an RSD of 1.5% (n = 5), in comparison with the RSD of 0.05% (n = 5) obtained with IPCC-MS3, and the separation from the serum components

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**Table 1. Patients with GBS Poisoning in Whom Serum MPN Concentrations Were Determined**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex</th>
<th>Age</th>
<th>GBS consumed</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>2</td>
<td>50—60</td>
<td>Vomiting and impairment of consciousness from 3 h after ingestion. At 3 and 4 h, for 5 min each, appearance of general tonic–clonic convulsions. Symptoms ameliorated after diazepam and vitamin B(_6) preparations were given.</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>1 yr 8 mo</td>
<td>20</td>
<td>Vomiting and impaired consciousness 6 h after intake. From 6 to 9 h after, repetitive general tonic–clonic convulsions. Symptoms ameliorated after diazepam and vitamin B(_6) were given.</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>1</td>
<td>15</td>
<td>At 2 and 7 h after intake, general tonic–clonic convulsions for 3 min. Symptoms ameliorated after administration of vitamin B(_6) preparation.</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>38</td>
<td>60</td>
<td>Continual vomiting after intake. One episode of tonic–clonic convulsions about 12 h after. Vitamin B(_6) was then given, and symptoms ameliorated.</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>2</td>
<td>20</td>
<td>Vomiting and one episode of general tonic–clonic convulsions for 10 min after intake. Diazepam and vitamin B(_6) were then given, and symptoms ameliorated.</td>
</tr>
</tbody>
</table>
was also inadequate. In addition, it is possible that this method, which employs the volatile substance IPCC-MS3, can function as preparative chromatography to purify MPN.

As for the standard curve for MPN, obtained with fluorescence detection at an excitation wavelength of 290 nm and an emission wavelength of 400 nm, a regression line was given as \( y = 70.0x + 3788.7 \) within the HPLC injection range of 10 ng—10 pg (in terms of the injected sample concentration, range: 1.0 \( \mu \)g/ml—1 ng/ml), and the correlation coefficient was 0.999. When the signal-to-noise ratio was taken to be 5 and the amount injected onto HPLC was 5 pg (in terms of the concentration of the injected sample, 500 pg/ml), a detection limit even lower than the lower limit of 0.5 ng so far reported in the literature for HPLC was possible. It is suggested that one reason why such sensitive detection has become possible is that, with the mobile phase using IPCC-MS3, the base noise was small and the shape of the MPN peak could be made sharper.

For the standard solutions of pyridoxine, pyridoxal, pyridoxamine, and 4-pyridoxic acid, similar standard curves were obtained. However, since the vitamin B6 group of molecules other than pyridoxamine are unfortunately not well separated by means of deproteinization alone from the interference peaks in serum, it was necessary to find a more appropriate pretreatment technique to carry out simultaneous analysis.

**Recovery** Figure 2 shows the chromatograms of the reference serum alone (upper tracing) and of the reference serum containing MPN added to give a concentration of 0.01 \( \mu \)g/ml (lower tracing). MPN was effectively distinguished from interfering peaks in reference serum.

Table 2 shows the results of the recovery of MPN added to reference serum to give concentrations of 1.0, 0.1, 0.01, and 0.002 \( \mu \)g/ml. With the HPLC method of Yagi et al., the mean recovery of MPN added to a concentration of 0.1 \( \mu \)g/ml was 96%, while with our previous method, the mean recovery of MPN added to the level of 0.01 \( \mu \)g/ml was 92.9% and the RSD was 6.5%; the GC/MS method \( ^6 \) yielded 86.1% mean recovery, with RSD=17.0%. With the present method, after MPN addition to the 0.01 \( \mu \)g/ml level, a mean recovery of 95.9% was achieved, with an RSD of 4.9%. Not only are these figures superior to those of the conventional methods, but also a good percentage was recovered when the final concentration of the added MPN was only 0.002 \( \mu \)g/ml, which no other study reported attempting; thus quantitation was possible at this very low level with the present method.
Finally, the time required for one analysis was 30 min, which compares extremely favorably with the conventional solid-phase extraction technique.

**Application 1: MPN Determination in the Serum of Patients with GBS Poisoning**

Figure 3 shows a typical chromatogram of the serum of a patient in whom toxic symptoms appeared after the consumption of GBS. This HPLC technique has made possible the satisfactory isolation of MPN from the components of the serum of an actual patient.

Figure 4 presents curves plotting the serum concentrations of MPN in 5 patients and the times elapsed since their consumption of GBS. In patient 1, convulsions occurred for 5 min both 3 and 4 h after GBS intake. The serum level of MPN during the convulsions at the 4-h point in this patient was 1.28 μg/ml. In patient 2, from the 6th to the 9th h after GBS intake, convulsions occurred repeatedly, and 8 h after, the concentration of MPN was 0.46 μg/ml. Patient 3 had convulsions 2 and 7 h after GBS consumption, and the serum MPN concentration 3 h after was 0.58 μg/ml. Patient 4 had convulsions 2 and 7 h after GBS consumption, and the serum MPN concentration 3 h after was 0.58 μg/ml and at 7 h after 0.42 μg/ml. Patient 4 experienced convulsions 12 h after consumption, at which point the MPN level was 0.24 μg/ml; patient 5 had convulsions 4 h post consumption, with the serum MPN level at 5.4 h post consumption being 0.65 μg/ml. Thus, in these 5 cases, the serum level of MPN ranged from 0.24 to 1.28 μg/ml.

As far as we are aware, in addition to our 5 cases, there have been 8 others of GBS poisoning in whom serum MPN concentrations were determined. In 6 of those reported cases, there was no clear indication of the relationships between either the symptoms or the serum MPN levels and the passage of time, but of the remaining 2, 1, reported by Kajiyama et al., showed a convulsive reaction 9 h after the intake of 50—60 GBS, and an MPN level of 0.36 μg/ml there after; while the other, a patient reported by Ishii et al., who had consumed 50 GBS, had convulsions 4.5 h later and a serum MPN concentration of 0.48 μg/ml. Serum MPN concentrations in those two cases were compatible with our results.

After tests conducted by Yagi et al. in domestic rabbits to which a toxic dose (30 mg/kg) of MPN was administered, it was found that the lower limit of the blood level of MPN for the appearance of toxic symptoms was 1 μg/ml, but the results of our present study suggest that it is possible for convulsions to occur in humans at even lower MPN concentrations.

In one of our cases, patient 4 had a blood MPN level 18 h after eating GBS which had actually increased since the 12th h. Convulsions in this patient also occurred 12 h after GBS intake, which was later than in our other cases, which may have resulted from a delay of unknown cause in MPN absorption. The patient concerned was an adult, and she may have eaten more food together with the GBS than other patients who were children did, but at this stage we can only speculate on the reason.

It will be necessary to clarify the kinetics of the elimination of MPN from the blood by accumulating analytical data on more cases of GBS poisoning. With this method, the lower detection limit is 500 pg/ml, which is superior from the microanalytical standpoint to the values yielded by conventional techniques, and it is possible to calculate the elimination rate variable reliably down to a low concentration. Also, by investigating the relationship between serum MPN levels and degrees of severity of poisoning in a large number of patients, it may well be possible to predict repeated occurrences of convulsions in advance and to treat GBS poisoning on the basis of scientific findings.

**Application 2: Determining the Content of Free MPN in GBS**

Figure 5 shows an HPLC chromatogram showing an analysis of the water extract of GBS collected in Tokyo. The present method is capable of clearly separating MPN...
from other components of GBS. Table 3 lists the results of analysis of GBS collected in eight regions of Japan for the purpose of resale.

The data on the free MPN content per gram of dried GBS used for analysis gave widely scattered results, with mean values of 246.0 μg and an RSD of 36.5% (n=5). In the GBS analyzed by Wada et al., 100 μg of free MPN was found per gram of dried material, whereas the mean amount detected in the present study was about twice that amount. By region, the mean MPN content in GBS collected in Osaka was significantly (p=0.05) greater than that in other regions of Japan, and thus there may be differences in MPN content depending on the provenance. Scott et al. 17) reported that, apart from free MPN itself, MPN glucosides that are hydrolyzable by β-glucosidase are also present. We are now intending to measure not only free-form MPN, but also total MPN content depending on the provenance.

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**Table 3. Free MPN Contents of GBS Collected in Various Regions of Japan**

<table>
<thead>
<tr>
<th>Region of collection</th>
<th>Dry weight per seed (Mean±S.D. (g))</th>
<th>MPN content per seed (Mean±S.D. (μg))</th>
<th>MPN content/dry weight (Mean±S.D. (μg/g))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RSD (%)</td>
<td>RSD (%)</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>Osaka</td>
<td>0.95±0.04</td>
<td>382.6±67.7</td>
<td>404.2±80.3</td>
</tr>
<tr>
<td>Fukuoka</td>
<td>0.77±0.04</td>
<td>242.0±24.7</td>
<td>221.9±43.1</td>
</tr>
<tr>
<td>Tokyo</td>
<td>0.80±0.15</td>
<td>211.9±43.1</td>
<td>168.3±48.4</td>
</tr>
<tr>
<td>Aomori</td>
<td>0.79±0.13</td>
<td>218.6±84.3</td>
<td>134.4±40.5</td>
</tr>
<tr>
<td>Nagano</td>
<td>1.01±0.06</td>
<td>214.9±43.1</td>
<td>106.5±19.8</td>
</tr>
<tr>
<td>Niigata</td>
<td>0.65±0.10</td>
<td>218.6±84.3</td>
<td>149.8±76.3</td>
</tr>
<tr>
<td>Okayama</td>
<td>0.61±0.05</td>
<td>218.6±84.3</td>
<td>127.4±80.3</td>
</tr>
<tr>
<td>Asahikawa</td>
<td>0.87±0.08</td>
<td>218.6±84.3</td>
<td>127.8±86.8</td>
</tr>
</tbody>
</table>

Ten GBS collected and sold in the markets in each region were used for analysis. Examination of the differences of the mean MPN contents per gram of dry weight of GBS was carried out using multiple comparison ANOVA, and then ANOVA was performed with Scheffe’s procedure. The level of significance was 5%.

