Simultaneous Analysis of Ibotenic Acid and Muscimol in Toxic Mushroom, *Amanita muscaria*, and Analytical Survey on Edible Mushrooms

(Food Hygienic Studies of Toxigenic Basidiomycotina. I)

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Koujun TSUNODA^{*1}, Noriko INOUE^{*1}, Yasuo AOYAGI^{*2}
and Tatsuyuki SUGAHARA^{*3}

(*¹Suginami City Institute of Public Health Research, Tokyo: 3-20-3, Takaidohigashi, Suginami-ku, Tokyo 168, Japan; *²Kagawa Nutrition Junior College, 3-24-3, Komagome, Toshima-ku, Tokyo 170, Japan; *³Kagawa Nutrition College, 3-9-21, Chiyoda, Sakado-City, Saitama 350-02, Japan)

A convenient analytical method for ibotenic acid (IBO) and muscimol (MUS) in a toxic mushroom, *Amanita muscaria* (*A. muscaria*), was developed. IBO and MUS in the mushroom were extracted with 70% methanol. After filtration, IBO and MUS in the extract were determined by high performance liquid chromatography (HPLC) with a UV detector set at 210 nm. The HPLC system adopted was ion-pair chromatography in the reverse-phase mode on an IRICA RP-18 (C₁₈) column (4.0 mm×25 cm) with sodium dodecyl sulfate as a counter ion. Recoveries of IBO and MUS added to the sample were more than 98%, and the minimum detectable concentration of IBO or MUS was about 1 ppm. The concentrations of IBO and MUS in *A. muscaria* ranged from 258 to 471 ppm and from 18 to 27 ppm, respectively. Neither of the compounds was detected in commercial edible mushrooms.

Key words: ibotenic acid; muscimol; toxic mushroom; *Amanita muscaria*; ion-pair liquid chromatography; spectroscopic analysis; NMR

Introduction

Uncultivated (wild) vegetables and mushrooms are extremely popular as foodstuffs in Japan. However, food poisoning by wild mushrooms including *A. muscaria*^{1)~3)} occurs every year⁴⁾. *A. muscaria* contains α -amino-3-hydroxy-5-isoxazoleacetic acid, (ibotenic acid, IBO) and its decarboxylated derivative, 5-aminomethyl-3-hydroxyisoxazole (muscimol, MUS), which are hallucinogenic^{5)~7)}. As a first step to prevent food poisoning caused by the mushroom, *A. muscaria*, it is necessary to develop a convenient analytical method for IBO and MUS. The analytical methods so far reported are time-

consuming^{8)~10)}, and IBO may decompose to MUS during the analysis. We have developed an analytical method for IBO and MUS and used it to evaluate the levels of IBO and MUS in *A. muscaria* and to follow the conversion of IBO to MUS during storage under various conditions.

Experimental

Materials

MUS was purchased from Sigma Chemical Co. Stock solutions of IBO and MUS (50 ppm) were prepared by dissolving IBO and MUS in 70% methanol, and standard solutions for quantitative analysis were made by stepwise dilution of the stock solutions. All other materials, unless

otherwise specified, were of reagent grade or the highest available grade from usual commercial sources.

Samples

1. The fruit bodies of *A. muscaria* were collected in Nagano Prefecture during summer 1987. The fresh fruit body was divided into two groups, which were kept in cool boxes, one (frozen sample) at -20°C and the other (fresh sample) at 4°C . A part of the frozen sample was freeze-dried (freeze-dried sample).

2. Commercial mushrooms (edible mushrooms) were purchased at supermarkets in Tokyo.

Preparation of IBO

Authentic IBO was prepared by extraction from the frozen sample. Purification and identification were conducted as follows; the sample (15 kg) was homogenized and extracted with about 70% methanol, and the solution was filtered through a filter paper No. 5A. The filtrate (about 45 L) was concentrated to about one-twentieth of the initial volume with a flash evaporator, and subjected to chromatography on column-I (Amberlite IR-120, acidic form, 5 cm $\times$ 90 cm, Rohm and Haas Co., U.S.A.) after defatting with ether (5 L $\times$ 2). IBO was eluted with 2 *N* ammonia (10 L) after the column-I had been washed with water (15 L). The eluate was concentrated to dryness under reduced pressure at 45°C , and the residue was dissolved in a small portion of water. The solution was placed on column-II (Dowex 1 $\times$ 4, acetate form, Dow Chemical Co., U.S.A.), and eluted with 2 *N* acetic acid (AcOH, 5 L). The eluate was concentrated, the residue was dissolved in water, and the solution was adjusted to pH 2.2 with hydrochloric acid. The solution was placed on column-III (Dowex 50 w $\times$ 4, pyridine form, 5 cm $\times$ 40 cm), which was fractionated by linear gradient elution between pyridine-AcOH buffers 0.2 *M* (pH 3.1, 2 L) and 2.0 *M* (pH 5.0, 2 L). The eluate fractions which were ninhydrin-positive were concentrated, and the residue was dissolved in water. The solution was placed on column-II again, and elution was done with a linear gradient prepared from AcOH solutions of 0.1 *N* (1.5 L) and 2.0 *N* (1.5 L). The eluate containing IBO

was concentrated, and the residue was dissolved in water. The solution was applied to column-III again, and eluted with 0.2 *M* pyridine-AcOH buffer (pH 3.1, 2 L). The eluate containing IBO was concentrated, and the residue was dissolved in water. The solution was applied to column-II, and eluted with 1 *N* AcOH (5 L). The eluate containing IBO was concentrated to a small volume. IBO was crystallized by adding ethanol to the solution, purified by recrystallization from aqueous ethanol, and dried in vacuo at 45°C . Its purity was confirmed by thin layer chromatography, spectrophotometry and high performance liquid chromatography.

Thin layer chromatography (TLC)

Analytical TLC was conducted on silica gel plates (Kieselgel 60 F254, Merck) with the following solvent systems: 1, *n*-propanol-10% ammonium water (95 : 5, v/v); 2, *n*-butanol-AcOH-water (60 : 20 : 20, v/v). After the development, spots were detected by spraying ninhydrin solu-

Fig. 1. Absorption spectra of ibotenic acid and muscimol in 25% methanol
A: muscimol (50 ppm); B: ibotenic acid (50 ppm)

Fig. 2. Chromatograms of ibotenic acid and muscimol in *A. muscaria* extracts, and authentic standards (12.5 ng)
IBO: ibotenic acid; MUS: muscimol

Table 1. Recoveries of Ibotenic Acid and Muscimol Added to *A. Muscaria*

	Added ($\mu\text{g/g}$)	Found ($\mu\text{g/g}$)					Mean ($\mu\text{g/g}$)	Recovery (%)	M. D.
		No. 1	2	3	4	5			
Ibotenic acid	0	187.2	185.1	187.6	186.4	184.9	186.2	98.7	0.8
	40	226.7	224.9	226.8	225.5	225.0	225.7		
Muscimol	0	10.7	10.7	11.0	10.8	10.7	10.7	98.5	0.7
	40	50.4	49.6	50.1	50.3	50.4	50.1		

M. D.: Mean deviation

Table 2. Changes of Concentration of IBO and MUS during Storage

Periods		First day (ppm M. D.)		Second week (ppm M. D.)		First month (ppm M. D.)		Second month (ppm M. D.)		Third month (ppm M. D.)	
Experiment-1 at -20°C	IBO	379	9.3	382	7	388	11	376	17	38.2	1.1
	MUS	21	1	20	3	20	1	20	1	2.0	0.1
Experiment-2 at cool and dark	IBO	418	2	414	3	409	7	406	8	41.5	0.5
	MUS	22	1	21	1	22	2	23	1	2.0	0.1
Experiment-3 at 4°C in MeOH	IBO	326	0	324	2	327	9	310	9	29.9	1.3
	MUS	17	0	18	1	16	1	18	1	3.0	0.1

Values represent the data from three samples.

M. D.: Mean deviation; IBO: ibotenic acid; MUS: muscimol

tion.

High performance liquid chromatography (HPLC)

Analytical HPLC was performed with the isocratic solvent system described below, using an IRICA RP-18T (C₁₈) (4.0 mm i. d. × 250 mm) column at a flow rate of 0.6 ml/min at 45°C. The solvent system used for isocratic elution consisted of degassed water-acetonitrile-methanol mixture (65 : 20 : 15) containing sodium dodecyl sulfate (SDS, 2.1 mM) and phosphoric acid (4 mM) (pH 2.2).

Since the absorption maxima of IBO and MUS in 25% methanol are 211 nm and 207 nm, respectively (Fig. 1), the HPLC eluates were monitored with a Tosoh Co. CCPE UV8010 UV detector set at 210 nm. Analytical spectrophotometry (350~195 nm) of IBO and MUS was conducted with the same system.

Extraction and analysis

The freeze-dried sample (2.5 g) was homogenized (Nippon Seiki Co AM-7 homogenizer) in 70 ml of 75% methanol, which was made up to 100 ml with 75% methanol after being filtered through a filter paper (Toyo Roshi No. 5A). This solution was further filtered through a membrane filter (Toyo Roshi DISMIC-25, 0.45 μm), and a 5 μl aliquot was subjected to analysis by HPLC. In the case of the fresh sample, 25 g was homogenized in 50 ml of methanol, and the homogenate was filtered and made up to 100 ml with methanol. Subsequent procedures were the same as described above.

Recovery experiments

The following recovery experiments were performed five times. The frozen sample (100 g) was homogenized, and the concentrations of IBO and MUS in the homogenate were determined by this method. Then 400 μg each of IBO and MUS were blended in the homogenate (10 g), and the concentrations were again determined. The recoveries of added IBO and MUS were calculated by subtraction of the known concentration in the frozen sample.

Storage experiments

The following three experiments were performed to examine the changes of concentration

Table 3. Contents of Ibotenic Acid and Muscimol in *A. muscaria* and Commercial Edible Mushrooms

Sample	IBO (ppm)	MUS (ppm)
<i>Amanita muscaria</i>		
1) Benitengutake	471	27
2) "	339	18
3) "	417	27
4) "	338	25
5) "	258	18
<i>Lentinus edodes</i>		
1) Shiitake	ND	ND
<i>Tricholoma matsutake</i>		
1) Matsutake	ND	ND
<i>Pleurotus ostreatus</i>		
1) Hiratake	ND	ND
2) "	ND	ND
3) "	ND	ND
<i>Lyophyllum ulmarium</i>		
1) Bunashimeji	ND	ND
<i>Grifola frondosa</i>		
1) Maitake	ND	ND
<i>Kuehneromyces nameko</i>		
1) Nameko	ND	ND
2) "	ND	ND
<i>Flammulina velutipes</i>		
1) Enokitake	ND	ND
2) "	ND	ND
<i>Agaricus bisporus</i>		
1) Mushroom	ND	ND
2) "	ND	ND
<i>Auricularia auricula-Judal</i>		
1) Kikurage	ND	ND

IBO: ibotenic acid; MUS: muscimol; ND: not detected

Other names of the mushrooms

<i>Pleurotus ostreatus</i>	: 1) Shimeji, 2) Shirosimeji, 3) Oo-shimeji
<i>Lyophyllum ulmarium</i>	: 1) Hon-shimeji
<i>Kuehneromyces nameko</i>	: 2) Yama-nameko
<i>Flammulina velutipes</i>	: 2) Tora-enokitake
<i>Agaricus bisporus</i>	: 2) Brown mushroom

of IBO and MUC in the mushroom during storage.

1. The sample was divided into three groups for a triplicate experiment (each group has six portions of 10 g apiece). Fresh samples (10 g) were kept at -20°C after being chopped into small pieces.

2. Fresh samples (10 g) were kept in metha-

Fig. 3. ¹H-NMR spectrum of α-amino-3-hydroxy-5-isoxazoleacetic acid (ibotenic acid) in deuterium oxide

a, b: proton (H); d: dioxane (internal standard); e: H₂O (moisture)

Fig. 4. ¹³C-NMR spectrum of α-amino-3-hydroxy-5-isoxazoleacetic acid (ibotenic acid) in deuterium oxide

3, 4, 5, 6, 7: carbon (C); d: dioxane (internal standard)

nol (40 ml) at 4°C after homogenization.

3. Freeze-dried samples (1 g) were kept in a desiccator at room temperature in the dark after pulverization.

Results and Discussion

1. Extraction

Since IBO is said to be unstable under usual conditions, being decarboxylated to MUS, care is necessary during analysis. Decarboxylation of IBO is catalyzed by the enzyme decarboxylase to yield MUS. We selected 75% methanol as the solvent for extraction, since it deactivates

decarboxylase in mushroom, and eliminates oily substances.

2. HPLC

In order to select a suitable column for good base-line separation by HPLC, we examined alumina, silica gel and so on. Among them, the C₁₈ column seemed to be most suitable for our purpose, with the solvent system described above (Fig. 2).

3. Recovery test

The average recovery of IBO and MUS from

A. muscaria spiked at the level of 40 ppm was 98% with 0.8 and 0.7 mean deviation, respectively, the detection limit being 1 ppm.

4. Changes of concentration of IBO and MUS during storing

As shown in Table 1, decomposition of IBO to MUS did not occur during storage for 3 months under the conditions described above, suggesting enzymatic activity in mushroom is the main cause of the decomposition.

5. IBO and MUS in mushroom obtained from supermarkets

We analyzed mushrooms purchased from super markets and detected no IBO or MUS (Table 2). In *A. muscaria* collected in Nagano prefecture, however, we detected IBO at levels of 258 ppm to 471 ppm and MUS at levels of 18 ppm to 27 ppm.

6. Spectroscopic analysis¹¹⁾

IBO in *A. muscaria* was extracted with methanol, purified by ion exchange column chromatography and crystallized. Its identity was confirmed by TLC, HPLC (UV detection) and ¹H-NMR and ¹³C-NMR spectroscopy (JEOL FX270 spectrometer).

1) ¹H-NMR: The ¹H-NMR chemical shifts of two methine protons of 4-isoxazole in dioxane were δ 6.01 ppm and δ 4.94 ppm (Fig. 3), in agreement with reported values¹²⁾.

2) ¹³C-NMR: Five carbon signals attributable to C₃-hydroxyl (δ 185 ppm), C₄-methine (δ 115 ppm), C₅-isoxazole (δ 181 ppm), C₆- α -carbon (δ 82.9 ppm) and C₇-carboxyl (δ 192 ppm) were clearly detected (Fig. 4).

These data confirmed the molecular structure of IBO as α -amino-methyl-3-hydroxyisoxazoleacetic acid (monohydrate)⁵⁾.

Conclusion

A sensitive simultaneous determination of IBO and MUS in *A. muscaria* was developed, allowing analysis of IBO without decomposition^{5), 8) 10)}. IBO and MUS in the samples were stable at below 4°C in methanol, at below -20°C or at room temperature in a desiccator after

freeze-drying.

Methanol extraction of IBO and MUS provided a suitable sample solution for HPLC, which was performed in the reversed-phase mode on an ODS-column packed with end-capped silica gel using SDS as a counter ion. Baseline resolution of IBO and MUS was achieved with high sensitivity. Recovery was about 98% with a mean deviation below 0.8. The detection limit of IBO and MUS was about 1 ppm.

Fresh *A. muscaria* contained 258~471 ppm of IBO and 18~27 ppm of MUS. The concentration of MUS in the mushroom was lower than has been found by other investigators^{8), 10)}. IBO and MUS were not detected in commercial edible mushrooms.

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