Gas Chromatography–Mass Spectrometry Analysis of 4-O-Methylpyridoxine (MPN) in the Serum of Patients with Ginkgo Seed Poisoning

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Abstract

The 4-O-methylpyridoxine (MPN) present in the seeds of the Ginkgo biloba (maidenhair tree) has anti-vitamin B6 actions, and ginkgo seed poisoning can induce convulsions. We developed a specific quantitative method using gas chromatography–mass spectrometry for the analysis of MPN in human serum. The trifluoroacyl (TFA) derivative of MPN was obtained by treating MPN with trifluoroacetic anhydride at 50°C for 5 min and remained stable for 6 h. The calibration curve of standard MPN obtained in the selective ion mode using the base ion (m/z 343) was linear between 100 pg and 10 ng, and the detection limit was 50 pg. The full mass spectrum of 100 pg of the TFA derivative of MPN was obtained easily. MPN was extracted from the serum with the use of a C18 solid-phase extraction cartridge. The recovery rate of MPN added to the serum at a concentration of 0.1 pg/ml was 90.0%.

Introduction

Ginkgo seeds have been consumed in Japan and China as both medicine and food since ancient times. Ginkgo seed poisoning was recorded by China's Li Shizhen in 1596 in "Bencao Wangmu" and by Ikiken Kaibara in 1708 in "Yamato Honso" (1). Cases of poisoning due to the consumption of ginkgo seeds were frequent in the period after World War II, when there was a shortage of food, and some reportedly resulted in death (2). Since then, with the improvement in the availability of food in Japan, ginkgo seed poisoning has decreased, but it has not yet been eliminated, especially in children (1). In countries other than China and Japan, however, ginkgo seed poisoning is frequent, and few reports on it have been published. The principal symptoms of poisoning due to ginkgo seeds are vomiting and clonic or tonic convulsions, or both, which can at first sight easily be misdiagnosed as having epilepsy. The convulsions typically occur 1–12 h after ingestion of the ginkgo seeds, and the attacks occur repeatedly in many cases (3). For a long time, the agent that was responsible for the intoxication could not be identified, but was revealed by the work of Wada et al. (4) to be the antivitamin B6 4-O-methylpyridoxine (MPN). It is supposed that its mechanism of action is through inhibition of the production of γ-aminobutyric acid (GABA), which is an inhibitory neurotransmitter in the brain (1). Currently, the only method of assaying MPN is by high-performance liquid chromatography (HPLC) with fluorescence detection (5). However, in cases in which the information on ginkgo seed ingestion is unclear, there has been an awareness of the need to develop a method of mass spectrometry offering a high degree of qualitative analysis in order to demonstrate the presence of ginkgo seed poisoning.

Until now, the methods of derivatizing vitamin B6 compounds, which have a similar structure to MPN, using gas chromatography–mass spectrometry (GC–MS) analysis were acyl derivatization with acetic anhydride and pyridine, as reported by Hachey et al. (6), tert-butyldimethylsilyl derivatization using N-methyl-N-(tert-butyldimethylsilyl) trifluoroaceticamide and pyridine, and trimethylsilyl (TMS) derivatization using N(O-bis(trimethylsilyl) trifluoroacetamide (BSTFA), trimethylchlorosilane (TMCS), and pyridine together, both of which were reported by Leyland et al. (7).

In the present study, we employed a C18 cartridge to extract MPN from human serum; then it was rapidly converted to the trifluoroacetyl (TFA) derivative using trifluoroacetic anhydride (TFAA), and carried out analysis by GC–MS.

Materials and Methods

Chemicals and reagents

MPN was synthesized by the method described by Harris (8). The purity of the synthesized MPN was checked by HPLC method (5), and was 99%.
The vitamin B₆ compounds were purchased: pyridoxine from Sigma Chemical Co. (St. Louis, MO); pyridoxal hydrochloride, pyridoxamine dihydrochloride hydrate, and 4-pyridoxic acid from Aldrich Chemical Co. (Milwaukee, WI); and pyridoxal-5-phosphate monohydrate from ICN Biomedicals Inc. (Aurora, OH).

TFAA was obtained from Tokyo Kasei (Tokyo, Japan). The other solvents used were special-grade reagents obtained from Wako (Osaka, Japan). For the recovery tests, a reference serum containing no MPN (lot no. 49H8929, Sigma Chemical) was used.

**GC–MS**

A Shimadzu GC17A GC and QP-5050A MS with a Shimadzu Class5000 computer system were employed. Chromatographic conditions for these analyses were as follows: a HP-5MS fused-silica capillary column (15 m x 0.25-mm i.d., 0.25-μm film thickness, J&W Scientific, Folsom, CA); helium carrier gas at 2 mL/min; GC oven temperature program, 20°C/min from 80°C, held for 2 min at 300°C; injector temperature, 250°C; split/splitless injector, splitless mode for 2 min; and injection volume, 1 μL. The MS was operated in the EI or in the CI mode using methane as reagent gas at a pressure that has a 2:1 ratio for m/z 29 to m/z 17. The scan range of both the EI mode and the CI mode was from 50 to 450 u at 1 s/scan. The manifold temperature was 250°C.

**Extraction of MPN from serum**

A 500-mg Isolute® C18 cartridge (International Solvent Technology, Ltd., Mid Glamorgan, U.K.) was conditioned by washing with 3 mL of methanol followed by 3 mL of distilled water at a flow rate of 1 mL/min.

To 1 mL of serum, 0.2 mol/L tartaric acid solution and distilled water were added to give a total volume of 6 mL at a final pH of 5, and the resulting solution was introduced into the cartridge. After washing with 3 mL of distilled water and then 3 mL of diethyl ether, MPN was eluted with 6 mL of methanol. The solvent was evaporated to dryness under reduced pressure at 40°C in a vial.

**Derivatization**

For the TFA derivatization of MPN, 100 μL of TFAA was added to the reagent that had been evaporated to dryness in the vial. The vial was then tightly stoppered, and heated at 50°C for 5 min. After the vial had cooled to room temperature, the solvent was removed carefully under a stream of nitrogen, 100 μL of ethyl acetate was added to the residue, and 1 μL was used for GC–MS.

**Recovery of MPN in serum**

Ten microliters of solution containing 0.1 or 0.01 μg of MPN was added to 1 mL of standard serum (containing no MPN). Extraction was performed as described, and the recovery was calculated. The procedure was repeated five times for each sample.

**Case History**

A 17-month-old Japanese girl, weighing 9.8 kg, consumed 10 pan-fried ginkgo seeds. Until then, she developed normally, and had experienced no seizures. Three hours later, emesis occurred, and this was followed after another 2 h by generalized tonic convulsions lasting for 10 min; pyrexia was not observed. The patient was admitted to our hospital 8 h after in-

![Figure 1](https://academic.oup.com/jat/article-fig/figure/1)

**Figure 1.** EI mass spectrum (top) and CI mass spectrum (bottom) of the TFA derivative of MPN.
gestion of the ginkgo seeds. After admission, she was diagnosed as having ginkgo seed intoxication. Pyridoxal phosphate was administered intravenously for treatment, after which the patient had no further recurrence of the convulsions, and her general physical condition also improved. She was discharged on the next day without any neurological sequelae. The serum that was subjected to analysis was drawn approximately 8 h after she had consumed the ginkgo seeds and was kept frozen at -20°C until it was analyzed.

Results

Conditions for derivatization

The reaction dynamics of the TFA derivatization of 1 μg of MPN using TFAA were examined at room temperature, 50°C and 100°C. The results showed that the TFA derivatization reaction of MPN proceeded to completion in 20 min at room temperature, and in 5 min at both 50°C and 100°C. The TFA-derivatized MPN gave a single, isolated peak in TIC chromatograms. The coefficient of variation (CV) of the area of the TIC peaks when 1 μg of MPN was derivatized five times and subjected to GC–MS analysis was 2.0%, and the reproducibility of the derivatization was good. Moreover, TFA-derivatized MPN is stable for 6 h at room temperature. Next, when the five substances in the vitamin B₆ group, pyridoxine, pyridoxal hydrochloride, pyridoxamine dihydrochloride hydrate, 4-pyridoxic acid, and pyridoxal-5-phosphate monohydrate, which are present in the body and which resemble MPN in chemical structure, were subjected to TFA derivatization under the same conditions, four of them—all except pyridoxal-5-phosphate monohydrate—gave peaks on chromatograms.

Mass spectra

The mass spectra of the TFA derivatization of MPN in El mode and CI mode are presented in Figure 1. In the El mode, what are considered the molecular ions or the quasi-molecular ions of the TFA derivative of MPN, that is, m/z 375 (M) and m/z 374 (M-1) were not detected. However, in the CI mode, m/z 376 (M+H), considered to be the quasi-molecular ion, was detected, and m/z 404 was also identified as the quasi-molecular ion [M+C₂H₅]. Consequently, it was considered that, in the TFA derivative of MPN, the two hydroxyl radicals are TFA-derivatized (Figure 2). Also, in the El mode, the base ion m/z 343 was identified as [M-32(OCH₃+H)]; the fragment ion 274, as [343-69(CF₃)]; the fragment ion 261, as [M-113(OCOCF₃)-H]; and the fragment ion 246, as [343-97(COCF₃)].

GC–MS conditions

Our method of GC–MS, which used an HP-5 capillary column, clearly separated the TFA derivatives of MPN and the vitamin B₆ group substances (Figure 3). The identity of the MPN in the mass chromatogram was confirmed by visual or
computerized comparison of the peak underlying mass spectra with reference spectra recorded this study. Quantitative determination was performed in the selective ion mode using m/z 343, that is, the base ion at [M-32].

The m/z 343 base ion was not affected by the fragment ions of the vitamin B₆ group that were TFA-derivatized together with MPN.

The calibration curve for standard MPN analyses was linear over the range 100 pg-10 ng (y = 481975x - 136699, r = 0.999) by the absolute calibration method using analysis software (Class 5000, Shimadzu, Kyoto, Japan). The detection limit of MPN at a signal-to-noise ratio of 5 was 50 pg. The full mass spectrum of 100 pg MPN was easily detected.

The reproducibility of the present method was assessed by analyzing 1 ng of standard MPN five times per day for five consecutive days. The average coefficients of variation of within-day and between-day assays were 2.0 and 2.4%, respectively.

### Table I. Rate of Recovery of MPN from Serum

<table>
<thead>
<tr>
<th>Added* (ng/mL)</th>
<th>Recovery (%)</th>
<th>CV (%)</th>
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<tbody>
<tr>
<td>100</td>
<td>90.04 ± 8.86</td>
<td>9.84</td>
</tr>
<tr>
<td>10</td>
<td>86.08 ± 14.67</td>
<td>17.04</td>
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</table>

* Amounts are expressed as nanograms per milliliter of serum.

1 Values are mean ± standard deviation, n = 5.

Extraction conditions and recovery

The mean recovery rates from an Isolute C18 cartridge of MPN added to human serum are shown in Table I.

The effects of pH on absorption and desorption on an Isolute C18 cartridge were investigated. When the recovery rates of MPN at 0.1 μg per 1 mL serum were examined at pH 3.0, 4.0, 5.0, 6.0, and 7.0, it was found that the recovery rates in all conditions at pH 3.0 and 7.0 were stable at least 90.04% (CV = 9.84% or less), and no effect attributable to the pH of the sample was seen on the adsorption and desorption of MPN onto the Isolute C18 cartridge. Therefore, we used a method that adjusted the serum samples to a pH of 5.0. The minimum amount of methanol necessary to elute the MPN from the Isolute C18 was 5 mL.

Analysis of TFA-derivatized MPN of patient with ginkgo seed poisoning

Figure 4 shows the chromatogram and mass spectrum of the TFA derivative extracted from the serum of a patient with ginkgo seed poisoning about 8 h after the ingestion of ginkgo.
seeds. The MPN-TFA peak was clearly identified in the m/z 343 base ion chromatogram (Figure 4B), and its detection using the full mass spectrum was easy. Using the m/z 343 ion, quantitation was performed. The serum concentration of MPN was 0.10 μg/mL.

Discussion

MPN, which was synthesized as an analogue of vitamin B₆, has been clearly shown to have a potent antivitamin B₆ action in both animals and humans. As a result of the report of Wada et al. (4), it is now also recognized as being the substance to which ginkgo seed poisoning can be attributed. In the past, the only method developed for the analysis of MPN was fluorescence HPLC (5). However, qualitatively, this method is inadequate for identifying MPN. Accordingly, in the clinical sphere, especially for cases in which the patients do not know whether they have consumed any ginkgo seeds, there has been a demand for the establishment of a specific method of mass spectral analysis.

In order to analyze MPN by GC, we performed a number of studies on its derivatization. As a result of using six derivatization reagents or reagent combinations—TFAA alone, TFAA + pyridine, BSTFA alone, BSTFA + pyridine, BSTFA + TMCS (99:1), and BSA + TMCS + TMSI (3:2:3)—in separate experiments to derivatize MPN with TFA or TMS, we ascertained that TFAA alone and BSTFA alone yielded single-peak MPN derivatives in TIC chromatograms. The El mass spectra and structure of the TMS-derivatized MPN are shown in Figure 5.

Although BSTFA alone required a reaction time of 60 min at 50°C, it was possible with TFAA to achieve derivatization in the much shorter time of 5 min at the same temperature. In addition, the reaction time with TFAA alone at room temperature was only 20 min. Moreover, the advantages of TFA derivatization over TMS derivatization when BSTFA was used were that, after the derivatization reaction, unreacted TFAA could be simply removed with a stream of nitrogen and little contamination of the detector due to the injection of excess unreacted reagent occurred. In the TFA derivative of MPN, TFA has replaced the two hydroxyl groups of MPN. On analysis in the El mode, the m/z 343 base ion could be easily monitored without any obstruction by the vitamin B₆ group compounds TFA-derivatized simultaneously with the MPN, specifically pyridoxine, pyridoxal hydrochloride, pyridoxamine dihydrochloride hydrate, 4-pyridoxic acid, and pyridoxal-5-phosphate monohydrate.

The results of this method are satisfactory: the detection limit of the TFA derivative of MPN is 50 pg, and the detection of 100 pg of that derivative with a full mass spectrum is easy. Also, in relation to extraction using a C₁₅ solid-phase extraction cartridge, the mean recoveries of MPN added to blood serum (n = 5) was 86.1% with a CV(%) of 17.0 when the serum MPN concentration was 0.01 μg/mL and 90.0% with a CV(%) of 9.8 with a serum MPN level of 0.1 μg/mL. Thus, quantitatively also, these results are good.

Many reports state that the number of ginkgo seeds that causes poisoning in children varies from 7 to 150, in adults, from 40 to 300 (3). In experiments using rabbits, the blood levels of MPN required to induce a convulsive state were considered to be at least 1 μg/mL (5). However, in cases of ginkgo seed poisoning in humans, on ingestion of about 50 ginkgo seeds by a 21-month-old boy, in whom tonic convulsions arose 2 1/2 h later, the level of MPN found in the serum 8.5 h after ingestion was 0.09 pg/mL (5), and in a boy of 2 years 1 month who had ingested more than 50 ginkgo seeds and suffered convulsions 4 h 40 min later, no MPN was detected in the serum 19 h after ingestion (9).

When patients are brought to hospital in such cases, the blood levels are less than 1 pg/mL. In the case we analyzed, the MPN concentration in the blood 8 h after ingestion was low: 0.10 μg/mL. GC–MS is also an adequate technique for handling cases such as those requiring microanalysis, and is a useful analytical method for use in clinical practice.

Ginkgo seed poisoning in children is common, and many cases of ginkgo seed ingestion are clarified by the statements of the mother. However, there is a report of an adult man being discovered by his family with a depressed level of consciousness and in general convulsions after he had eaten ginkgo seeds (10). The capacity for full mass spectral identification offered by our method makes it a highly suitable method.
of analysis for the diagnosis of patients who are not aware that they have consumed ginkgo seeds.

References


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