資料10. Swiss mice等の毒性実験のまとめ

Parameter	Material or compound tested in the cancer study (reference)					
	Fresh raw mushroom (Toth and Ericson, 1986)	Dry-baked mushroom (Toth et al., 1997a)	Freeze-dried mushroom (Toth et al., 1998)	CPH (McManus et al., 1987)	GCPH (Toth, 1986a)	HMBD (Toth et al., 1982)
Daily exposure per mouse	4.7 g	5.57 g	1.25 g*	1250 mg/l drinking water for life	1400 mg/kg body weight /l day weekly for 52 weeks	Single dose 400 mg/kg body weight
Fraction of tumour-bearing animals with the highest increase of a specific tumour (type of tumour)	38% (forestomach in males)	20% (forestomach in females, glandular stomach in males)	22% (lung in males)	38% (aorta and large arteries in males)	26% (subcutaneous tissue in males)	32% (glandular stomach in males)

Table 18. Fraction of treated animals with the most pronounced increase of a specific tumour.

資料11. マッシュルーム及び関連毒性物質のリスク評価

Parameter	Material or compound tested in the cancer study (reference)					
	Fresh raw mushroom (Toth and Ericson, 1986)	Dry-baked mushroom (Toth et al., 1997a)	Freeze-dried mushroom (Toth et al., 1998)	CPH (McManus et al., 1987)	GCPH (Toth, 1986a)	HMBD (Toth et al., 1982)
Daily exposure per mouse	4.7 g	5.57 g (males)	1.25 g*	1250 mg/l drinking water for life	1400 mg/kg body weight /l day weekly for 52 weeks	Single dose 400 mg/kg body weight
Daily exposure per kg body weight (mouse)	189 g	229 g	50 g	204 g	149 g	0.82 mg
Daily human intake of mushroom per kg body weight	0.1 g (0.025 g)	0.1 g (0.025 g)	0.1 g (0.025 g)	0.1 g (0.025 g) (containing 10-11 mg CPH/kg)	0.1 g (0.025 g) (containing 16-42 mg GCPH/kg)	0.1 g (0.025 g) (containing 0.6-4 mg HMBD/kg)
Human cancer risk due to lifelong exposure	193×10^{-6} (52×10^{-6})	86×10^{-6} (23×10^{-6})	211×10^{-6} (56×10^{-6})	$1.8\text{-}2.0 \times 10^{-6}$ (0.5×10^{-6})	$2.7\text{-}7.0 \times 10^{-6}$ ($0.7\text{-}1.9 \times 10^{-6}$)	$23\text{-}150 \times 10^{-6}$ ($6\text{-}40 \times 10^{-6}$)

* 1.25 g freeze-dried mushroom approximately corresponds to 12.5 g raw mushroom

Table 19. Estimated life-time human cancer risk from the intake of Agaricus bisporus in the Nordic countries (average of intakes in Danmark, Iceland, Norway and Sweden). (Finnish figures withun brackets.)

研究成果の刊行に関する一覧表

雑　誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Nagaoka M.H, Akiyama H., Maitani T.	Binding patterns of vanadium to transferrin inhealthy human serum studied with HPLC/high resolution ICP-MS.	The Analyst	129	51-54	2004
Kondo K., Watanabe A., Iwanaga Y., Abe I., Tanaka H., Nagaoka M.H, Akiyama H., Maitani T.	Analysis of agaritine in mushrooms and in agaritine-administered mice using liquid chromatography-tandem mass spectrometry	*J.Chromatography B*	834	55-61	2006
Kondo K., Watanabe A., Iwanaga Y., Abe I. , Tanaka H., Nagaoka M.H., Akiyama H., Maitani T.	Determination of genotoxic phenylhydrazine agaritine in several mushrooms using liquid chromatography-electrospray ionization tandem mass spectrometry	*Food Additives and Contaminants Accepted*			
Akiyama H., Toida T., Sakai S., Amakura Y., Kondo K., Sugita-Konishi Y., Maitani T.	Determination of cyanide and thiocyanate in Sugihiratake mushroom using HPLC method with fluorometric detection.	*J. Health Science*	52	73-77	2006
Nagaoka M.H., Nagaoka H., Kondo K., Akiyama H., and Maitani T.	Measurement of a genotoxic hydrazine, agaritine, and its derivatives by HPLC with fluorescence derivatization in the Agaricus mushroom and its products	*Chemical and Pharmaceutical Bulletin* in press			

研究成果の刊行物・別刷り

ARTICLE

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Binding patterns of vanadium to transferrin in healthy human serum studied with HPLC/high resolution ICP-MS

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Vanadium (V) is an essential metal for mammals. It has different valence states. In blood, V is bound to transferrin (Tf), a glycoprotein that has two metal-binding sites (C-lobe site and N-lobe site). In the present study, the binding patterns of V to serum Tf were analyzed by combined on-line HPLC and high-resolution ICP-MS (HPLC/HR-ICP-MS). The levels of ^{51}V, ^{56}Fe and ^{32}S, which are interfered with polyatomic ions such as $^{35}Cl^{16}O^+$, $^{38}Ar^{13}C^+$ and $^{37}Cl^{14}N^+$, $^{40}Ar^{16}O^+$ and $^{40}Ca^{16}O^+$, and $^{16}O_2^+$, respectively, when using quadrupole ICP-MS, could be monitored simultaneously by HR-ICP-MS at a resolution of $m/\Delta m$ = 4000. Sample (a 1 ml portion of serum from a healthy person or 2 mg of human serum Tf (hTf)) was directly subjected to HPLC equipped with an anion-exchange column. V in human serum without any *in vitro* V spike was detected as V_C–Tf (V bound to C-lobe site of Tf) and $metal_2$–Tf. Since V(III) was most favorable in terms of the binding to hTf in the presence of bicarbonate and V bound to the C-lobe site of hTf was detected only in the case of V(III) among the three valence states of V, it was suggested that a part, at least, of V in the V_C–Tf in healthy human serum may be present as V(III), in addition to the generally accepted V(IV).

Introduction

Vanadium (V) is an essential metal for mammals,[1,2] although its role in humans has not been elucidated yet. Recently, it has been reported that V compounds lower the blood glucose level in rodent models of diabetes mellitus.[3–6] Therefore, much attention has been focused on the activity of V in the body.

Transferrin (Tf) is a glycoprotein of about 80 kDa with the characteristic property of requiring a synergistic anion.[7] Although its principal role in the blood is to transfer iron (Fe) to cells,[8] it can bind a large number of other metals. Vanadium is bound to Tf in the blood.[9,10] It has two homologous lobes for metal-binding (hereafter called the N-lobe site and the C-lobe site) connected by a single short peptide.[11] Although the amino acid sequence of the two lobes reveals extensive identity[12] and the ligands for each Fe-binding site consist of two tyrosines, one histidine, one aspartic acid and a bidentate anion such as carbonate,[1] the binding affinity of the two lobes is not identical.[13,14] The preferential site depends on the kinds of Tf, metal and synergistic anion.[15] For example, we reported the preferential binding of Fe and aluminium (Al) to the N-lobe site in the presence of bicarbonate as the synergistic anion.[16,17]

Vanadium ions are present in several valence states including V(III) (vanadic), V(IV) (vanadyl) and V(V) (vanadate). Since V(IV) and V(V) ions are present as oxovanadium ions (VO^{2+} and VO_2^+, respectively), they are bulky. Moreover, the oxo group(s) of oxovanadium ions may supply ligands supplied usually by synergistic anions.[18] The fact that V(V) binding to Tf does not require the presence of bicarbonate[19,20] may support this explanation, although the synergistic anion is required for the binding of V(IV) to Tf.[21,22] Thus, the preferential binding lobe and the binding affinity of V ion to Tf differ markedly depending on its valence state.

An on-line combination of HPLC and metal-detection spectrometry is widely used for the speciation of metals in biological samples. For detecting metals, ICP-MS has an extremely high sensitivity,[23] which is preferable for trace metals in biological samples. However, polyatomic-isotope interference from argon gas, atmospheric gas and biological materials can cause serious errors in the determination with common quadrupole ICP-MS instruments.[24] In the case of V in biological samples, $^{35}Cl^{16}O^+$ (*m/z* 50.964 u), $^{38}Ar^{13}C^+$ (*m/z* 50.966 u) and $^{37}Cl^{14}N^+$ (*m/z* 50.969 u) interfere with the detection of ^{51}V (*m/z* 50.944 u and the natural abundance is 99.75%). Moreover, in the measurement of Fe, which is essential for the study of Tf, the detection of ^{56}Fe (*m/z* 55.935 u and natural abundance is 91.72%) is affected by $^{40}Ar^{16}O^+$ (*m/z* 55.957 u), because an argon plasma is used in the ICP spectrometry. However, by using a double focusing high-resolution ICP-MS (HR-ICP-MS), the analyte signal is separated from the interference.[25]

In our previous study, the binding of V ions to two lobes of apo-human serum Tf (hTf) in the presence and absence of bicarbonate was studied in three different valence states (V(III), V(IV) and V(V)), using an on-line combination of HPLC and HR-ICP-MS (HPLC/HR-ICP-MS) with an anion-exchange column.[26] The three ions ranked V(III) > V(IV) > V(V) in affinity to hTf in the presence of bicarbonate.

In the present study, we studied the binding patterns of both V and Fe in healthy human serum. Since the V level was quite low, a 1 ml sample was required to obtain the V peak even with HR-ICP-MS. The established new analytical system and the new finding on the valence state of V in the serum are reported in the present study.

Experimental

Reagents

Apo-hTf (> 97%) was purchased from Sigma (St. Louis, MO) and used without further purification. Vanadium(III) chloride, ferric chloride hexahydrate, sodium citrate and sodium bicarbonate were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). The calibration solution for ICP-MS measurements was obtained from SPEX (Metuchen, NJ) as a multi-element standard stock solution (10 μg ml^{-1}). Nitric acid of ultra-pure analytical reagent grade (TAMAPURE-AA-10) was purchased from Tama Chemicals Co., Ltd. (Kawasaki, Japan). Other chemicals were of reagent grade or of the highest grade commercially available. All laboratory ware used was immersed in about 2 M HNO_3 for at least one night and rinsed with ultra-pure water (> 18 MΩ cm), which was prepared with a Milli-Q SP Reagent Water System (Millipore, Bedford, MA), to avoid contamination from various ions. The ultra-pure water was bubbled with nitrogen gas for 30 min to remove dissolved oxygen completely and used throughout the experiment.

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Sample preparation for HPLC/HR-ICP-MS

Blood collections from the healthy volunteers were carried out following the guidelines of the ethics regulations of our institute. Serum was obtained by centrifugation at 3000 *g*.

Apo-hTf was dissolved in 10 mM Tris–HCl (pH 7.4) containing 25 mM $NaHCO_3$ as the synergistic anion. Each solution was added to the apo-hTf solution (2 mg apo-hTf ml^{-1}) and the mixture was allowed to stand for the indicated times. After preparing each reagent solution, all tubes and bottles were sealed under a nitrogen gas atmosphere.

HPLC/HR-ICP-MS

An HPLC apparatus (LC-10Ai, Shimadzu, Kyoto, Japan) equipped with an anion-exchange column (TSKgel BioAssist Q analytical column (4.6 mm id × 50 mm, TOSOH, Tokyo, Japan) (column 1) or two TSKgel BioAssist Q preparative columns (20 mm id × 40 mm each, TOSOH, two columns were directly connected) (column 2) was connected directly with an HR-ICP-MS instrument (ELEMENT, Finnigan MAT, Bremen, Germany). A 100 μl sample (serum or 0.2 mg hTf) and a 1 ml sample (serum or 2 mg hTf) were applied to column 1 and column 2, respectively. Two solvents were used for gradient elution: solvent A (50 mM Tris–HCl (pH 7.4)) and B (A + 0.25 M ammonium acetate). HPLC conditions 1 and 2 for columns 1 and 2, respectively, are shown in Table 1. The eluate was transferred to a UV detector (at 280 nm, SPD-10AVi, Shimadzu) and then introduced into the Meinhard nebulizer of the HR-ICP-MS. Under HPLC condition 1, the levels of ^{32}S, ^{51}V and ^{56}Fe were monitored continuously in the medium resolution mode ($m/\Delta m$ = 4,000). Under HPLC condition 2, the levels of ^{34}S, ^{51}V and ^{54}Fe were monitored. After each analysis, the column was washed to eliminate the V ions adsorbed by the column according to the clean-up procedures reported previously.[26] The operating parameters for HR-ICP-MS were as follows: radio frequency power, 1.3 kW: argon gas flow rate, 15 l min^{-1} (outer), 0.85 l min^{-1} (intermediate), 0.93 l min^{-1} (carrier). Mass calibration was performed with a 1 ng ml^{-1} multi-element standard solution prepared from the standard stock solution, the ultra-pure nitric acid and ultra-pure water. The detection limit (S/N = 3) estimated for ^{51}V in the medium resolution mode was 10 pg ml^{-1}.

HR-ICP-MS data acquisition parameters are as follows: E-scan, mass window, 100%; search window, 60%; integration window, 60%; acquisition time, 2.22 s per each run.

Table 1 HPLC conditions

HPLC condition 1 for column 1		
Time/min	B (%)	Flow/ml min^{-1}
0–0.1	0–8	1
0.1–8	8	1
8–22	8–18	1
22–35	18	1
HPLC condition 2 for column 2		
Time/min	B (%)	Flow/ml min^{-1}
0–2	0	0.7
2–3	0	0.7–1
4–7	0–7	1
7–19	7	1
19–20	7	1–3
20–28	7	3
28–33	7–9	3
33–36	9–14	3
37–38	14	3–1
38–95	14	1

Results and discussion

Binding patterns of V in human serum (column 1)

Previously we could detect Al bound to Tf in serum from a healthy person with column 1 under HPLC condition 1. Fig. 1 (left panel) shows the HPLC/HR-ICP-MS chromatograms for serum from a healthy person with column 1 under HPLC condition 1. Though 100 μl serum was subjected to column 1, no V peak was detected, probably due to the low level of V in serum. To ascertain that V peaks can be detected if the amount of V is sufficient, V(III) was added to the serum. As shown in Fig. 1 (right panel), the major three V peaks were detected at 16, 19 and 26 min, which were ascribed to V_C–Tf, $metal_2$–Tf and V_N–Tf, respectively, in accordance with our previous report.[26]

However, the heights of V peaks in the serum sample were different from those in the hTf sample of our previous report. Namely, V peaks ranked $metal_2$–Tf > V_C–Tf > V_N–hTf for the serum sample in Fig. 1 (right panel) and ranked $metal_2$–hTf > V_N–hTf > V_C–hTf for the hTf sample. Therefore, it was considered that various compounds in serum such as citrate, oxalate, *etc.* may have affected the binding affinity of V to Tf in the serum sample. In fact, though citrate is a non-synergistic anion, it is present in serum at the concentration of 0.1 mM and forms chelates easily with V(III).

Establishment of HPLC condition 2 with column 2

To apply the 1 ml portion of the serum sample to the HPLC column and to separate all peaks of metal-binding Tf and apo-Tf, we adopted the preparative column and devised the gradient conditions as shown in Table 1. The major three Fe-binding hTfs and apo-hTf were detected at 53, 59, 67 and 79 min in Fe-supplemented apo-hTf and were assigned to Fe_C–hTf, $metal_2$–hTf, apo-hTf and Fe_N–hTf, respectively, based on the comparison with the chromatogram from column 1.

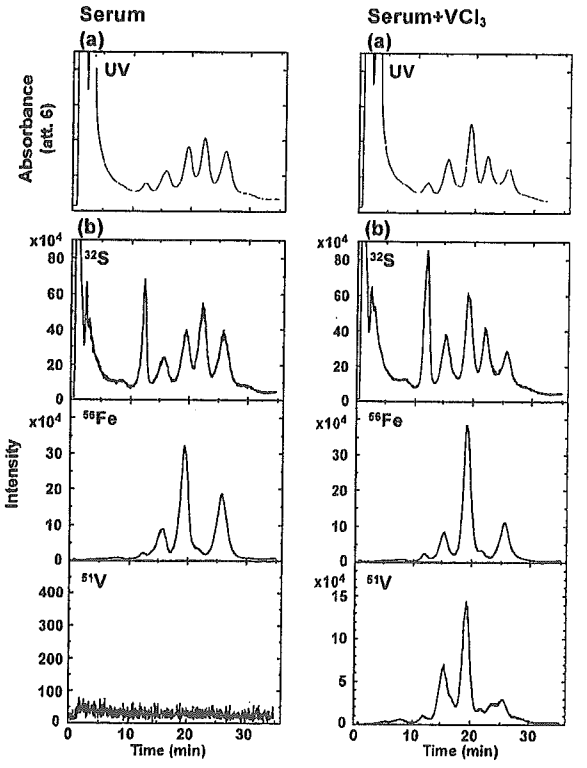

Fig. 1 HPLC-UV (280 nm, top) (a) and HPLC/HR-ICP-MS (^{32}S (top), ^{56}Fe (middle) and ^{51}V (bottom) levels) (b) chromatograms for serum from a healthy person before (left) and after (right) the addition of VCl_3. The respective sample solutions were sealed under a nitrogen gas atmosphere and allowed to stand for 2 h before analysis. Column 1 and HPLC condition 1 were used.

In our previous study,[17] beside the Fe_C–hTf peak at 16 min, a small Fe peak was observed at 10 min and it was ascribed to Fe bound to hTf with smaller numbers of sialic acid molecules, as sialidase-treated hTf eluted faster than non-treated hTf.[27] Therefore, in establishing HPLC condition 2, it was ensured that Fe_C–hTf could be distinguished from Fe bound to disialo- or trisialo-hTf. Thus, the HPLC condition 2 was prudently established to perfectly separate the peaks of V bound to native-hTf and that of V bound to disialo- or trisialo-hTf.

Since the 1 ml portion of the sample, which is ten times that used with column 1 in Fig. 1, was analyzed, the levels of ^{34}S (*m/z* 33.968, natural abundance 4.21%) and ^{54}Fe (*m/z* 53.940, natural abundance 5.80%) were monitored instead of ^{32}S (*m/z* 31.972, natural abundance 95.02%) and ^{56}Fe (*m/z* 55.935, natural abundance 91.72%), respectively. The eluate from 0 to 38 min was rich in ionic and organic compounds and so the flow rate under HPLC condition 2 was frequently changed. The HPLC apparatus was thus not combined with the HR-ICP-MS instrument until 38 min to avoid damage to the HR-ICP-MS instrument. Hence, the chromatograms started at 38 min in Fig. 2.

Binding patterns and total amount of V in serum

Fig. 3 represents the HPLC chromatograms obtained by UV absorption (280 nm) and HR-ICP-MS (^{34}S, ^{54}Fe and ^{51}V) for a 1 ml portion of serum from a healthy person. Since Milli-Q water was introduced to the HR-ICP-MS instrument until 38 min, V levels (about 10 cps) detected between 0 and 38 min were considered to be due to Milli-Q water or instrumental noise. In the V-level chromatogram, only one peak was apparently detected at the retention time of Fe_C–Tf (53 min) and was ascribed to V_C–Tf. Although not definite, very small peaks were also observed at the retention times of $metal_2$–Tf (59 min) and Fe_N–Tf (79 min).

The V concentration in the serum sample was measured with a 20 fold diluted sample. The V concentration in the serum was 140 pg ml^{-1}. The total area of V from 38 to 95 min corresponded to about 60% of the injected V content. The peak area of V_C–Tf peak occupied 42% of the total V area. Therefore, it was considered that most of the total V in the serum was detected by HPLC/HR-ICP-MS. The reported serum V concentrations were 50 pg ml^{-1} from electrothermal atomic absorption spectrometry,[28] and 31 pg ml^{-1} from radiochemical neutron activation analysis[29] and reported to be 10–760 pg ml^{-1} with HR-ICP-MS.[30] The serum V concentration in the present study (140 pg ml^{-1}) was comparable with the reported values.

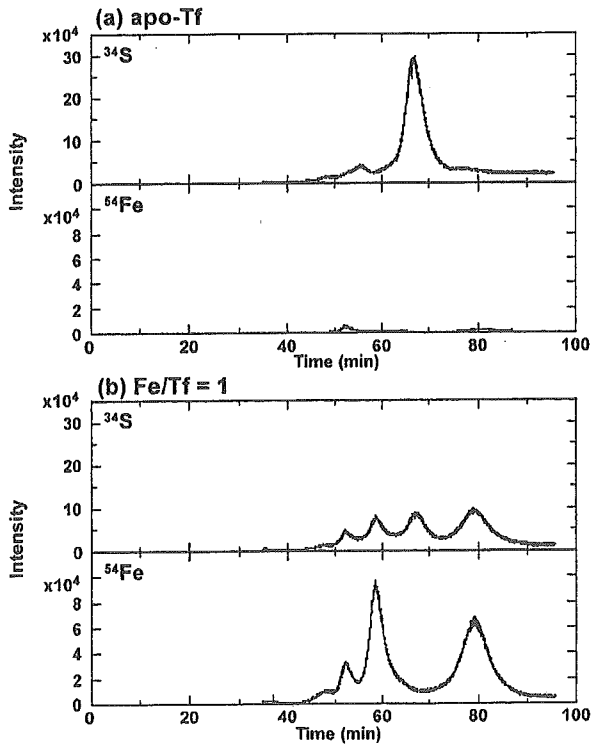

Fig. 2 HPLC/HR-ICP-MS (^{34}S and ^{54}Fe levels) chromatograms for apo-hTf solution (a) and apo-hTf solution supplemented with Fe-citrate (1:1) (Fe/hTf molar ratio = 1) (b) in the presence of bicarbonate. The mixed solution was allowed to stand for 24 h. Column 2 and HPLC condition 2 were used.

Binding patterns of V added as V(III) or V(IV) in serum from a healthy person and valence state of V in serum

To confirm the binding site of V bound to Tf *in vivo*, V was added as V(III) or V(IV) to serum from a healthy person. Fig. 4 depicts the HPLC/HR-ICP-MS (^{34}S, ^{54}Fe and ^{51}V) chromatograms for healthy human serum without any V spike (top) and supplemented with VCl_3 (middle) or $VOSO_4$ (bottom).

In Fig. 4 (top), two obvious V peaks were observed at the retention times of V_C–Tf (53 min) and $metal_2$–Tf (59 min) in the chromatogram for serum without any V spike. In the previous study, the N-lobe site was the preferential binding site of Fe in the presence of bicarbonate.[16] On the other hand, V(III) preferred both the C-lobe and N-lobe as affirmed by the Urea–PAGE.[26] Therefore, it seems plausible that V(III) binds to the C-lobe in serum and $metal_2$–Tf is V_C,Fe_N–Tf.

When the serum was supplemented with V(III) at the molar ratio (V/Tf) of 1, two major peaks were observed at the same retention times of Fe_C–Tf (53 min) and $metal_2$–Tf (59 min). On the other hand, V(IV) gave a peak at a slightly longer retention time (55 min) as well as the peak of N-lobe in an "open form"[26] at the retention time of 71 min. The observed slightly longer retention time (55

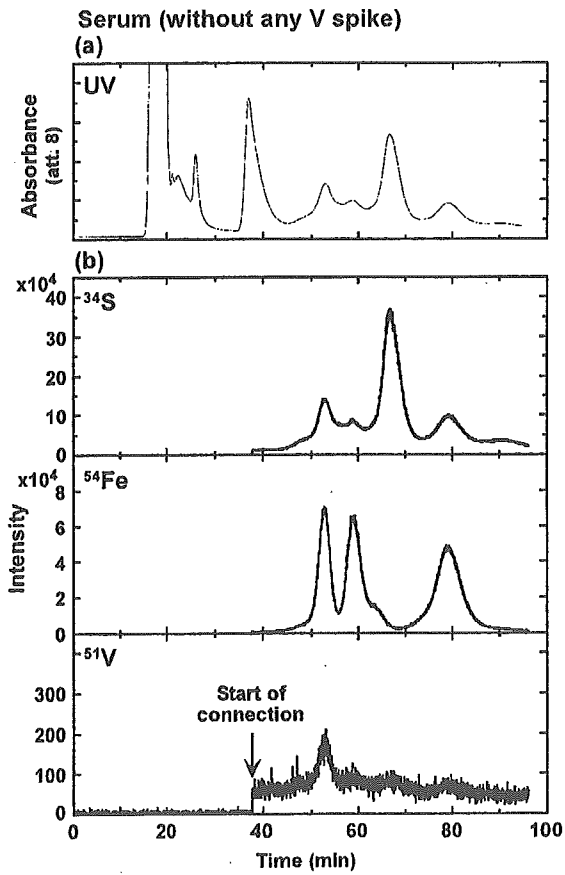

Fig. 3 HPLC-UV (280 nm, top) (a) and HPLC/HR-ICP-MS (^{32}S (top), ^{56}Fe (middle) and ^{51}V (bottom) levels) (b) chromatograms for serum from a healthy person without any V spike. Column 2 and HPLC condition 2 were used.

min) may suggest that when bound to the C-lobe site, bulky V(VI) (VO^{2+}) is bound to Tf in an "open form" as also observed in the previous study[26].

In the previous study,[26] V_C–hTf in the "closed form" corresponding to the peak at 53 min in this study could not be detected by the HPLC/HR-ICP-MS after the addition of V(IV) or V(V) ion to apo-hTf in the presence of bicarbonate. Moreover, a part of the V ions was removed from V–hTf during the column procedure.[26] It was also reported that V(V)–hTf binding was dissociated during the elution in the anion-exchange column procedure.[31] Therefore, the possibility that the V bound to the C-lobe site of Tf in the serum detected by the HPLC/HR-ICP-MS is V(IV) and/or V(V) seems low.

Consequently, it was considered that the V detected as V_C–Tf in the "closed form" was V(III). Thus, the interpretation that the V peak detected in the serum from a healthy person is of V(III) bound to the C-lobe site was confirmed by the standard addition experiment. This is the first report that demonstrates the V-binding lobe of Tf in healthy human serum.

Recently, it has been demonstrated that V compounds show insulin-like activity.[3–6] The effectiveness of V(IV) and V(V) compounds has been reported previously.[32] Since V(III) has a much higher affinity for hTf, the possibility that V ions are transferred in serum as V(III) may have to be studied further to clarify the action of V in the body.

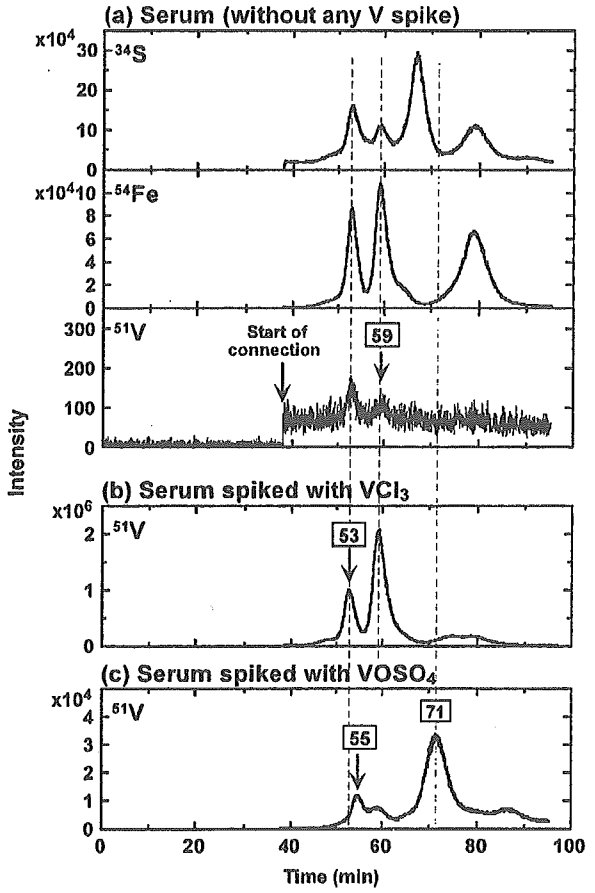

Fig. 4 HPLC/HR-ICP-MS (^{34}S, ^{54}Fe and ^{51}V levels) chromatograms for serum from a healthy person without any V spike (a), serum spiked with VCl_3 (b) or $VOSO_4$ (c) at 25 μM. The mixed solution was allowed to stand for 2 h. Column 2 and HPLC condition 2 were used.

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Analysis of agaritine in mushrooms and in agaritine-administered mice using liquid chromatography–tandem mass spectrometry

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Abstract

A sensitive and specific method for quantifying a genotoxic hydrazine, agaritine, has been developed using liquid chromatography–electrospray ionization tandem mass spectrometry (MS). Synthetic agaritine was structurally assigned by ^{1}H, ^{13}C and two-dimensional nuclear magnetic resonance (NMR) analysis (heteronuclear multiple-bond correlation [HMBC] and heteronuclear multiple-quantum coherence [HMQC]), high-resolution fast-atom-bombardment (HR-FAB) MS. Agaritine was separated on an ODS column using 0.01% AcOH–MeOH (99:1) as an eluent with a simple solid-phase-extraction cleanup for mushroom samples and with acetonitrile and methanol deprotenization for plasma samples. There were no interference peaks in any of the mushrooms or mouse plasma samples. The recoveries of agaritine from the spiked mushroom samples and spiked mouse plasma were 60.3–114 and 74.4%, respectively. The intra-day precision values for the spiked mushrooms were 5.5 and 4.2%, and the inter-day precision values were 15.0 and 23.0%, respectively. The limit of quantification was 0.01 μg/g (in mushrooms) and 0.01 μg/ml (in plasma). A precursor ion scan confirmed that agaritine derivatives, which can exert a similar toxicity, were absent. These results indicate that this analytical method for quantifying agaritine could help to evaluate the risk of mushroom hydrazines to humans.

Keywords: Phenylhydrazine; Agaritine; Mushroom; Genotoxic; LC–MS–MS

1. Introduction

The cultivated mushroom *Agaricus bisporus* contains large quantities of aromatic hydrazines. Amongst these, the most abundant is agaritine [β-*N*-(γ-L(+)-glutamyl)-4-(hydroxymethyl)phenylhydrazine], the concentration of which is approximately 165–475 μg/g [1,2] or 1.7 mg/g in fresh mushrooms [3]. Toth and co-workers [4,5] demonstrated that the administration of uncooked mushrooms to mice induced a significant increase in the number of bone and forestomach tumors in both sexes, and in the occurrence of lung tumors in males [4,5]. Ethanolic and aqueous extracts from *A. bisporus* led to mutagenicity in the Ames test [6]. The direct-acting mutagenicity in this study was not attributed to agaritine, but rather to phenols and quinines that might behave as reactive oxygen species [7,8]. The direct mutagenicity was not affected by baking the mushrooms at 225 °C for 10 min [9]. Compounds leading to mutagenicity appear to be strong against heat. There are, however, also reports showing that agaritine does have a direct mutagenic effect. The mutagenicity of agaritine can be attributed directy to 4-(hydroxymethyl)phenylhydrazine (HMPH) and/or the 4-(hydroxymethyl)benzenediazonium ion (HMBD), both of which are formed by the eenzymatic degradation of agaritine that results in the loss of the γ-glutamyl group [10–13]. HMPH and HMBD are highly unstable and are carcinogenic [10,12,14].

Price et al. reported that agaritine was metabolized to compounds that covalently bond to proteins [15]. Shephard et al. demonstrated covalent bonding between agaritine and DNA [16]. These results have led to the hypothesis that mechanisms of the mutagenicity of agaritine and the *A. bisporus* mushroom might be similar.

A report evaluating the risk posed by phenylhydrazines in cultivated mushrooms (*A. bisporus*) to humans was recently published [17]. This report states that agaritine is thought to

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be converted to free hydrazine HMPH by γ-glutamyl transpeptidase, which is abundant in the kidney. This is consistent with a previous study that found that the mutagenicity of agaritine incubated with kidney homogenate and with kidney plus liver homogenates more than doubled and tripled, respectively [13]. These results indicate that agaritine might be converted to HMPH and then to HMBD, which can then be transformed into a radical compound that is potentially mutagenic. However, no evidence has been obtained of the presence of either HMPH or HMBD [13,18], as no suitable detection method had been identified and the molecules are unstable. Therefore, the *N*-acetyl derivative of HMPH must generally be used for toxicological studies.

Agaritine was first isolated and identified by Levenberg [19,20] and Kelley et al. [21]. This L-glutamic acid-containing hydrazine is susceptible to oxidation in the air. The stability of this molecule was examined by Hajšlová et al., who demonstrated that agaritine degrades within 48 h in tap water and that the degradation appeared to be oxygen-dependent. The best analytical method reported to date for the detection of agaritine is based on high performance liquid chromatography (HPLC) equipped with an ultraviolet (UV) detector (237 or 254 nm) using an ODS column [1,2,13]. In this method, only the commonly eaten *A. bisporus*, which contains substantial amounts of agaritine, can be analyzed. Agaritine cannot be detected in other mushroom species that contain only small quantities of agaritine due to multiple unspecific peaks in the HPLC. For toxicological studies, mouse and rat plasma have been analyzed for agaritine and its metabolites by liquid scintillation counting after the administration of radio-labeled agaritine [22]. The addition of radio-labeled agaritine was necessary because the concentration of agaritine in the plasma samples was too low to be detected by a UV method. There are no previous reports of a sensitive and specific agaritine detection method that is applicable to both food and biological samples [22]. A widely applicable analytical method for the detection of agaritine and its metabolites is required in order to assess the risk posed by phenylhydrazine agaritine to humans. The presence of agaritine degradation products in food and agaritine metabolites in plasma from agaritine-administration in mice or rats, as the toxicities of these molecules, remains unclear, although the presence of unidentified agaritine metabolites were reported in both of these experimental cases [13,15].

In this study, an analytical method for the quantification of agaritine using liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) for several edible and processed mushroom species and agaritine-administered mouse plasma is described. This method is comprised of an extraction with methanol, a simple solid-phase-extraction (SPE) cleanup for the mushroom samples or deproteinization by acetonitrile and methanol for the plasma samples, and electrospray mass spectrometry in the negative mode in conjunction with HPLC. An agaritine standard was synthesized and structurally elucidated using two-dimensional (2D) nuclear magnetic resonance (NMR) techniques such as heteronuclear multiple-bond correlation (HMBC) and heteronuclear multiple-quantum coherence (HMQC), as well as high-resolution (HR) fast-atom-bombardment (FAB) MS.

2. Experimental

2.1. Food samples

Fresh samples of *A. bisporus*, the Shiitake mushroom *Lentinus edodes*, the Maitake mushroom *Grifola frondosa*, and dried samples of the Himematsutake mushroom (*Agaricus blazei* Murill) were purchased from supermarkets in Tokyo, Japan. The Sugihiratake mushroom *Pleurocybella porrigens* was collected from in northern Japan. All of the fresh mushrooms were freeze-dried for 2 days using a vacuum freeze dryer (FD-81; EYELA, Kawasaki, Japan).

2.2. Chemicals

The methanol used in the sample preparation and in the LC/MS/MS analysis was obtained from Kanto Chemicals (Tokyo, Japan). MilliQ water was also used in this study. All of the other chemicals were of the highest grade available. Agaritine was synthesized according to the method described by Datta et al. with minor modifications [23]. The purity of the synthetic agaritine was >95% based on HPLC (254 nm). A standard stock solution was prepared in methanol and stored at below −20 °C before use.

2.3. Mouse plasma

Mouse plasma was taken from Slc:ddY mice (25–30 g, male, 8W; Japan SLC, Shizuoka, Japan) after agaritine administration (4.0 mg/kg mouse).

2.4. NMR Measurements

^{1}H, ^{13}C and 2D NMR (HMBC and HMQC) spectra were recorded on an ECA-500 (JEOL, Japan) in CD_3OD and $CDCl_3$ (^{1}H at 500 MHz and ^{13}C at 125 MHz), respectively. Chemical shifts (δ) are described in ppm using tetramethylsilane (TMS) as a reference. Coupling constants (J) are given in Hz. The samples to be measured were prepared under a nitrogen atmosphere to avoid oxidation in air.

2.5. HRMS

To identify the synthetic agaritine, FABMS in the positive mode (JMS-700; JEOL, Japan) was used.

2.6. LC/MS conditions

LC/MS/MS measurements were performed using a PE SCIEX (Concord, ON, Canada) model API 3000 triple-quadrupole mass spectrometer coupled to an Agilent 1100 series HPLC system with a G1315 photodiode-array detector (Palo Alto, CA). The HPLC system was equipped with a 3-μm Shiseido CAPCELL PAK AQ column (2.1 mm × 250 mm and

2.1 mm × 150 mm; Yokohama, Japan). The gradient conditions ranged from 99% water containing 0.01% AcOH with 1% MeOH to 90% MeOH with 10% water containing 0.01% AcOH. The flow rate was 0.2 ml/min and the column temperature was 35 °C.

The analytes were detected using electrospray ionization (ESI) in the negative mode. Multiple-reaction-monitoring (MRM) was performed using the characteristic fragmentation ions *m/z* 266 → 248 and 266 → 122 for agaritine. Optimization of the ionization and fragmentation conditions in the ESI mode for agaritine was achieved by the infusion and flow-injection analysis of agaritine. The optimization was performed several times to determine parameters such as the collision gas to be used and the focusing potential (FP). The parameters for the LC/MS/MS analysis of agaritine were as follows: ionspray voltage = −4500 V; collision gas = 6; focusing and entrance potentials = 60 and 20 V, respectively, and temperature = 500 °C. A switching valve led the column eluents to the mass spectrometer as the analytes were being eluted.

Precursor-ion and neutral-loss scans were performed to analyze the agaritine derivatives. The collision energy (CE) was changed from −2 to −40 to obtain better resolution. The data were acquired and calculated using Analyst 1.4.1 software (PE SCIEX).

2.7. Sample preparation

Freeze-dried mushrooms (1.0 g) and processed foods (1.0 g) made from *A. blazei* Murill were extracted with MeOH (3 × 30 ml) by shaking for 20 min. After filtration with a paper filter, the samples was evaporated to dryness and the residue was dissolved in 3 ml of 0.01% AcOH–MeOH (9:1). Bond Elut C_{18} cartridges (500 mg/3 ml; Varian, Palo Alto, CA) were conditioned with MeOH followed by 0.01% AcOH–MeOH (9:1). The sample solutions (1 ml) were loaded onto the cartridge and an additional 2 ml of 0.01% AcOH–MeOH (9:1) was added. The eluent was collected removing the yellow pigments and lipid-soluble materials. The final sample solutions (10 μl) were injected into the LC/MS/MS system. Agaritine was quantified using a linear calibration function that was established using the agaritine standard at concentrations of 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1.0 and 5.0 μg/ml (r^2 = 0.993–0.999).

Mouse plasma from agaritine-administered mice was prepared as follows; blood was collected 20 min after agaritine administration to mice and immediately placed on ice before centrifugation (10,000 rpm, 2 min). After centrifugation, the plasma (200 μl) was deproteinized by acetonitrile and methanol, and then diluted with mobile phase to 600 μl. Agaritine was quantified using a linear calibration function as described above (0.001, 0.005, 0.01, 0.05, 0.1, 0.5 and 1.0 μg/ml).

2.8. Accuracy and precision

The amount of agaritine recovered from the mushrooms spiked with agaritine (5 μg/g) using the above methods was determined. Intra-day precision was obtained by measuring three replicate samples that were spiked with 5 μg/g of the agaritine standard on the same day. Inter-day precision was estimated by measuring three replicate samples on different days. The quantification limit was determined using fortified samples based on an S/N ratio of 10:1.

The recovery of agaritine from mouse plasma spiked with agaritine (0.25 μg/ml) was determined based on the results from 11 mice.

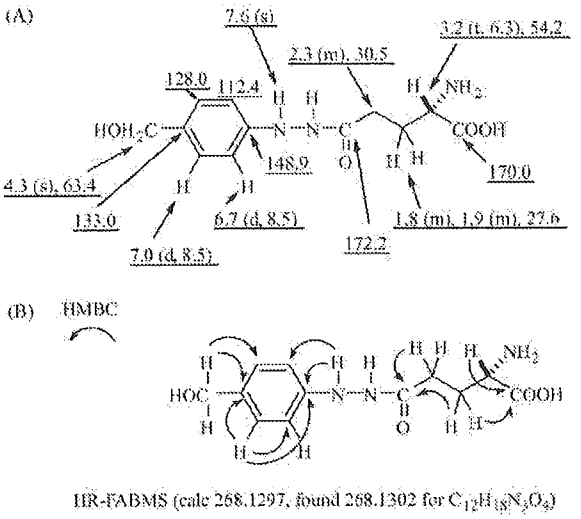

Fig. 1. Structure and NMR spectral data of agaritine. All signals were assigned by ^1H, ^{13}C and 2D NMR (HMBC and HMQC) spectra. (A) Chemical shifts (δ) of the synthesized agaritine; (B) HMBC correlation of the signals in agaritine. δ = 4.3 (s) and 7.6 (s) correspond to the HOH_2C– group on the benzene ring and NH– of the hydrazine group, respectively.

3. Results and discussion

3.1. Agaritine

Agaritine is not commercially available so this compound must be synthesized for research purposes. However, a complete structural assignment for this compound has not been reported to date.

We fully assigned the structure of agaritine that we synthesized. The structure and chemical shifts (δ) of agaritine are shown in Fig. 1A. The characteristic signals are δ = 4.3 (singlet) and 7.6 (singlet), corresponding to the HOH_2C– group on the benzene ring and the –NH– of the hydrazine group, respectively. The agaritine structure was confirmed by ^1H, ^{13}C, 2D NMR (HMQC and HMBC), and HR-FABMS experiments. The HMBC correlations around the benzene ring are shown in Fig. 1B, which supports the agaritine structure. HR-FABMS result confirmed the identity of agaritine ($[M+H]^+$, calc. 268.1297; found 268.1302).

3.2. LC/MS/MS

The protonated $[M+H]^+$ (*m/z* 268) and deprotonated $[M-H]^-$ (*m/z* 266) molecular ions of agaritine were detected using ESI. Peaks at *m/z* 250 in the positive-ion and 248 in the negative-ion modes, corresponding to the expected fragmenta-

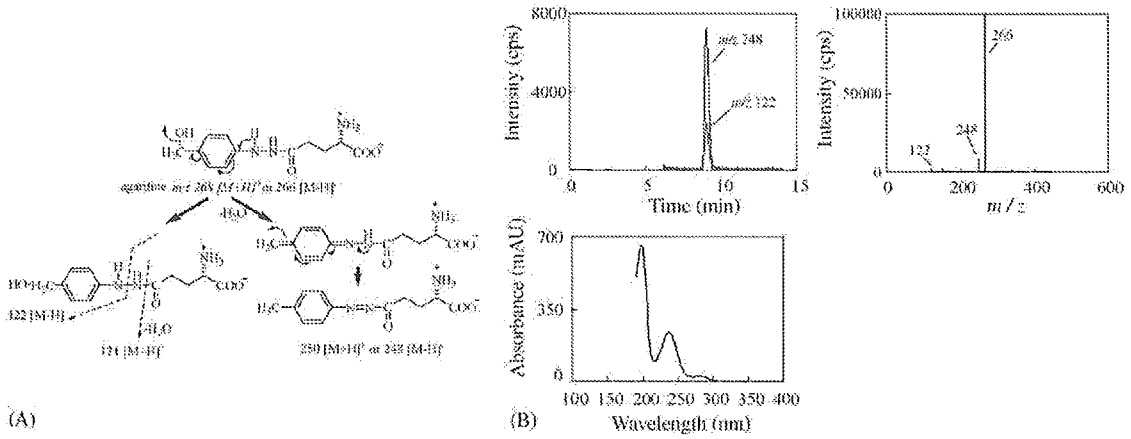

Fig. 2. Fragmentation mechanisms of agaritine in the positive- and negative-ion mode, MRM chromatograms of agaritine, and mass and UV spectra of agaritine. (A) The first fragmentation (loss of H_2O over the benzene ring) occurrs easily. High collision energy causes the second fragmentation, resulting in the loss of the glutamyl moiety; (B) MRM (*m/z* 266–248 and 266–122) chromatograms of the agaritine standard (0.05 μg/ml); (C) mass spectrum of the agaritine standard. Two fragment ions are observed (*m/z* 122 and 248); (D) UV spectrum of the standard.

tion pattern (loss of H_2O over the benzene ring), were observed. The high collision energy gave additional fragment ions that were observed at *m/z* 121 in the positive and 122 in the negative modes, which are the result of the loss of the glutamyl group. The agaritine standard and mushroom samples spiked with agaritine were analyzed in both the positive and the negative modes to compare the sensitivity, specificity and baseline stability of the two modes. Based on these results, MRM at both *m/z* 248 and *m/z* 122 in the negative mode was used for the quantification of agaritine, as the background noise level in the negative mode was lower than that in the positive mode, and the baseline was more stable. The peak area and height ratio of *m/z* 248 to *m/z* 122 was a constant value. This was then used as away to confirm that the observed peaks were agaritine. The fragmentation mechanisms of agaritine are illustrated in Fig. 2A. Representative mass chromatograms at *m/z* 248 and 122, along with mass and UV spectra of the agaritine standard (0.05 μg/ml) are shown in Fig. 2.

3.3. Agaritine in mushrooms

To date, the agaritine content has been determined by HPLC coupled to a UV detector (237 or 254 nm). *A. bisporus* contain large amounts of agaritine (165–475 μg/g [1,2] or 1.7 mg/g fresh mushroom [3]) and these concentrations permit agaritine detection by the HPLC–UV method, although the specificity is poor. We initially attempted to determine the presence of agaritine using HPLC equipped with a DAD detector in several species of mushrooms such as *A. bisporus*, *A. blazei* Murill, *L. edodes*, *G. frondose* and *P. porrigens*. Agaritine was detected in two *Agaricus* samples by UV (254 nm) and at least one peak overlapped with that of agaritine in the DAD data (190–400 nm scan; data not shown). It was clear that the agaritine peak on the sample chromatograms was not a single compound. Furthermore, the presence of agaritine could not be determined in the other species spiked with 5.0 μg/g due to the presence of large interference peaks (Fig. 3). The chromatograms presented in red show UV (237 nm)-monitored traces of two mushrooms spiked with agaritine. Compared to the chromatograms in black (nonspiked mushrooms), there is no significant difference between the two. Around the retention time (RT = 9 min) at which agaritine is supposed to be eluted, large interference peaks exist. This indicates that agaritine determination by UV detection is problematic in mushrooms.

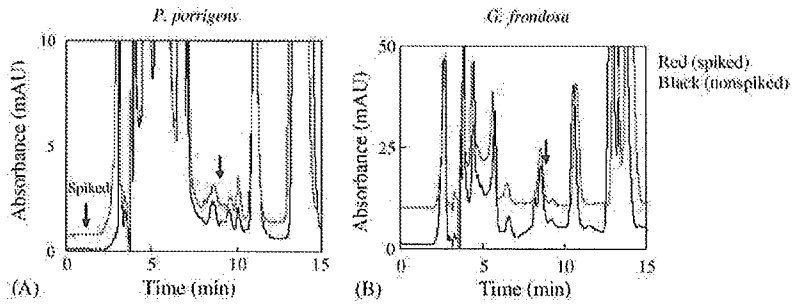

Fig. 3. Typical chromatograms of two mushroom species. Freeze-dried mushrooms were extracted with methanol and cleaned up using a C_{18} cartridge. The samples were analyzed with a UV detector (254 nm). Chromatograms of *P. porrigens* and *G. frondose* are shown in black. Chromatograms of two mushrooms spiked with the agaritine standard (5.0 μg/g) are in red. Small amounts of agaritine (less than μg/g level) could not be detected by the UV method. The spiked agaritine is predicted to appear at the retention times indicated by the arrows. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

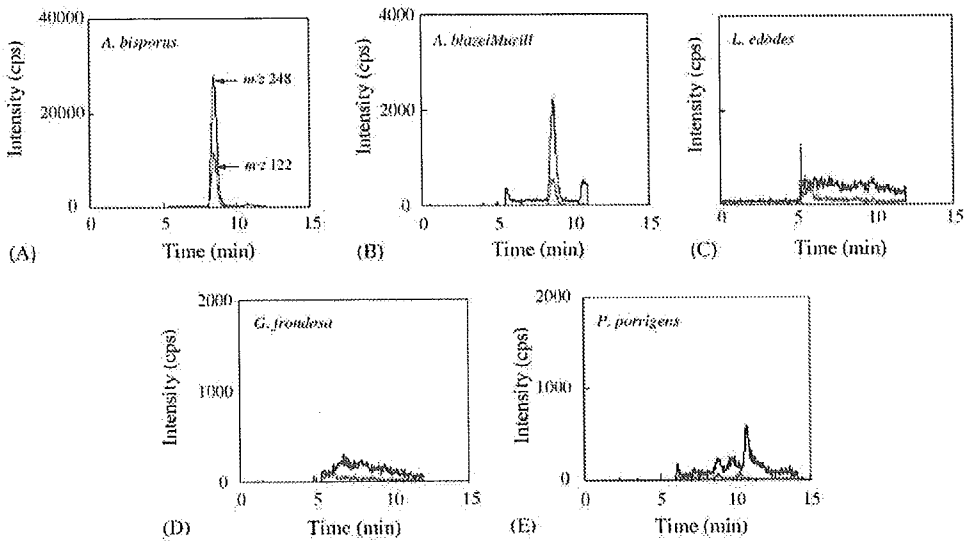

Fig. 4. Representative MRM chromatograms of five mushroom species. Two fragment ions were monitored simultaneously (*m/z* 266–248 in blue, 266–122 in red). None of the mushrooms, with the exception of the *Agaricus* spp., contained agaritine. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Thus, an analytical method using LC/MS/MS has been developed to determine agaritine with specificity and sensitivity. Five mushrooms, *A. bisporus*, *A. blazei* Murill, *L. edodes*, *G. frondose*, *P. porrigens*, were analyzed. There were no peaks hindering agaritine determination (Fig. 4). Only *Agaricus* spp. Were found to contain agaritine, and it was not detected in the other species. *A. bisporus* and *A. blazei* Murill had an agaritine content of 198 μg/g wet and 2,017 μg/g dry. The limit of quantification (LOQ) of this method was 0.01 μg/g, although the mobile phase of only 1% organic solvent (methanol) was disadvantageous to the sensitivity during the MS analysis. The peak height ratio of *m/z* 248 to *m/z* 122 was always constant between the agaritine standard and the samples. This indicates that this LC/MS/MS method is highly sensitive and specific for agaritine determination, and can be applied to other foods and biological samples such as plasma.

In addition to these results, the presence of an agaritine derivative that consists of HMPH condensed with aspartic acid (agaritine-Asp) was investigated using a precursor ion scan of 122 and MRM (*m/z* 252–234 and 252–122). Agaritine-Asp may exhibit a similar toxicity to agaritine, because it is also capable of generating HMPH. A precursor ion scan of dried *A. blazei* Murill revealed three peaks (Fig. 5A). Peak 2 was identified as agaritine (Fig. 5D), while the other two peaks could not be identified (Fig. 5C and E). MRM experiments confirmed that the remaining two peaks were not agaritine-Asp. If agaritine-Asp was present, a peak would appear at both *m/z* 252–234 (in blue) and 252–122 (in red) at the same retention times as peaks 1 and 3 in the precursor ion scan. The fourth peak (RT = 18 min) in Fig. 5A was background noise, which was also observed in the control sample (solvent alone). The results of neutral loss scans (losses of 130 for agaritine-Asp and 144 for agaritine) confirmed that agaritine-Asp was not present (data not shown). Based on these results, only *Agaricus* spp. mushrooms synthesize agaritine, which arise from the condensation of HMPH with glutamic acid. This is the first time that agaritine derivatives have been analyzed.

3.4. Agaritine in mouse plasma

The analytical method for mushrooms was applicable for mouse plasma. The agaritine content in the plasma was analyzed using MRM at *m/z* 266–248 given the higher sensitivity and lower background level was lower. Agaritine was detected (0.06 μg/ml) in the mouse plasma 20 min after agaritine administration (4.0 mg/kg). As shown in Fig. 6, agaritine was determined in mouse plasma without any unspecific peaks in the MRM chromatogram. Together with the results of the mushroom samples, it is clear this LC/MS/MS method would be very useful in quantifying agaritine in both mushrooms and plasma.

3.5. Accuracy, precision and recovery

Following the development of this new method for the determination of agaritine, the novel technique was validated. Table 1 summarizes the recoveries of agaritine from the spiked mushroom samples. The recoveries of agaritine from *Agaricus* product, *L. edodes* and *P. porrigens* were 114, 60.3 and 91.6, respectively. A matrix standard was used in the *P. porrigens* samples due to a suppression of the ionization in the *P. porrigens* samples.

Table 1
Recoveries of agaritine from three mushrooms and *Agaricus* product ($n = 3$)

	Recoveries (%)
Product A from *Agaricus blazei* Murill	114
Product B from *Agaricus blazei* Murill	92
Shiitake (*Lentinula edodes*)	60.3
Sugihiratake (*Pleurocybella porrigens*)	91.6

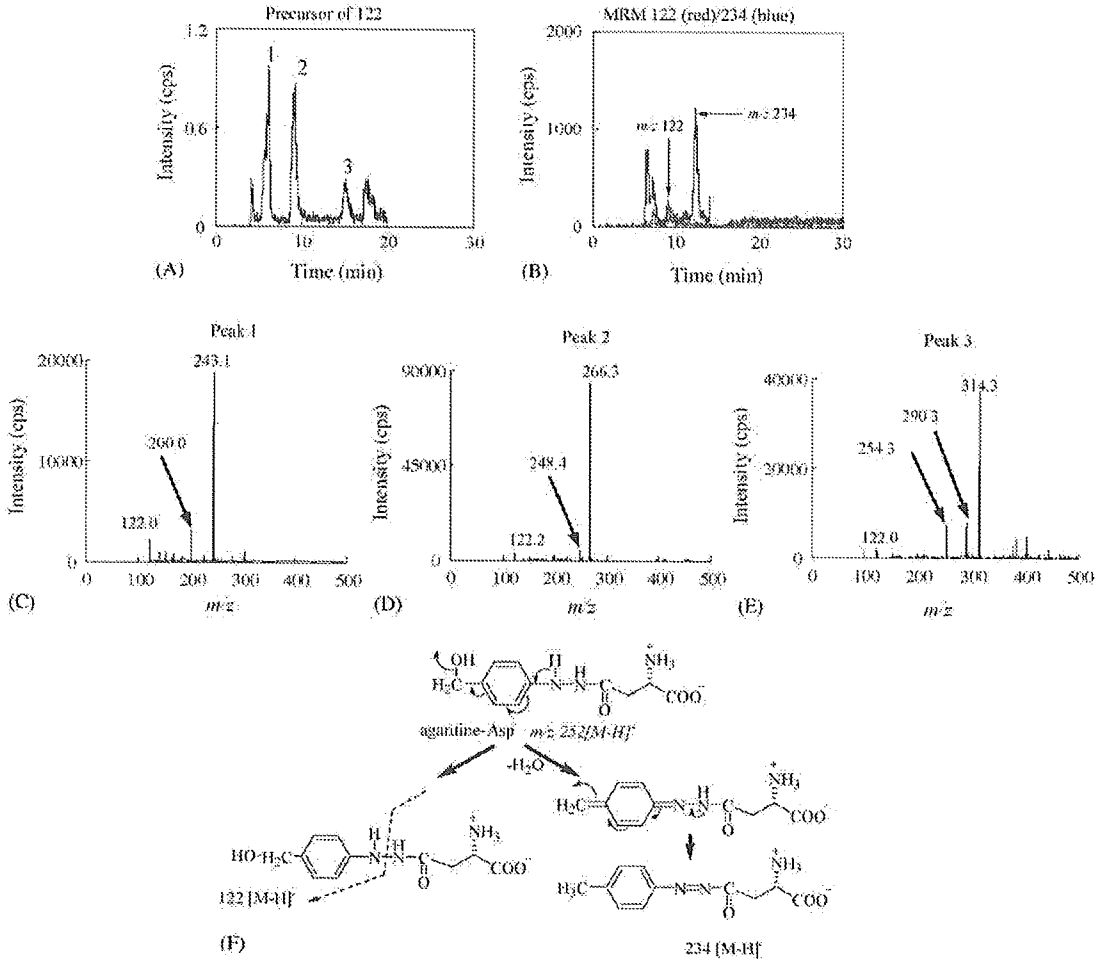

Fig. 5. A precursor ion scan of *m/z* 122 to investigate the agaritine derivatives. The fragment ion of *m/z* 122 was produced by cleavage of the N—N bond and elimination of the glutamyl moiety. The fragment ion is not dependent on the right part of agaritine like glutamic acid (F). Thus, agaritine-Asp will be detected by the precursor ion scan if it is present. An MRM analysis (*m/z* 252–234 in blue, 252–122 in red) was carried out to confirm the presence or absence of agaritine-Asp. Collision energies of −20 and −30 eV were required to obtain these results. (A) Precursor ion scan (*m/z* 122) of *A. blazei* Murill; (B) MRM chromatograms; (C–E) Mass spectra of peaks 1–3 in the chromatogram shown in (A); (F) fragmentation of agaritine-Asp. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Accuracy and precision for the determination of agaritine in mushroom samples (data are based upon assay of triplicate on three different days)

	Added (μg/ml)	Founded (μg/ml)	Intra-day R.S.D. (%)	Inter-day R.S.D. (%)	Accuracy (%)
Product C from *Agaricus blazei* Murill	5.0	4.4	4.2	15.0	−11.5
Maitake mushroom (*Grifola frondose*)	5.0	4.1	5.5	23.0	−18.1

Table 2 shows the intra- and inter-day accuracy and precision values for two mushrooms. The intra-day precisions values for the *Agaricus* product (Product C) and *G. frondosa* were 4.2 and 5.5%, and the inter-day precisions values for the two species were 15.0 and 23.0%, respectively. The accuracy varied from −11.5 to −18.1%. These values were acceptable for the labile hydrazine agaritine in mushrooms.

The recovery of agaritine from agaritine-administered mice is shown in Table 3.

Table 3
Recoveries of agaritine from agaritine-administered mice ($n = 11$)

	Recoveries (%)
Mouse plasma	73 ± 4.4

The value is shown as mean ± S.D.

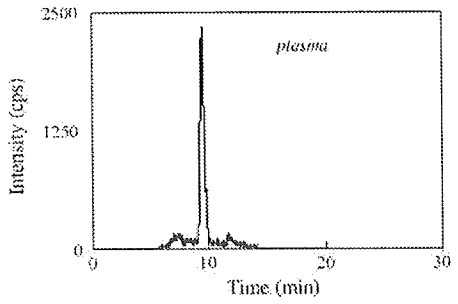

Fig. 6. A representative MRM chromatogram of mouse plasma 20 min after agaritine administration. The fragment ion of *m/z* 248 was monitored to quantify the agaritine in agaritine-administered mice.

4. Conclusion

The LC/MS/MS method described here demonstrated a high sensitivity and specificity for the quantification of agaritine in both various species of mushrooms and deproteinized mouse plasma. Even a trace amount of agaritine in both samples can be determined using this method. Additionally, we investigated for the first time agaritine-Asp, which is an agaritine derivative, and HMPH, which is a degradation product. No evidence was found of the presence of these compounds in the mushrooms in this study. The high specificity and versatility of this method make it a valuable tool for further identification-based research.

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Determination of Cyanide and Thiocyanate in Sugihiratake Mushroom Using HPLC Method with Fluorometric Detection

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A novel type of encephalopathy occurred in patients with chronic kidney diseases, which was associated with the ingestion of the Sugihiratake mushroom during the fall of 2004 in Japan. We attempted to investigate whether cyanide and thiocyanate are present in the Sugihiratake samples and determined the cyanide and thiocyanate levels in fifteen samples collected from different Japanese districts using HPLC with fluorometric detection. The cyanide ions and thiocyanate ions were detected in the ranges of N.D.–114.0 and N.D.–17.0 μg/g in the samples, respectively. This is the first study to quantitatively detect cyanide and thiocyanate in the Sugihiratake mushrooms. This result demonstrated that cyanide exposure could occur from the intake of Sugihiratake mushrooms in one's diet. Furthermore, we discussed the possible association between cyanide and the onset of encephalopathy.

Key words —— Sugihiratake, cyanide, thiocyanate, HPLC, encephalopathy

INTRODUCTION

Sugihiratake is the fungus *Pleurocybella porrigens*, which is a flat mushroom that grows on cedar and pine trees during the fall season, not only in the districts of northern Japan, but is also widely distributed in Japan.[1] It has a specific flavor, and many Japanese have been favorably consuming it in the processed foods of the highly popular miso (fermented bean paste soup) and the deep-fried food tempura. However, during the fall of 2004 in Japan, an outbreak of serious encephalopathy exclusively occurred in patients with chronic kidney diseases after the intake of this mushroom in many areas of Japan including the Akita, Yamagata, and Niigata Prefectures. Therefore, there have been some reports based on the clinical findings that encephalopathy was induced after the ingestion of this mushroom. The exact factors that induced the encephalopathy remain unclear and the association between the Sugihiratake mushroom intake and the onset of this novel type of encephalopathy is still currently controversial.

In the present study, we attempted to investigate the cyanide contents in wild Sugihiratake collected from several districts in Japan using a specific HPLC method with fluorometric detection, and were the first to detect cyanide in some of these samples. In addition, we discussed the possible association between cyanide intake and the onset of encephalopathy.

MATERIALS AND METHODS

Materials —— The Sugihiratake mushroom samples were collected from the local health environment centers and the prefectural institutes of the public health and environmental science in Japan through the Ministry of Health, Labor and Welfare (MHLW) of Japan.

Reagents —— A standard solution of potassium cyanide (0.1 M) was prepared by dissolving potassium cyanide (Wako Pure Chemicals, Osaka, Japan) in 0.1 M sodium hydroxide; the concentration of cyanide was calibrated by titration with silver nitrate using potassium iodide as the indicator according to the Liebig-Dènigès method.[2,3] A standard solution of potassium thiocyanate (Wako Pure Chemicals) was prepared using redistilled water. All other

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Table 1. Cyanide and Thiocyanate Contents of the Sugihiratake Mushroom Samples

Sample No.	Producing district	CN^- (μg/g dry weight)	SCN^- (μg/g dry weight)
1	Akita 1	12.7	1.1
2	Akita 2	25.5	4.6
3	Yamagata	0.7	0.2
4	Niigata 1	1.8	0.5
5	Niigata 2	56.2	17.0
6	Mie	3.1	1.6
7	Gifu 1	22.1	10.3
8	Gifu 2	114.0	9.4
9	Fukui 1	3.0	1.1
10	Fukui 2	0.9	1.4
11	Ishikawa	N.D.	0.1
12	Kyoto	1.2	0.2
13	Ibaraki	96.6	8.4
14	Fukushima 1	0.6	0.1
15	Fukushima 2	0.3	N.D.

N.D.: not detected.

chemicals were of analytical reagent grade.

Preparation of Sample Solution —— The freeze-dried Sugihiratake sample was ground to a fine powder using a grinder (Retsch GmbH, Haan, Germany), and a 500 mg test sample was extracted with 10.0 ml of 0.1 M sodium hydroxide by shaking overnight in a 50 ml centrifuge tube. A one ml portion was then placed in the outer well of the Conway cell and 1.0 ml of 0.1 M sodium hydroxide was placed in the center chamber. The Conway cell and ground-glass cover were coated with silicone grease, and a glass cover was placed on top of the microdiffusion cell, leaving a small space for the addition of the acidic solution. To the samples described above for the determination of cyanide, 1.0 ml of 1.2 M sulfuric acid was added to the outer chamber. Subsequently, the ground-glass cover was moved to seal the microdiffusion cell. These cells were carefully rotated in order to mix the solution in the outer chamber. The cells were then rotated every 30 min. Cyanide in the sample was allowed to diffuse for 4 hr at room temperature and the liberated hydrogen cyanide was absorbed into the sodium hydroxide solution in the center chamber. An aliquot from the center chamber solution was analyzed by HPLC.[4] For the recovery of thiocyanate from each sample during the pretreatment procedure, the Conway microdiffusion cell was kept for 24 hr and the collected thiocyanate in the center chamber was analyzed by HPLC. Using the pretreatment procedure described above, the spiked standard cyanide and thiocyanate at the 1 μmol level were recovered at 100.2 ± 3.2 and 95.4 ± 5.5%, respectively.

HPLC conditions[4] —— The HPLC system consisted of a double-plunger pump (PU-1580, Jasco., Tokyo, Japan), an intelligent fluorescence detector (FP-920S, Jasco) with a xenon lamp and 12-μl flow cell, a chromato-integrator (D-2500, Hitachi, Tokyo, Japan) and a sample injector (7725i, Reodyne, CA, U.S.A.). The HPLC conditions were as follows: column, a strong base anion exchange resin, TSK-Gel SAX (150 × 6 mm i.d., Tosoh Co., Tokyo, Japan); eluent, 0.1 M sodium acetate buffer (pH 5.0) containing 0.2 M sodium perchlorate (flow-rate, 1.0 ml/min); chlorination reagent, 0.1% chloramines T aqueous solution (flow-rate, 0.5 ml/min); pyridine-barbituric acid reagent, a mixture of barbituric acid (1.5 g), pyridine (15 ml), concentrated hydrochloric acid (3 ml) and redistilled water (82 ml) (flow-rate, 0.5 ml/min); the excitation and emission wavelengths of the detector were 583 and 607 nm, respectively.

RESULTS AND DISCUSSION

We attempted to investigate the cyanide content in the Sugihiratake samples and determined the cyanide in fifteen samples collected from different Japanese districts using HPLC with fluorometric detection. As shown in Table 1, we detected the cyanide ions and thiocyanate ions in the ranges of N.D.–

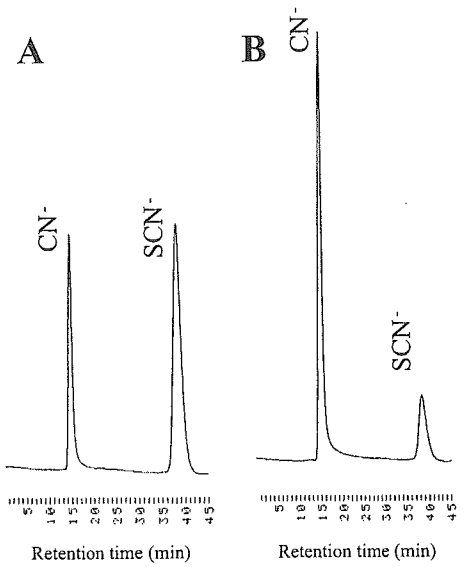

Fig. 1. Typical HPLC Chromatograms of Cyanide Ion and Thiocyanate Ion in a Sugihiratake Mushroom Sample
A; CN^- and SCN^- Standard (10 mM, respectively), B; Sample 5 (Niigata).

114.0 μg/g dry weight and N.D.–17.0 μg/g dry weight, respectively. These levels would not be lethal doses for acute toxicity even if 1 kg of the maximum level sample was consumed, because the lethal dose of cyanide is estimated to be 200–300 mg for an adult human. This result demonstrates that cyanide and thiocyanite exposures would occur from the intake of Sugihiratake mushrooms.

As for the determination of cyanide, the conventional spectrophotometric method has been previously used in forensic toxicology and waste water regulation.[5] However, it is known that thiocyanate could cause a serious false positive using the conventional method. Therefore, even forensic scientists have often mistakenly estimated cyanide because of the false positive caused by thiocyanate.[6] In the present study, we used the specifc and sensitive HPLC method of Toida *et al.*[4] after the pretreatment using the Conway cell. As shown in Fig. 1, we could simultaneously detect cyanide and thiocyanate using the HPLC system with an ion chromatographic column with non-interference determination. To our knowledge, this is the first report to accurately determine the content of cyanide and thiocyanate in Sugihiratake mushrooms. Since we confirmed the non-production of cyanide from linamarin, glucosidic cyanogens, using alkali solution (data not shown), we consider that the cyanide detected in the Sugihiratake samples would be present in the sodium form or potassium form.

Many food plants including agriculturally important species, such as cassava, flax, sorghum, alfalfa, peaches, almonds, and beans, are known to be cyanogenic.[7] Center African cassava flour contains sufficient quantities of cyanogens. When cassava is the staple part of the diet, the human daily consumption is equivalent to about one-half the lethal dose, which probably is thought to be the reason for the widespread and chronic neurological disorders called "konzo" found in this area.[7] In addition, the cyanide production has been observed in a wide range of fungi, such as *Phaeolepiota aurea*, *Rozites caperatus*, *Leucopaxillus giganteus*, and *Pleurocybella porringens* (Sugihiratake),[8] although there are no reports to describe the cyanide content in these fungi. Some reports suggested that the cyanide production in fungi could be associated with snow mold disease and fairy ring disease in some plants.[9]

To date, some clinical case studies involving the outbreak of acute encephalopathy that occurred in Japan during the fall of 2004 have already been reported.[10–13] All the cases were involved with the intake of Sugihiratake and the patients had varying degrees of renal dysfunction. The common clinical syndrome was characterized by weakness and involuntary movements of the extremities or dysarthria at the onset of the disease and subsequent intractable focal motor seizures, resulting in the generalized status of epilepticus or a comatose state. Some brain MRI examinations revealed that diffuse lesions in the basal ganglia and multiple ringed lesions in the cerebral cortex.

While there are some studies that cyanide could induce encephalopathy.[14–19] Smith *et al.* showed that comparatively small doses of cyanide given over long intervals can produce histological changes in the central nervous system of the rat.[14] As for the clinical study, Rachinger *et al.* showed that the toxicity of cyanide caused cerebral damage, primarily to the basal ganglia in the case report of patients that attempted suicide with cyanide.[17] This symptom appears to be consistent with those cases that occurred in the Akita Prefecture of Japan.

Furthermore, a recent study showed that cyanide and thiocyanate do accumulate in haemodialysis patients due to tobacco smoking.[20] Cyanide is known to be metabolized to thiocyanate by the enzyme rhodanese. This reaction is essential to life through its detoxification of cyanide, and thiocyanate synthesis can be accelerated under cyanide-loaded conditions such as tobacco smoking.[21] In addition, a

recent study showed that the risk of cerebral infarction stroke was significantly increased in individuals having high serum thiocyanate concentrations.[22] There also is some evidence that thiocyanate enhances the action of glutamate in a subclass of neuronal glutamate receptors which are involved in the neurodegenerative disorders.[23]

These results suggested that the ingestion of cyanide from foods could also induce the accumulation of cyanide and thiocyanate in the blood of patients with chronic kidney diseases and might be associated with the onset of encephalopathy. However, since the human capacity for detoxification and the toxicity of cyanide and thiocyanate from food exposure in haemodialysis patients have not been fully investigated, a further investigation is necessary in order to elucidate the relationship between the cyanide in Sugihiratake mushrooms and the onset of a new type of encephalopathy that occurred in Japan during the fall of 2004. Nevertheless, it should be noted that there could still be other factors and other substances that caused this novel type of encephalopathy in addition to the ingestion of cyanide from Sugihiratake mushrooms.

In conclusion, we were the first to determine the cyanide content in the Sugihiratake samples collected from certain areas of Japan during the fall of 2004. In addition, we showed that some samples could contain cyanide in the range of N.D.–114.0 μg/g, and suggested that its form in the Sugihiratake samples could be the sodium or potassium salt. This finding suggested that cyanide in Sugihiratake might be associated with the onset of a novel type of encephalopathy in patients with chronic kidney diseases, which occurred in Japan during the fall of 2004.

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