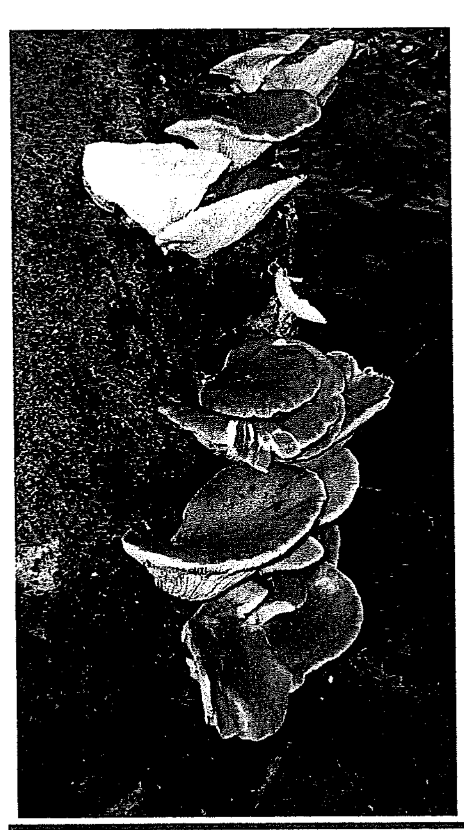
添付資料 5

ツキヨタケ *Omphalotus guepiniformis*（キシメジ科ツキヨタ属）

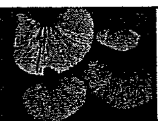
特徴	傘の大きさ	大型で 10～25cm 程度
	形と色	傘　：初め黄褐色で，成熟すると紫褐色～暗紫褐色。半円形，まれに円形で濃色の小鱗片を有する。 ひだ：白から薄い黄色で幅は広い。 柄　：太く短い柄が傘の側方に付くものが多いが，中央に付くものもある。ひだの付け根につば様の隆起帯がある。色は傘より淡色。肉の内部は暗紫色～黒褐色のしみがある。このしみは不明瞭なもの，ないものもある。 肉　：厚い。
	発生時期	夏～秋（特に秋）
	発生場所	ブナ、イタヤカエデなどのに重なり合って発生する。
	その他	目がかなり慣れれば，暗い場所ではひだが青白から蛍光緑にかすかに光る。 地方名：ワタリ、ワシタケ
	間違えやすい食用きのこ	ヒラタケ、ムキタケ、シイタケ
症状	食後３０分～１時間程で嘔吐，下痢，腹痛などの消化器系の中毒症状が現れる。 幻覚痙攣を伴う場合もあるが，翌日から 10 日程度で回復する。	
毒成分	イルジンＳ，イルジンＭ，ネオイルジン	



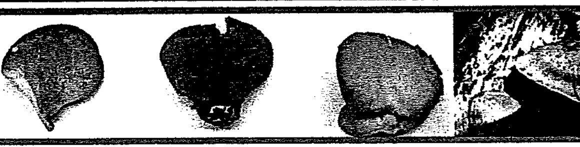
少し盛り上がったつばが柄の付け根にある

傘は通常半円形または扇型であるが，円形のものも報告されている（上）。

黒いシミがあるものが多い。黒いシミがほとんどないものもあるので注意が必要である。



暗闇で目が慣れれば，青白く見える。カメラで一定の露出時間で撮影すれば，蛍光緑色に確認できる（F5.6 で 1 時間 40 分）。



よく似ている食用きのこ
左から，ムキタケ，ツキヨタケ，ヒラタケ，シイタケ。

添付資料 5

1	(1) 毒成分 ＊1	illudin S、illudinM　（＝胃腸系の中毒） illudin S の毒性：LD_{50}：マウス（腹腔内）50 mg/kg dehydroilludin M, neoilludin A,B　（＝細胞毒）など illudin S　illudin M　dehydroilludin M Illudin M, illudin S には嘔吐作用がある。また，illudin 類は細胞毒性も強いと考えられており，その毒性を低減したアナログ体 irofulven は抗がん剤として検討されている。 irofulven DNA 合成阻害作用があり，固形癌などに対しても幅広いスペクトルの抗癌作用を示す。
	(2) 食中毒の型	胃腸系の中毒
	(3) 中毒症状	嘔吐、腹痛、下痢などの典型的な胃腸系の中毒を起こす ひどい場合は、痙攣、脱水、アシドーシスショックなどを起こす
	(4) 発病時間	食後 30 分～数時間

2 (1) 発症事例
＊2

(症例 1)
平成元年 10 月 19 日、惣菜店の店主が宮城県でシイタケだと思い採ってきたツキヨタケを、弁当の中にひじきと煮付けして 19 個販売した。摂食したものは 12 人。摂食後全員 2～2 時間 30 分の間に悪寒、激しい嘔吐を頻繁に繰り返した。嘔吐は多いもので 12 回、少なくでも 4 回起こし、このうち生後 11 ヶ月の乳児は摂食後 2 時間で 12 回もの嘔吐を繰り返す。3 家族 6 人は中毒症状が激しいため病院で治療を受けた。

(症例 2)
平成元年 10 月 28 日、山で採ってきたきのこを炒め物にして 3 切れ摂食。摂食 1 時間 30 分後、冷汗が出現、腹痛、軟便のため医療機関へ受診。初診時、所見は顔面蒼白、悪心、嘔吐、腹痛、鼻水、冷汗などが認められた。催吐により症状は軽快、外来処置のみで自宅療法となった。その後 4～5 日間は胃部不快感が持続したが、肝および腎機能に異常は認めなかった。

(その他)
平成 18 年 10 月 8 日、採取したキノコを飲食店で焼いたりスープにして摂取した。キノコを食べた 15 人中 13 人がすぐに嘔吐した。

平成 21 年 10 月 18 日、60 代女性が高知県で採取したキノコ 1 本を焼いて食べたところ、摂取約 1 時間半後に嘔吐など食中毒症状を示した。病院で治療後回復。

平成 21 年 10 月 14 日
鯖江市山中で採取したキノコを家族 4 名で食し、全員が嘔吐など食中毒症状を示した。

(2) 患者数

	発生件数	摂食者総数	患者数
2000 年	12 件	>69 人	69 人
2001 年	3 件	45 人	45 人
2002 年	19 件	110 人	91 人
2003 年	11 件	39 人	36 人
2004 年	16 件	53 人	52 人
2005 年	15 件	70 人	63 人
2006 年	17 件	65 人	61 人
2007 年	12 件	37 人	35 人
2008 年	18 件	63 人	59 人
2009 年	19 件	67 人	62 人

摂食者総数について，正確な人数が不明の場合は判明している人数以上という表示にしている（＞６９など)。

<table>
<tr>
<td></td>
<td>(3) 中毒対策</td>
<td>＜処置＞
激しい下痢症状のため下剤の投与は一般に行なわない。特に嘔吐、水溶性下痢が極度の場合、体液喪失による脱水、電解質異常に対する補液に十分気をつける。
(1)　催吐・胃洗浄
(2)　吸着剤投与
(3)　対症療法：補液
(4)　重症例には血液灌流（DHP:direct hemoperfusion）が有効</td>
</tr>
<tr>
<td>3</td>
<td>(1) 毒成分の分析法
1
＊3</td>
<td>(1) イルジンSの分析方法

均一化した食品試料　10 g
↓ ← MeOH 100ml
ホモジナイズ
↓ ― 残渣（MeOH 抽出　x2 回）
MeOH 抽出液（ 200ml）
↓
減 圧 濃
↓ ← 酢酸エチル　100ml
↓ ― 残渣（酢酸エチル抽出　x2 回）
酢酸エチル抽出液　(200ml) を無水硫酸ナトリウムで脱水
↓
減 圧 乾
↓ ← 酢酸エチル　1ml
↓ ← ヘキサン　4ml
フロリジルミニカラムに負荷
↓
酢酸エチル /ヘキサン (1:4)5ml で洗い流し、
酢酸エチル /ヘキサン (4:1)5ml で溶出後、減圧乾固
↓ ← アセトニトリル　1ml
↓ ← 純水　4ml
LC/MS 用　試験溶液

＜LC/MS の測定条件＞
カラム：L-column（1.2x150mm）
移動相：0.1% 酢酸/アセトニトリル
グラジェント：0-30-40min(10 -95-95%)
以上の条件で　12 分に溶出される

MS の測定モード：positive　mode
フラグメンター電圧：100v　（m/z265[M+H]$^+$イオンなどがえられた）</td>
</tr>
<tr>
<td></td>
<td>(2) 毒成分の分析法
2
＊4</td>
<td>Illudin 類が有機溶媒にも水にも溶解することから，溶媒抽出に代わり，固相抽出を用いた方法ある。

メタノール抽出 2 ml +水添加試料（20 ml）
↓　Oasis HLB (500 mg)</td>
</tr>
</table>

		保持後，20%メタノール 5 ml で洗浄 ↓ 5 ml で溶出 LC/MS/MS を用いて，MRM モードで，m/z 265 → 247 をモニターする。
4	諸外国での状況	朝鮮半島、ロシア極東地方、中国東北部に分布し、チベット南部の亜熱帯林に同属腫が発生している
5	その他の参考になる情報 ＊３	・ツキヨタケはヒダが暗所で蛍光黄緑色を発する特徴がある。以前は、このキノコに多く含まれるイルジン S やランプレオールと呼ばれる物質が発光体であるとされていた。しかし、現在ではこの蛍光体はランプテロフラビンであることが判明した。ランプテロフラビンは生物発光によってツキヨタケのヒダの外部に放出されること、またヒダの外部は弱酸性で中性のときよりは発光が強い、中性付近では同時に放出される加水分解酵素により、ランプテロフラビンは速やかにリボフラビンに返還される。 ランプテロフラビンの構造式　$C_{22}H_{28}N_4O_{11}$ Exact Mass: 524.2 ・ツキヨタケは食用のムキタケと同じ場所に混生して生えていることがあり、そのときに誤認して摂食してしまうことがある。 ＜ツキヨタケ同定の呈色反応＞ ・グアヤクチンキ反応 グアヤク脂 1 g を 70%エタノール 5 ml に溶解し、試薬を調整する・食用のムキタケは青緑色に変色するが、有毒のツキヨタケは変色しない。

		・硫酸バニリン反応 蒸留水 3 ml に濃硫酸 8 ml を加え、バニリン 1 g を溶解し、試薬を調整する。 食用のムキタケは赤紫になるが、有毒のツキヨタケは変色しない
6	間違えやすいキノコ	
	一般名	ムキタケ
	学名	*Panellus serotinus* （Pers.:Fr） Kühn
	特徴	
	発生場所	特に、ブナ、ミズナラ林に多く倒木上に多数重なり合って発生する。
	発生時期	秋
	形態	傘：粘性があるが、細毛に覆われている ツキヨタケに比べて表皮がむけやすい。肉は厚く、白い。 ヒダ：黄色～黄土褐色 柄：太く短い
	一般名	ヒラタケ
	学名	*Pleurotus ostreatus* (Jacq.:Fr) Kummer
	特徴	
	発生場所	広葉樹の古木、切株
	発生時期	秋～春
	形態	傘:はじめは黒色→灰青色→灰白色と傘が開いていくとともに色が薄くなる。肉は白色 ヒダ：はじめ白色→淡黄褐色をおびる 柄：白色、多数の個体が基部で融合する。根本には白毛状の菌糸が多数付く
	一般名	シイタケ
	学名	*Lentinula edodes* (Berk.) Pegler
	特徴	
	発生場所	広葉樹の倒木、切株
	発生時期	春、秋
	形態	傘：はじめ傘の周縁部に白い綿毛状の鱗片をつけるが、成長するにつれて鱗片が消失し、傘にひび割れが生じることが多い。肉は白色で弾力がある。 ヒダ：白色 柄：上部は白色で下部に向かって褐色に色づくこともある

添付資料5

ツキヨタケの写真

添付資料５

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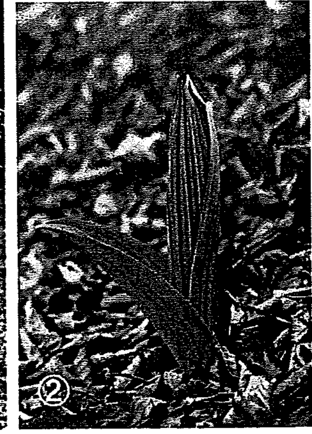
添付資料 6

バイケイソウ



①花期のバイケイソウ（有毒）
②芽出し期のバイケイソウ（有毒）
③芽出し期のオオバギボウシ（山菜）
④バイケイソウ（矢印）とオオバギボウシ（左）は一緒に生えていることがあるため注意が必要。

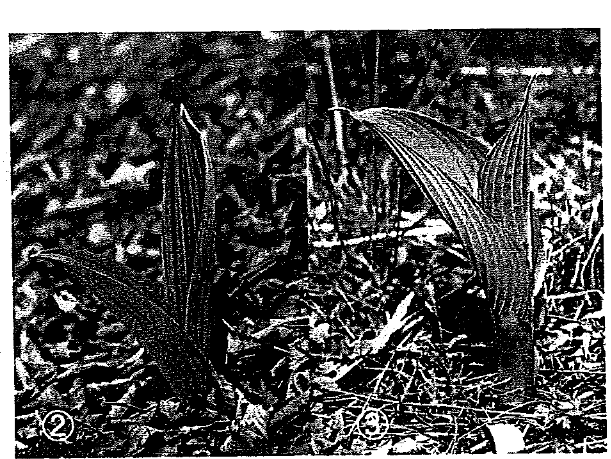
（写真提供：①〜③: 磯田　進、④長谷川幹夫）

和名	バイケイソウ　(ユリ科)
別名	ハエドクソウ、ハエノドク、ハエコロシ、シシノハバキ
茎高	100〜200 cm
特徴	太く直立した茎に楕円形の大きな葉をもち、初夏に緑白色の花を多数総状につける。新芽の時の形態が山菜のオオバギボウシ(地方名ウルイ)やギョウジャニンニク(地方名アイヌネギ)と似ており、中毒事故が多い。オオバギボウシの葉は葉柄があって、主脈から側脈が出るのに対し、バイケイソウの葉は無柄で、完全な平行脈であることなどで識別できるが、芽出し期にはわかりにくいため、少し葉が開いたもので確認するとよい。不快な苦みがあることも特徴。全草に有毒アルカロイドを含有し，加熱しても毒は消えない。誤食すると嘔吐，下痢，手足のしびれ，めまい等の症状が現れ、死亡する危険もある。根茎は吐剤や血圧降下剤として用いられたこともあるが、毒性が強く現在では用いられない。
有毒成分	アルカロイド（プロトベラトリン、ジェルビン、ベラトラミンなど）
分布	本州中部以北、北海道からユーラシア北東部に分布し、山地から高山の湿った場所に生える。

毒性

部位	茎・葉	花	地下部（根茎）
毒性	強毒	強毒	強毒
食用の可否	×	×	×

バイケイソウ

一般名	バイケイソウ
地方名	げりめき(新潟)、さきそー・さぎそー(熊本)、さつぷし(栃木)、ししのはばき(岐阜)、ずっくい(岩手)、はえどくそう（長野）、はえころし（長野）、はえのどく(長野、愛媛)、ばけけんそー(三重)、ゆりば(奈良)、どす（徳島）
分類	ユリ目 Liliales、ユリ科 Liliaceae、シュロソウ属 *Veratrum* (APG 分類体系ではユリ目、シュロソウ科、シュロソウ属)
学名	*Veratrum album L.* subsp. *oxypetalum* Hultén
英名	**white hellebore, false hellebore**
生育地	北海道、本州、四国、九州の低山から高山帯までの林内や湿った草原に生え、やや群生する。
形態	大型の多年草で、茎は高さ 100〜200cm に達する。地下茎は太く、短い。下部の葉は茎を囲んで鱗片状となり、茎の中部には長さ 20〜30cm、幅 20cm の楕円形の大きな葉をつける。7〜8 月に、茎頂に大型の花序を立て、直径 1〜3cm の緑白色の花をつけ、雄蕊は花被片より著しく短い。花の色や大きさ、形などに変異があり、細かく分類されることもある。 新芽の時の形態が山菜のオオバギボウシ(地方名ウルイ)やギョウジャニンニク(地方名アイヌネギ)と似ており、極めて中毒事故が多い。オオバギボウシの葉は葉柄があって、主脈から側脈が出るのに対し、バイケイソウの葉は無柄で、完全な平行脈であることなどで識別できるが、芽出し期にはわかりにくいため、少し葉が開いたもので確認するとよい。中毒事例が多いため、福島県、茨城県、群馬県、東京都、新潟県、富山県など、多くの自治体が機関のホームページで、写真やイラストを使って形態の特徴を示し、見分け方を解説している(下欄に掲載)。  ①花期のバイケイソウ ②芽出し期のバイケイソウ（有毒） ③芽出し期のオオバギボウシ（山菜） ④バイケイソウ（矢印）とオオバギボウシ（左）は一緒に生えていることがあるため注意が必要。

添付資料 7

	(写真提供: ①～③ 磯田　進、④長谷川幹夫)
毒成分	プロトベラトリン protoveratrine、ジェルビン jervine, シクロパミン cyclopamine , ベラトラミン veratramine などのアルカロイド。中毒事例にあるように、ゆでる、炒める、天ぷらにするなど熱を加えても毒成分は分解されず中毒を起こす。 jervine　veratramine cyclopamine　protoveratrine
中毒症状	吐き気、嘔吐、手足のしびれ、呼吸困難、脱力感、めまい、痙攣、血圧低下など。重症の場合は意識不明となり、死亡する。
発病時期	30 分～1 時間で発症
発生事例	（症例 1）2009 年 4 月 15 日に福島県いわき市の 59 歳の男性が市外でウルイ（オオバギボウシ）と間違えてバイケイソウを採取し、酢味噌和えにして食べたところ、めまいや嘔吐、血圧低下などの症状が現れ、市内の病院を受診し入院した。男性は回復し、16 日には退院した。 (2009 年いわき民報) （症例 2）2008 年 4 月 16 日午前に東京都内の飲食店営業者が福島県でオオバギボウシと思って採取した山菜を、同日午後 5 時 30 分頃天ぷらにして客に提供。さらにその山菜の天ぷらと酢味噌和えを営業者と従業員が試食。同日午後 6 時頃から午後 10 時にかけて、山菜の天ぷら等を食べた計 5 名が吐き気、嘔吐、血圧低下、手足のしびれ等の症状を呈し、2 ヶ所の医療機関に救急搬送され入院した。飲食店に残っていた山菜を 17 日に鑑別した結果、バイケイソウであることが判明した。患者の意識は明瞭で、全員回復した。 (2008 年東京都報道発表資料) （症例 3）2007 年 5 月 3 日午後、埼玉県飯能市の住民が知人と群馬県内で山菜採りを行った際、「ウルイ」と判断した植物を採取した。この植物を、5 月 4 日の夜、飯能市内の飲食店に持ち込み、従業員に調理を依頼し、油で炒めて、採取者及び友人 3 名（男 3 名、女 1 名、年齢 36 歳～58 歳）で喫食した。食後 30 分～4 時間で、4 名とも吐き気、嘔吐、めまい等の食中毒様症状を呈した。採取した植物の残品を県農林総合研究センターに鑑定を依頼したところ、バイケイソウであることが判明した。患者は全員回復した。 (2007 年埼玉県報道発表資料) （症例 4）2006 年 6 月 4 日（日）午前 8 時 30 分頃、知り合いからもらったバイケイソウをみそ汁の具にして家族 2 名で食べたところ、同日午前 9 時頃から吐き気、嘔吐、手足のしびれ等の食中毒様症状を呈し、2 名とも医療機関に

	入院した。栃木県北健康福祉センターが調査し、材料の残品を確認したところバイケイソウであることが判明した。患者は全員回復した。 （2006 年栃木県報道発表資料） （症例５）2005 年 5 月 4 日から 5 日にかけて、東京都足立区在住の男性 2 名が、栃木県上都賀郡の山中で“ウルイ”と判断したものを採取した。5 日夕方、山菜採りに行った男性２名を含む３名が「“ウルイ”の酢味噌和え」を食べたところ、同日 20 時頃からめまい、おう吐等の症状を呈し、2 か所の医療機関に救急搬送され、入院した。6 日、患者宅に残っていた“ウルイ”を東京都健康安全研究センターで鑑別した結果、バイケイソウであることが判明した。患者３名のうち１名は、血圧降下の症状を一時呈したが、全員回復した。 （2005 年東京都報道発表資料）
患者数（2003～2007 年）	年度　発生件数　患者総数　摂食者総数 2007 年度　1 件　4 人　4 人 2006 年度　6 件　24 人　26 人 2005 年度*　6 件　10 人　13 人 2004 年度　4 件　9 人　9 人 2003 年度　3 件　5 人　6 人 （*1 件はバイケイソウかコバイケイソウか特定されてない） (厚生労働省発表資料)
中毒対策	毎年のように中毒が起こるので、多くの県や市町村でパンフレットやホームページを使って山菜との見分け方を紹介し、注意を喚起している。複数のホームページの写真やイラストを見て、異なる時期の形態を参照し、採ったものを確認する。特に芽出し期はオオバギボウシと酷似しているので、少し葉が開き、葉脈や葉柄などの区別点がはっきりしたもので確認すると良い。群生して多量に採れるため、「おすそ分け」による被害がある。採取した山菜を他人に与えるのは避けた方が良い。
毒成分の分析法	LCでの分析が主流 １．Rapid Commun Mass Spectrom. 2007;21(6):869-79. Characterization and identification of steroidal alkaloids in the Chinese herb Veratrum nigrum L. by high-performance liquid chromatography/ electrospray ionization with multi-stage mass spectrometry. 植物のメタノール抽出液をLC/MSで分析。20種類以上の標準品を単離 【LC条件】 カラム：Zorbax XDBC18 (4.6mmID, 150mm, 5 µm, Agilent Technologies, MA, USA) 移動相：メタノール（移動相A）-ギ酸アンモニウム（2mM, 0.5% ギ酸含有）（移動相B）、グラジエント：A；20% (v/v)～40% /20 min, 40%～80%/70 min. 流速：0.4 mL/min カラム温度：30℃ 【MS条件】 ネブライザーガス：窒素（35 psi, 10L/min, 350℃） HV voltage：4000 V mass rangere：m/z 50-1300 ２．J Anal Toxicol. 2008 Nov-Dec;32(9):768-73. Accidental intoxication with Veratrum album. 中毒を起こした人の血清から液液抽出でプロトベラトリンAとプロトベラトリンBを検出、定量。しかし、Veratridine, cevadine, jervine は、検出できなかった。

添付資料 7

	３． J Anal Toxicol. 2001 Sep;25(6):481-5. LC-EI-MS determination of veratridine and cevadine in two fatal cases of Veratrum album poisoning. 中毒を起こした人の血液から液液抽出でveratridineとcevadineを検出、定量。
その他の参考になる情報	1) 茨城県林業技術センターホームページ『バイケイソウに御用心！ 食中毒を未然に防ぐ完全見分け方マニュアル』 http://www.pref.ibaraki.jp/bukyoku/nourin/ringyo/kinoko/sodan,sansai.htm 2) 群馬県ホームページ『山野の有毒植物』 http://www.pref.gunma.jp/cts/PortalServlet?DISPLAY_ID=DIRECT&NEXT_DISPLAY_ID=U000004&CONTENTS_ID=2412 3) 福島県ホームページ『山菜の食中毒に注意』 http://www.pref.fukushima.jp/forestry/oshirase/sansai_doku/sansai_doku.html 4) 東京都福祉保健局パンフレット『身近にある有毒植物』(PDF) http://hfnet.nih.go.jp/usr/kiso/pamphlet/dokushoku.pdf#search='バイケイソウ 中毒 東京都福祉保健局' 昔、根茎を殺虫剤として用いていたことから、「はえころし」などの地方名がある。
間違えやすい植物	新芽の時の形態が山菜のオオバギボウシ(地方名ウルイ)やギョウジャニンニク(地方名アイヌネギ)と似る。

作成：中田政司（富山県中央植物園）

別添４

研究成果の刊行に関する一覧表

雑誌

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The metabolisms of agaritine, a mushroom hydrazine in mice

Kazunari Kondo [a,b,*], Asako Watanabe [b], Hiroshi Akiyama [a,b], Tamio Maitani [b]

[a] *Division of Biosignaling, National Institute of Health Sciences, Kamiyoga, 1-18-1, Setagaya, Tokyo 158-8501, Japan*
[b] *Division of Foods, National Institute of Health Sciences, Kamiyoga, 1-18-1, Setagaya, Tokyo 158-8501, Japan*

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Abstract

The mushroom hydrazine agaritine was measured in mouse plasma and urine using LC/MS/MS, which is highly specific. Agaritine concentration peaked 20 min after oral administration to mice (4.0 and 40 mg/kg). The concentration gradually decreased and returned to the basal level in 100 min. The maximum concentration, the time to the maximum concentration, and the half life were 0.37 μg/ml plasma, 0.33 h, and 0.71 h, respectively after administration of agaritine at 40 mg/kg body weight. One agaritine metabolite was found in the plasma and the urine from agaritine-administered mice. The structure of metabolites of agaritine by γ-GT was next investigated using LC/MS. HMPH proved to be generated from agaritine. The oxidative stress marker 8-OHdG was detected in agaritine-administered mouse urine. After administration, the 8-OHdG level immediately tripled, and then decreased to the control level over 48 h. Its level then elevated again and remained high for 11 days. These results suggest that agaritine quickly metabolizes and disappears in the plasma, whereas DNA damage lasts for a long time after a single administration of agaritine to mice.
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Keywords: Agaritine; Mushroom; Plasma; Urine; LC/MS/MS; Phenylhydrazine; Metabolism

1. Introduction

Agaricus mushrooms (*Agaricus* spp.) including the cultivated mushrooms, *Agaricus bisporus*, contain substantial amounts of aromatic hydrazines. The most abundant is genotoxic agaritine, [β-*N*-(γ-L(+)-glutamyl)-4-(hydroxymethyl)phenylhydrazine] (Kelly et al., 1962; Gigliotti and Levenberg, 1964). This L-glutamic acid-containing phenylhydrazine is susceptible to oxidation. Toth and Erickson 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 (Toth and Erickson, 1986; Toth et al., 1998). Bladder implantation of methanol extracts of fresh mushrooms induced cancer in the bladder epithelium (Hashida et al., 1990). Toth et al. reported that backed mushrooms could induce tumors (Toth et al., 1997). Ethanolic and aqueous extracts from *A. bisporus* demonstrated mutagenicity in the Ames test (von Wright et al., 1982). The carcinogenicity and mutagenicity of these mushrooms can be attributed to agaritine and 4-(hydroxymethyl)phenylhydrazine (HMPH) or the 4-(hydroxymethyl)benzenediazonium ions (HMBD), both of which are believed to be formed by the enzymatic degradation of agaritine, resulting in the loss of the γ-glutamyl group (Toth et al., 1978, 1981; Toth and Nagel, 1981; Walton et al., 1997). It has been previously reported that HMPH and HMBD are highly unstable and exhibit carcinogenicity (Toth et al., 1978, 1981; Ross et al., 1982; Walton et al., 1997). HMPH and HMBD are thought to be potent components that exert genotoxicity. However, they are virtually impossible to detect, because of their instability. The formation of HMPH was speculated by a UV absorption shift from 273 to 325 nm in the presence of sodium glyoxylate (Gigliotti and Levenberg, 1964).

Ross et al. revealed the recovery of agaritine from the gastro-intestinal tract of 3 mg-agaritine-administered mice using the HPLC–UV method. They also showed the agar-

* Corresponding author. Address: Division of Foods, National Institute of Health Sciences, Kamiyoga, 1-18-1, Setagaya, Tokyo 158-8501, Japan.
E-mail address: kondo@nihs.go.jp (K. Kondo).

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itine contents in the stomach, small intestine, caecum, and large intestine were successfully determined, but failed to detect agaritine in the blood because of the low sensitivity of the UV method (Ross et al., 1982). Walton et al. reported an investigation of the agaritine content in blood and urine using a scintillation counting method after ^{14}C-labeled agaritine had been administered to rats and mice. In the mouse, radioactivity in the blood peaked after 30 min, and then gradually decreased over a period of 12 h (Walton et al., 2000). The ^{14}C-labeled-agaritine-based experiment, however, has a limitation in that the radioactivity detected in the blood cannot distinguish between intact agaritine and degradative products including ^{14}C-labeled moieties. The fate of agaritine has thus up to now remained unclear.

More recently, we have established a new method for agaritine determination in mouse plasma and urine using HPLC coupled with tandem mass spectrometry (LC/MS/MS) that has high sensitivity and specificity (Kondo et al., 2006a,b). In the present study, the agaritine content in the plasma and the urine from agaritine-administered mice was determined using this method in addition to LC/MS/MS investigation of agaritine metabolism and DNA adduct (Singh and Farmer, 2006), the structure of the degraded products, DNA damage by agaritine in mice. This should facilitate the assessment of the health risk to humans.

2. Materials and methods

2.1. Chemicals and LC/MS/MS conditions

Agaritine and agaritine-COOH ([β-N-(γ-L(+)-glutamyl)-4-carboxyphenylhydrazine]) were synthesized according to the methods of Wallcave et al. and Hoesch et al. with minor modifications (Wallcave et al., 1979; Hoesch and Datta, 1987). Briefly, reaction mixtures in the reduction reaction of carboxylic acid to hydroxymethyl group in *p*-hydrazinobenzoic acid moiety were recrystallized from acetone/diethyl ether (1:4) to remove byproducts. Total yield was 17%.

The purities of the synthetic compounds were >95% by HPLC (254 nm) and >95% by ^1H NMR (500 MHz, DMSO-d_6 and DMSO-d_6-D_2O). The synthetic agaritine and agaritine-COOH were stored at −80 °C under a N_2 gas atmosphere. Standard stock solutions were prepared in methanol and stored at below −20 °C before use. The both compounds were stable in methanol for 1 week at −20 °C.

The structures of synthetic agaritine and agaritine-COOH were examined using 2-D NMR and high-resolution (HR) mass analysis. The results of ^1H, ^{13}C, and 2D NMR (HMBC and HMQC) were recorded using an ECA 500-MHz FT NMR spectrometer (JEOL, Japan).

Chemical shifts (δ) are described in ppm using tetramethylsilane (TMS) as a reference. Coupling constants (J) are given in Hz. HR-FABMS was performed in positive mode to identify them described previously (Kondo et al., 2006).

LC/MS/MS measurements were performed using a PE SCIEX model API 3000 triple–quadrupole mass spectrometer coupled to an Agilent 1100 HPLC system, including a G1315 photodiode array detector and a 3-μm Shiseido Capcell Pak AQ column (2.0 × 250 mm). Gradient conditions ranged from 99% water containing 0.01%AcOH–1%MeOH to 90% MeOH–10% water containing 0.01% AcOH. The analyte was detected using electrospray ionization in negative mode. Multiple-reaction-monitoring (MRM) was performed using characteristic fragmentation ions (m/z 266 > 248 and 266 > 122) for agaritine. A full scan analysis (m/z 50–1000) was carried out to analyze agaritine metabolites in plasma and urine. The parameters for the LC/MS/MS analysis of agaritine were as follows. Ionspray voltage (IS) = −4500 V; collision gas = 6; focusing and entrance potentials (FP and EP) = −60 V and −20 V, and temperature (T) = 500 °C, respectively. A switching valve led the column eluents to the mass spectrometer while the analytes were being eluted. The data were acquired and calculated using Analyst 1.4.1 software (PE SCIEX). Metabolite ID 1.3 was used to analyze agaritine metabolites.

2.2. Animals

Male ddY mice (7 weeks, 26–30 g, average = 28.07 ± 0.81 g) were purchased from SLC (Shizuoka, Japan) and were housed for one week. Each cage contained three or four mice. The animals were kept on a 12 h light/dark cycle, at room temperature of 24 ± 1 °C, humidity of 55 ± 5%, with free access to food (CRF-1, Oriental Yeast Co Ltd.) and water. The animals were used according to the guidelines of National Institute of Health Sciences and the Ministry of Health, Labour and Welfare of Japan.

2.3. Agaritine in plasma and pharmacokinetic study

The synthetic agaritine in MilliQ water (4.0 and 40.0 mg/kg) was administered to male ddY mice (8 weeks) by gavage after overnight starvation. The 33 mice were used for the time course of agaritine content in mouse plasma. Three mice were anesthetized with diethyl ether and the blood samples were withdrawn from them to prepare the plasma every 20 min until 180 min after agaritine-administration. MilliQ water was administered to the control mice. The pharmacokinetic parameters were calculated using the PK program @PKANS (D Three, Japan).

Mouse plasma from agaritine-administered mice was prepared as follows; blood was collected 20 min after oral administration of agaritine to mice and immediately placed on ice before centrifugation (10,000 rpm, 2 min). After centrifugation, the plasma (200 μL) was deproteinized by acetonitrile (750 μL), and then evaporated the solvent. The residue was dissolved with mobile phase (600 μL).

2.4. Agaritine in urine

For the analysis of agaritine and its metabolites in mouse urine, 20 mice were divided into five groups (one control and four agaritine-administration groups). Each group of four mice was housed in a metabolic cage immediately after oral administration of agaritine or MilliQ water to mice, and then urine was collected in 50 ml Falcon tubes every 12 or 24 h. Urine and fecal matter were separated by metabolic cages. Falcon tubes were covered with aluminum foil to avoid oxidative degradation.

2.5. Oxidative stress

Forty mice were divided into 5 control and 5 agaritine administration groups. Each group comprised 4 mice. The synthetic agaritine was dissolved in MilliQ water prior to this experiment. Mice were put in metabolic cages immediately after agaritine or MilliQ water administration. Mouse urine was then collected at indicated times (12, 24, 48, 72, 216, 264 h). Urine samples were diluted to 1:10 for ELISA. Creatinine contents were also measured by an enzymatic method. Oxidative damage to mice was evaluated by quantifying an oxidative stress marker 8-OHdG (8-hydroxy-2′-deoxyguanosine) according to the manufacturer's instruction. This marker was measured using a high sensitivity 8-OHdG ELISA kit (Japan Institute for the Control of Aging, Shizuoka, Japan). MDA (malondialdehyde) levels were also measured using HPLC after the reaction of MDA with thiobarbituric acid (TBA).

2.6. Agaritine degradation by γ-glutamyltranspeptidase (γ-GT)

Sodium glyoxylate (23.5 mg, Sigma, St. Louis, MO) and bovine γ-GT (29.2 mg, Sigma EC 2.3.2.2) were added to agaritine (3.6 mg) in 2.0 ml of

PBS. The mixture was allowed to stand for 180 min at room temperature. UV spectra were recorded at intervals. The reaction mixture was membrane-filtered (Millipore, Microcon YM-3, Bedford, MA) to separate the low molecular weight fraction from the high molecular weight fraction. The low molecular weight fraction was analyzed using LC/MS coupled with a photodiode array detector (PDA). Similarly, agaritine-COOH (0.086 mg) in 2.0 ml PBS was subjected to the same reaction using sodium glyoxylate (4.6 mg) and γ-GT (4.5 mg).

2.7. Agaritine degradation in microsomes

Pooled male human liver microsomes and male CD-1 mouse liver microsomes were purchased from Charles River Laboratories Japan. Microsomes (1 mg/ml) were incubated with NADPH regenerating system (Oriental Yeast Co Ltd.), uridine 5′-diphosphoglucuronic acid (UDPGA, Sigma), or 3′-phosphoadenosine 5′-phosphosufate (PAPS, Sigma) at 37 °C for 60 min. Decrease in agaritine content was monitored using LC/MS/MS.

3. Results

3.1. Determination of agaritine in mouse plasma and urine

To date, agaritine in blood samples has been determined by a scintillation counting method after administration of ^{14}C-radio labeled agaritine due to its higher sensitivity than the UV method (Walton et al., 2000). The total amounts of intact agaritine and its metabolites were given as a level of radioactivity. More recently, we have developed a sensitive and specific method for determining agaritine in mushroom samples using LC-coupled electrospray ionization tandem mass spectrometry (LC/MS/MS) (Kondo et al., 2006a,b). Determination of agaritine in mouse plasma and urine was also carried out using this method. As shown in Fig. 1B, there were no interference peaks on the chromatograms of the blank mouse plasma. The recovery of agaritine from the plasma spiked with 0.25 and 2.50 μg/ml of the synthetic agaritine was 77% ($n = 11$) and 90% ($n = 3$), respectively, and the spiked sample provided a distinct agaritine peak on the chromatogram (Fig. 1C). Application of our LC/MS/MS method to the mouse urine samples showed agaritine to be similarly distinctly identified without any interference peaks in the urine samples (data not shown). Multiple-reaction-monitoring (MRM) proved possible using two characteristic fragmentation ions (*m/z* 266 > 248 and 266 > 122) for agaritine. We used MRM (*m/z* 266 > 248) for the mouse plasma and urine samples because of their lower background.

We have next investigated the time course of the agaritine content in the mouse plasma after a single administration of the synthetic agaritine to mice. Agaritine appeared in the plasma 5 min after administration and the agaritine level peaked after 20 min. It then gradually decreased to the basal level over 100 min, as shown in Fig. 2. The limit of quantification using this method was 0.005 μg/ml plasma. Two different doses were administered: 4 and 40 mg/kg body weight. The time-courses were virtually the same. The pharmacokinetic parameters were calculated from these experiments. The maximum concentrations of agaritine in blood were 0.06 μg/ml (4.0 mg/kg p.o.) and 0.37 μg/ml (40.0 mg/kg p.o.), and the time to the maximum concentration was very fast (0.33 h) in both cases. The AUCs (area under the blood concentration–time curve) showed 0.06 μg ml^{-1} h (4.0 mg/kg p.o.) and 0.50 μg ml^{-1} h (40.0 mg/kg p.o.), which were different from the AUC

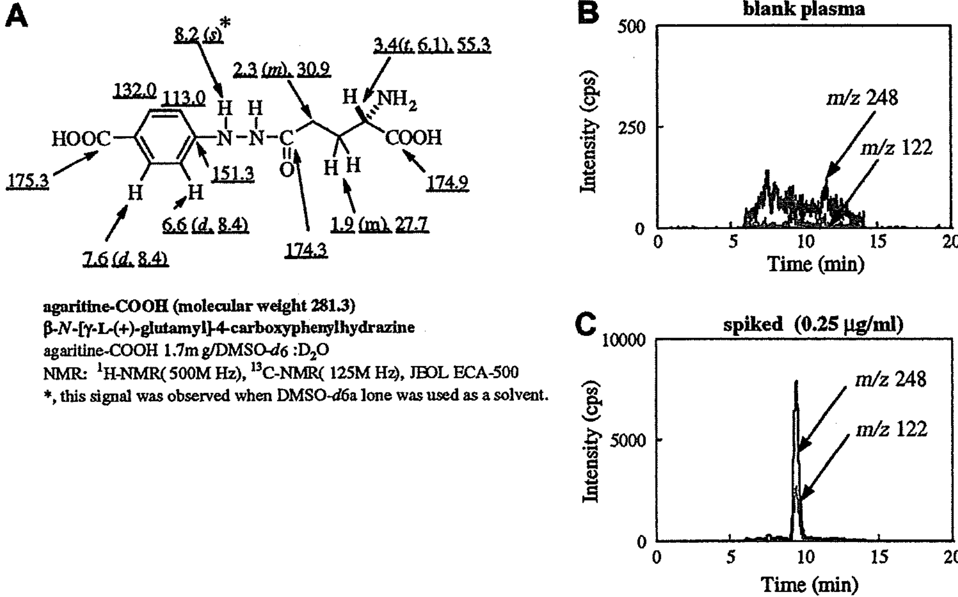
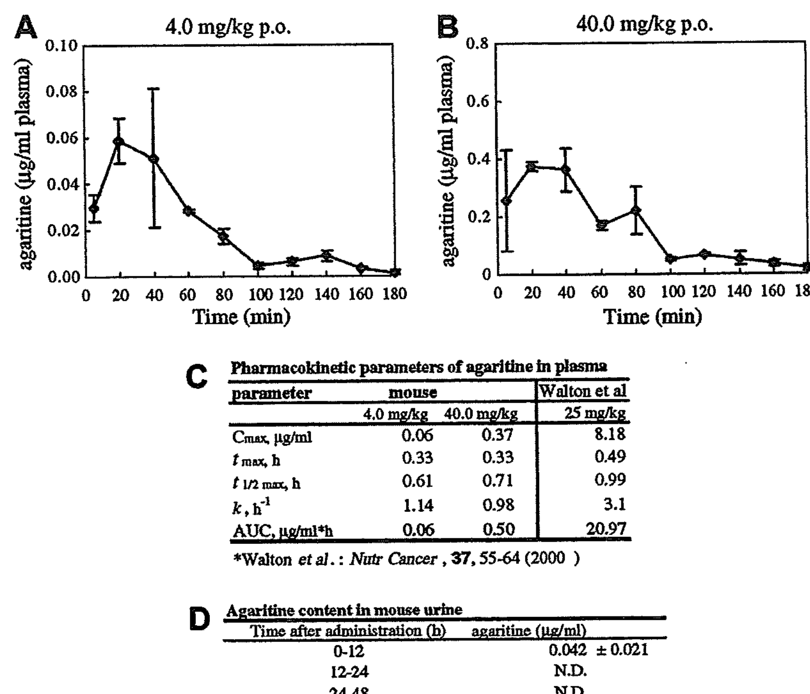


Fig. 1. Structure of agaritine-COOH and typical chromatograms of agaritine: (A) Structures and ^{1}H and ^{13}C NMR chemical shifts (δ). NMR measurements of agaritine-COOH in DMSO-d_6/D_2O were carried out using ECA-500; (B) MRM chromatogram of blank plasma; (C) MRM chromatogram of the plasma spiked with the synthetic agaritine (0.25 μg/ml). Two fragment ions were monitored simultaneously (*m/z* 266–248 in blue, *m/z* 266–122 in red). MRM (*m/z* 248) was used to determine agaritine content in mouse plasma. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)



C Pharmacokinetic parameters of agaritine in plasma

parameter	mouse		Walton et al
	4.0 mg/kg	40.0 mg/kg	25 mg/kg
C_{max}, μg/ml	0.06	0.37	8.18
t_{max}, h	0.33	0.33	0.49
$t_{1/2\,max}$, h	0.61	0.71	0.99
k, h^{-1}	1.14	0.98	3.1
AUC, μg/ml*h	0.06	0.50	20.97

*Walton *et al.*: *Nutr Cancer*, **37**, 55-64 (2000)

D Agaritine content in mouse urine

Time after administration (h)	agaritine (μg/ml)
0-12	0.042 ± 0.021
12-24	N.D.
24-48	N.D.

Fig. 2. Time course of agaritine content in the plasma and agaritine content in the urine collected from agaritine-administered mice. Thirty three mice were used. (A and B) Synthetic agaritine (4.0 mg/kg or 40.0 mg/kg) was orally administered to 8-week male ddY mice. After administration, blood was withdrawn every 20 min from three mice to prepare plasma samples. Agaritine concentration in the plasma peaked 20 min after administration. Values are means ±SD from three mice. (C) Pharmacokinetic parameters of agaritine in mice. C_{max}, maximum concentration; t_{max}, time to maximum concentration; $t_{1/2}$, half life, k, absorption rate constant; AUC, area under the blood concentration–time curve. (D) Agaritine concentration in the urine. Twenty mice were housed in 5 groups in metabolic cages (each group comprised 4 mice). The 0–12 h urine after agaritine administration contained agaritine. Values are means ±SD from 5 groups of mice.

value 20.97 μg ml^{-1} h (25 mg/kg p.o.) reported by Walton et al. The results are summarized in Fig. 2.

We also analyzed the agaritine content in the urine. Agaritine was detected in the urine collected between 0 and 12 h after agaritine administration (0.042 μg/ml urine). In the urine between 12 and 48 h or later after administration, no agaritine was detected as shown in Fig. 2D Taken together, these results suggest that agaritine is rapidly metabolized in mice.

3.2. Agaritine metabolites

Agaritine metabolites in the plasma and urine samples were investigated using LC/MS (total ion scans) coupled with DAD (total wavelength scans). The plasma samples (control and agaritine administration groups) were analyzed and the data acquired were compared to each other using Analyst 1.4.1. Fig. 3A and B show total ion chromatograms (TIC) and total wavelength chromatograms (TWC) of the plasma from the control group and from the agaritine-administered group, respectively. The TIC showed no difference between the two groups. To identify metabolites from the TIC of the two groups, we used Metabolite ID software (Applied Biosystems). However, no agaritine metabolites were found. On the other hand, one metabolite peak was detected in the TWC of the plasma samples 20 and 60 min after agaritine administration as shown in Fig. 3B. The metabolite peak became clearer on the chromatograms taken at UV 270 nm (Fig. 3C) and its UV spectrum was presented in Fig. 3D.

The urine samples were similarly investigated. The urine collected between 0 and 12 h after agaritine administration showed a distinct metabolite peak. The urine samples collected between 12 h and 48 h or later after administration did not show any such peak (Fig. 3E). The UV spectrum of the peak (λ_{max} 282 nm) was the same as that of the metabolite peak shown in the plasma, suggesting that the same metabolites are formed from agaritine in both mouse plasma and urine (Fig. 3F). However, the structure of the compounds remains to be solved, since the product was present only in trace quantities.

3.3. Oxidative stress

Agaritine is a phenylhydrazine derivatives, but the hydrazine moiety of agaritine (–NH–NH–) is masked by glutamic acid. Phenylhydrazine (Ph-NHNH2) can produce free radicals through free iron release, which causes anemia (Ferrali et al., 1997), suggesting that agaritine or its metabolites could subject mice to oxidative stress. We therefore investigated the effect of agaritine on oxidative stress in mice by measuring an oxidative stress marker 8-OHdG

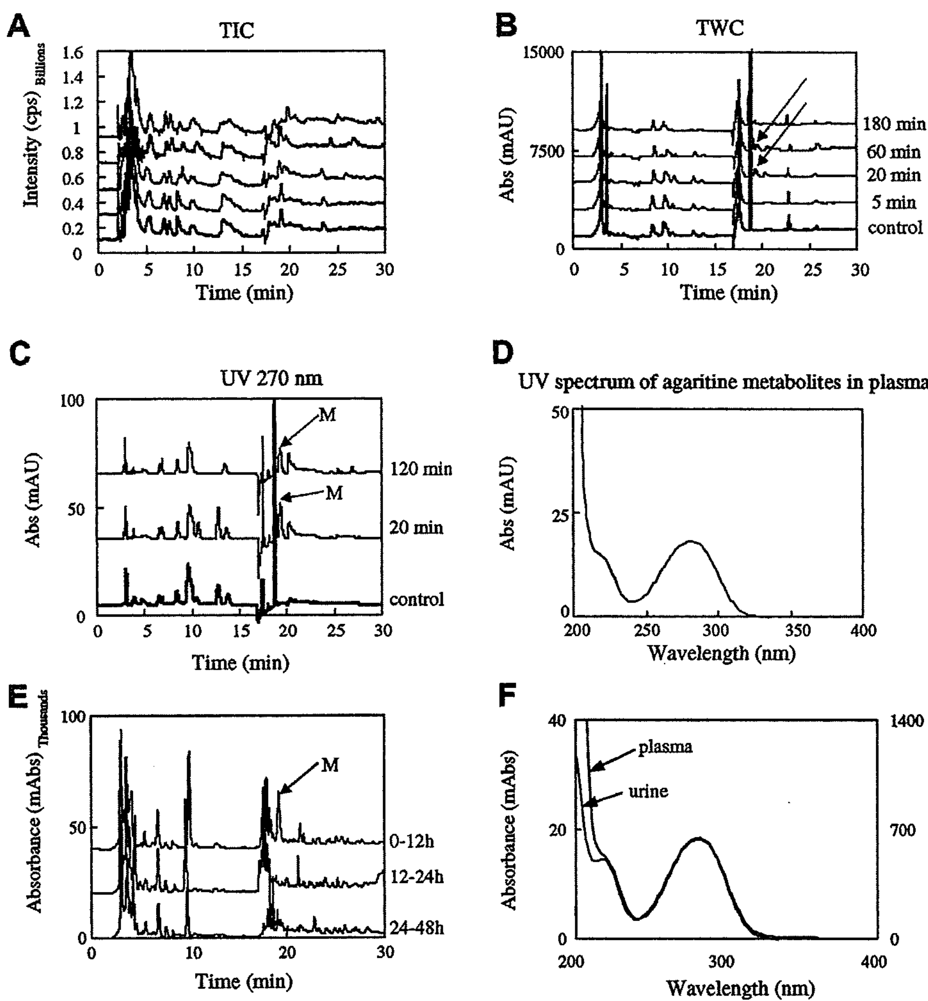


Fig. 3. Analyses of agaritine metabolites in mouse plasma and urine. LCMS coupled with PDA were used to investigate agaritine metabolites. (A and B) TIC and TWC of the plasma prepared from agaritine-administered ddY mice. The plasma samples after administration were analyzed (5, 20, 60, 180 min); the agaritine metabolite peaks were indicated by the arrows. (C) Chromatogram at 270 nm of the plasma samples (M = metabolite). (D) UV spectrum of agaritine metabolite in the plasma. (E) Chromatogram at 270 nm of the urine samples 12, 24, 48 h after administration. (F), UV spectrum of agaritine metabolite in the urine.

(8-hydroxy-2′-deoxyguanosine). As shown in Fig. 4, 8-OHdG levels in the urine collected between 0–12 h after administration almost tripled, then transiently decreased to the control level at 48 h after administration. The level then increased again and remained high for 11 days, suggesting that a single administration of agaritine may induce continuous oxidative DNA damage. In contrast, levels of malondialdehyde (MDA), another oxidative stress marker, in the urine were not significantly different between the control and the agaritine administration groups. The results of the two markers were not paralleled.

The changes in daily urine output between control and agaritine-administered mice were examined over 2 weeks. There was no significant difference in the daily urine volume, but there was a distinct tendency for the agaritine-administered group of mice to show lower urine output than the control mice during the experiments (Fig. 4A). The body weights of both groups steadily increased every week.

3.4. Degradation of agaritine in vitro

Agaritine is thought to be degraded by an enzyme γ-glutamyltranspeptidase (γ-GT), present at high levels in the kidney, to form 4-(hydroxymethyl)phenylhydrazine (HMPH), which can be a potent carcinogens. However, due to its instability, there have been no reports describing the direct detection of HMPH. We investigated agaritine degradation in mouse and human liver microsomes using LC/MS. Our results showed no decrease in intact agaritine, and no metabolites were found during one hour-incubation of agaritine in those microsome systems, suggesting that agaritine is stable in the liver microsome systems (data not shown).

In contrast, agaritine was degraded by γ-GT to form a conjugated product with sodium glyoxylate, which showed UV absorption at 325 nm (Fig. 5). This result is in good agreement with the data previously published else where (Gigliotti and Levenberg, 1964). The enzymatic reaction

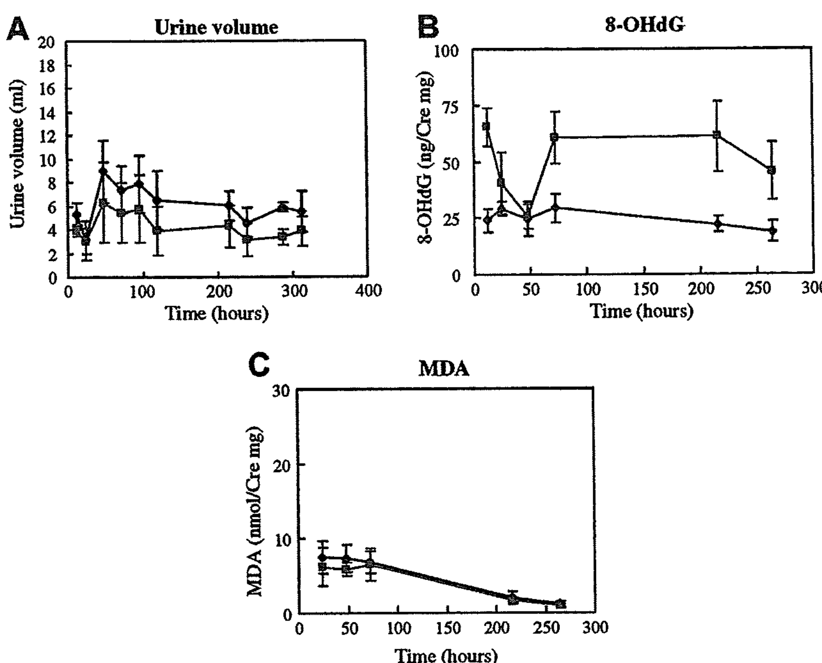


Fig. 4. Effect of agaritine on oxidative stress. Forty mice were divided into 5 control and 5 agaritine administration groups. Each group comprised 4 mice. Mouse urine was collected using metabolic cages. (A) Urine volume per day during the experiments; (B) 8-OHdG level in the urine. Oxidative stress marker 8-OHdG was measured in the urine collected from agaritine-administered mice. The urine was collected 12, 24, 48, 72 h, 216, 264 h after administration; (C) MDA level in the urine. Values are means ±SD ($n = 5$ groups). ◆ (black), control; ■(red), agaritine administration group. 8-OHdG and MDA levels are described as ng or nmol per 1 mg creatinine. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

completed within 180 min. However, there is as yet no data on where the absorption comes from. No investigations have been carried out. The reaction mixture was therefore separated through a membrane filter to form two fractions, low and high molecular weight fractions. The UV spectra were then recorded separately. The low molecular weight fraction showed UV absorptions at 292 and 325 nm, suggesting that a UV-absorbing substance, newly formed by the enzymatic reaction, was derived from a low molecular compound, not from a high molecular weight compound like γ-GT. Agaritine-COOH was similarly examined, showing that this compound was also degraded by γ-GT.

Moreover, when the low molecular weight fraction was subjected to LC/MS analysis, no peak with the absorption at 325 nm was detected (data not shown). It is possible that the high pressure in the HPLC system may decompose the product. Flow injection analysis (FIA) was carried out and one absorption peak at 325 nm was observed (Fig. 6). This peak had the same UV spectrum as that in Fig. 5 and, in addition, showed an m/z of 195 in LC/MS analysis, indicating that HMPH was indeed produced from agaritine by γ-GT though hydrazone structure.

4. Discussion

Agaritine is a major hydrazine rich in *Agaricus* spp. of mushrooms. This compound is believed to be degraded to metabolites such as HMPH and HMBD, which show carcinogenicity (Toth et al., 1978, 1981; Toth and Nagel, 1981; Walton et al., 1997). However, there has been no direct evidence of HMPH and HMBD formation from agaritine, and agaritine metabolism is still unclear. Ross et al. failed to detect agaritine in blood (Ross et al., 1982) due to the lack of sensitivity of their method. Walton et al. reported agaritine metabolism in the mouse and rat. Agaritine content in the blood was measured using liquid scintillation counting. After the administration of a single dose of radio-labeled agaritine, radioactivity peaked in 0.49 h and then gradually decreased over the next 12 h (Walton et al., 2000). The radioactivity in the plasma showed the total amounts of agaritine and its metabolites. They, however, failed to detect intact agaritine in the plasma or urine. We have measured agaritine in blood and urine samples using the LC/MS/MS method, which is highly specific. Agaritine appeared 5 min after agaritine administration to ddY mice, peaked in 20 min and then decreased to base level. The pharmacokinetic parameters calculated were similar to those reported by Walton et al. However, the maximum concentration of agaritine ($C_{max} = 0.37$ vs. 8.18) and area under the blood concentration–time curve (AUC = 0.50 vs. 20.97) were different. We analyzed agaritine in mouse plasma whereas Walton et al. measured radio-labeled agaritine in blood. The recovery rates of agaritine from mouse plasma were 77% and 90% as mentioned previously (Kondo et al., 2006a). We also analyzed agaritine in mouse whole blood, which showed a similar pattern of recovery (data not shown). Thus, the agaritine content we detected in the plasma is equal to