労災疾病等 13 分野医学研究・開発、普及事業

分野名「化学物質の曝露による産業中毒」

「産業中毒の迅速かつ効率的な診断法に係る研究・開発、普及」

研究報告書

平成２５年１２月

独立行政法人　労働者健康福祉機構

産業中毒の迅速かつ効率的な診断法に係る研究・開発、普及

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Ⅰ　研究の概要

「産業中毒の迅速かつ効率的な診断法に係る研究・開発、普及」

本研究は産業化学物質による健康障害を収集し、それらの診断法や診断・治療に役立つ曝露指標の研究・開発、普及を目的としている。そのため産業中毒研究センター及び環境医学研究センターに寄せられた相談事例及び症例を検討し、特異的診断法の検討および問題となる化学物質の曝露指標を特定し、種々の分析技術を活用することにより、診断項目および検査項目として確立するとともに、これらの成果と化学物質に関する情報を労災疾病研究のホームページから発信した。

1）症例の収集

主な症例収集としては、低濃度化学物質曝露による健康障害を訴えてシックハウス診療科を受診した患者を対象とした。受診者に対し、問診、血液生化学検査、精神心理検査、神経眼科的検査、免疫学的検査、及び付加的に吸入ばく露負荷試験を実施した。関西労災病院の受診者 391 名におけるこれらの検査の結果などを 2 期に分けてまとめた。検査受診者数とその診療受診者に占める割合を括弧内に示した。各項目の%は検査者に占める割合である。

対象者	期　　間	人　数			平均年齢
		男	女	合計	
第 1 期	2005 年 6 月から 2008 年 9 月末まで	62	195	257	42±15
第 2 期	2009 年 4 月から 2012 年 12 月末まで	46	88	134	47±15
合計	2005 年 6 月から 2012 年 12 月末まで	108	283	391	45±15

検査結果

①　血液検査

項目	第一期 (N=195、76%)		第二期 (N=65、49%)		合計 (N=260、66%)	
	N	%	N	%	N	%
FreeT3	49	26	3	5	52	20
総コレステロール	46	25	19	32	65	25
IgE	42	21	19	39	61	23
白血球数	25	12	3	5	28	11
ヘマトクリット値	23	11	12	18	35	13

② 精神心理検査-不安

STAI 特性不安	第一期 (N=239、93%)		第二期 (N=122、91%)		合計 (N=361、92%)	
	N	%	N	%	N	%
非常に不安傾向が強い	81	32	38	31	119	33
不安傾向がある	82	32	36	30	118	33
不安傾向なし	75	29	48	39	123	34

③ 精神心理検査-POMS

POMS の判定	第一期 (N=234、91%)		第二期 (N=116、87%)		合計 (N=350、90%)	
	N	%	N	%	N	%
専門医の受診を考慮	41	16	41	35	82	23
他の訴えと合わせて専門医の受診を考慮	158	61	52	45	210	60
健常	36	14	23	20	59	17

④ 瞳孔反応検査

項目	第一期 (N=194、76%)		第二期 (N=79、60%)		合計 (N=273、69%)	
	N	%	N	%	N	%
最小縮瞳潜時	9	5	3	4	12	4
63%回復潜時	18	9	8	11	26	10

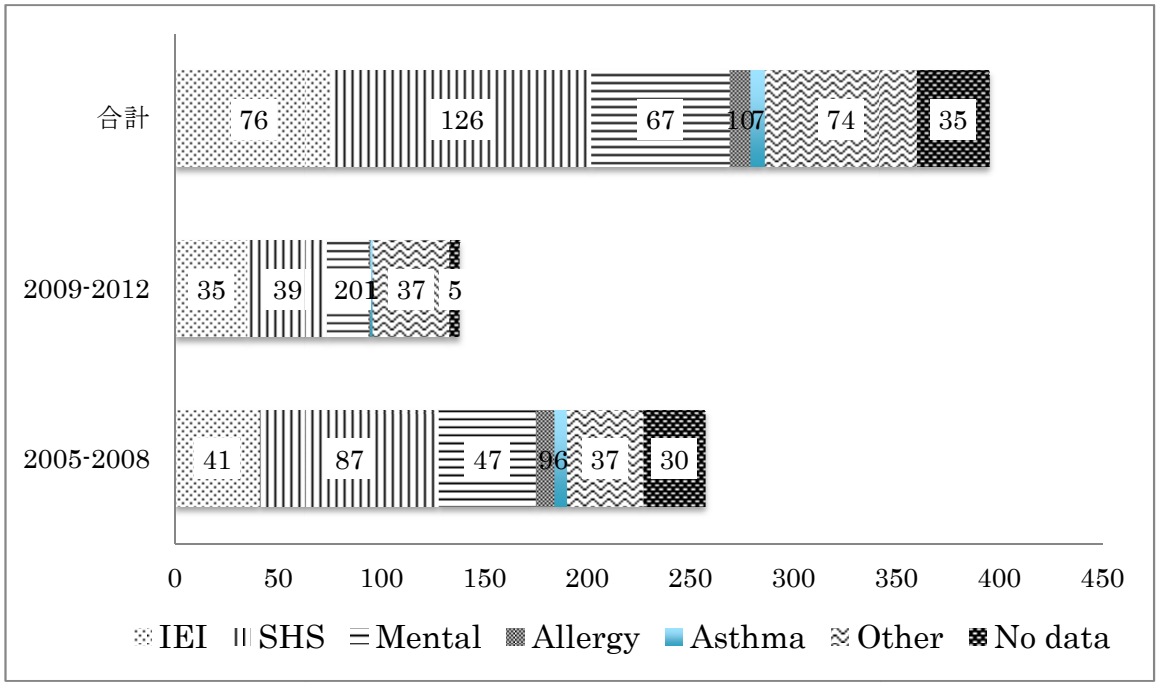
⑤ 視標追跡検査

項目	第一期 (N=198、77%)		第二期 (N=64、48%)		合計 (N=262、67%)	
	N	%	N	%	N	%
0.3 Hz	4	2	4	6	8	3
0.5 Hz	6	3	1	2	7	3

⑥　アレルギー検査

アレルゲン	第一期 (N=200、78%)		第二期 (N=68、51%)		合計 (N=268、69%)	
	N	%	N	%	N	%
スギ	90	45	43	63	133	50
ブタクサ	80	40	5	7	85	32
ダニ	65	32	35	51	100	37
オオアワガエリ	39	19	27	41	66	25
ハルガヤ	39	19	20	30	59	22

診断結果



症例収集のまとめ

•受診者は 70%が女性、平均年齢は 45 歳と中年女性が主な受診者であった。

•精神心理検査では、不安傾向のある患者が半数を超え、専門医の要受診者が 20%を占めており、メンタル対応が必要と考えられた。

•眼球運動検査の陽性率は非常に低く、特異診断とは考え難い。

•総 IgE 高値や環境アレルゲンの陽性率が高く、アレルギーとの相加作用が示唆された。

•全体を通した診断結果は、シックハウス症候群疑い 32%、特発性環境不耐症（IEI）疑い 19%、精神疾患疑い 17%、その他 32%であったが、第 1 期に比べ、第 2 期の受診者総数は半減していた。

症例報告

1-1) ホルムアルデヒド(FA)特異的 IgE 関連蕁麻疹と考えられた患者に室内環境濃度測定や吸入負荷試験を実施した結果、負荷試験は陰性であったが、気中 FA 濃度は指針値を超えており、パッチテスト陽性、FA-IgE 陽性、総 IgE 高値、そしてその後の換気による症状の消失から室内気中FAによるアレルギー性蕁麻疹と診断した事を報告した(Jpn J Occup Med Traumatol, 57:125-129, 2009)。

Case Report

Formaldehyde-specific IgE-mediated Urticaria Due to Formaldehyde in a Room Environment

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Abstract

Formaldehyde is a primary skin-sensitizing agent inducing allergic contact dermatitis, and may induce immunological contact urticaria. However, there have been few reports on allergy associated with IgE. We describe here a case of formaldehyde-specific IgE mediated urticaria due to formaldehyde in a room environment.

A 36-year-old woman exhibited urticaria on her legs and face. Laboratory examination revealed elevated formaldehyde-specific IgE and non-specific IgE. Patch testing with formaldehyde was positive. The concentrations of formaldehyde in the rooms of her apartment were high.

Although inhalation challenge test was negative, we suspected that her urticaria resulted from IgE-mediated allergic reaction to formaldehyde in the rooms of her apartment.

Her symptoms gradually disappeared with thorough ventilation of the rooms of her apartment.

(JJOMT, 57: 125—129, 2009)

—Key words—
formaldehyde, IgE, urticaria

Introduction

Recently, health problems such as allergy, headache, and sore throat due to chemical substances derived from building materials have been reported[1-4]. These problems are usually termed 'sick building syndrome' and formaldehyde is a representative cause of this syndrome[5].

In patients with allergic reaction thought to be due to formaldehyde, elevated formaldehyde-specific IgE levels are seldom observed. Moreover, there have been few reports on allergic reaction associated with formaldehyde-specific IgE even in individuals handling formaldehyde occupationally. We report here a case of formaldehyde-specific IgE-mediated urticaria due to formaldehyde in a room environment.

Case

Ten months after a 36-year-old woman moved to a new apartment, she sometimes noted urticaria on her legs and face when she was at home. The frequency of urticaria gradually increased, and she also noted swollen lips and slight dyspnea. She experienced no symptoms outside of her apartment.

She underwent a medical examination by dermatologists and was diagnosed with stress-related urticaria. However, on medical examination by another physician, laboratory examination revealed elevated formaldehyde-specific IgE and non-specific IgE levels. She was a housewife and had not handled formaldehyde occupationally. Since her symptoms occurred after her move, she visited our hospital because she believed they resulted from her indoor environment.

Although no members of her family, including her husband and child, recognized a smell in the rooms of

Table 1 Results of indoor air quality investigation

	Formaldehyde (μg/m³)
Japanese-style room	150
Western-style room 1	90
Western-style room 2	130
Living room	100
Guideline value	< 100

Table 2 Two-color CD4/CD8 analysis

		Reference values
CD4(−)CD8(+)	42.3%	230−810
CD4(−)CD8(−)	13.0%	140−350
CD4(+)CD8(−)	44.3%	280−580
CD4(+)CD8(+)	0.4%	< 70
CD4/CD8 ratio	1.05	0.40−2.30

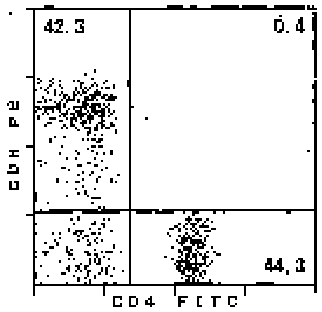

Fig. Two-color CD4/CD8 analysis (Dot plot). The vertical axis shows the fluorescent strength of CD4 and the horizontal axis shows the fluorescent strength of CD8.

Table 3 Results of measurement of hypothalamic-pituitary-adrenal axis hormones, interleukins and substance P.

		Reference values
CRF	23.7 pg/ml	
ACTH	24.4 pg/ml	7.4−55.7
Cortisol	11.5 μg/dl	4.0−18.3
IL-1β	14 pg/ml	(< 10)
IL-2	< 0.8 U/ml	
IL-4	6.1 pg/ml	(< 6.0)
IL-5	< 5.0 pg/ml	(< 4.0)
IL-6	0.5 pg/ml	(< 4.0)
Substance P	25 pg/ml	

the apartment, and none exhibited symptoms after the move, the formaldehyde concentrations in the rooms of her apartment were found to be higher than the guideline values for indoor air concentration of formaldehyde in Japan (Table 1).

From examination results at our hospital, she had no abnormal findings, including those of dermatological examination. Chest X-ray and electrocardiogram were both normal. WBC count was 3,800/μl (eosinophils 4.2%) and CRP was negative. Biochemical findings and results of urinalysis were all normal. However, non-specific IgE was elevated to 317IU/ml (normal range: <173IU/ml) and formaldehyde-specific IgE (FEIA) was also positive (1.08UA/ml). Among other specific IgEs, only IgE for cedar pollen was positive on the MAST-26 assay.

Lymphocyte surface markers (two-color CD4/CD8), some chemical mediators such as interleukin (IL)-1β, 2, 4, 5, and 6, and hypothalamic-pituitary-adrenal axis hormones were measured. The results of two-color CD4/CD8 analysis were normal (Fig. and Table 2). The results of measurement of ILs and substance P are shown in Table 3. Although IL-1 and IL-4 levels were slightly elevated, levels of other ILs were within reference ranges. Substance P level was almost the same as normal levels previously reported[4]. CRF, ACTH, and cortisol levels were 23.7pg/ml, 24.4pg/ml, and 11.5μg/dl and not abnormal.

After receiving informed consent, we performed a formaldehyde inhalation test at 100μg/m³ for 15 minutes in a room with the area of 5m². During the inhalation test, she noted a slight smell and headache, but exhibited no cutaneous symptoms during or after inhalation. Moreover, on pulmonary function testing, there were no significant differences in forced expiratory volume or the ratio of forced expiratory volume in one second before to after the challenge test (Table 4).

We also performed a patch test using 0.01% formaldehyde solution, with positive results after both 48 and 72 hours.

She had a past history of chronic eczema from childhood and took an H_1 blocker. However, she had experienced no symptoms of eczema for several years.

Although she exhibited no symptoms on examination, we diagnosed formaldehyde-specific IgE-mediated urticaria due to formaldehyde in a room environment on the basis of the following findings: 1) the formaldehyde levels in the rooms of her apartment were relatively high, and her symptoms appeared after moving to

Table 4 Pulmonary function tests

			Actual value	Predicted value	Percentage of predicted value
Pre-inhalation	VC	L	3.48	3.69	93.0%
	FVC	L	3.36	3.62	92.6%
	FEV1.0	L	3.02	3.45	87.0%
	FEV1.0%	%	89.81	92.71	102.7%
Post-inhalation	VC	L	3.48	3.69	93.0%
	FVC	L	3.25	3.69	87.0%
	FEV1.0	L	3.07	3.45	89.0%
	FEV1.0%	%	97.97	92.71	105.7%

the new apartment, 2) sensitization to formaldehyde was recognized, 3) there were no other substances to which sensitization was noted except cedar pollen, and 4) she was a housewife and has not handled formaldehyde occupationally.

Her symptoms gradually disappeared with thorough ventilation of the rooms of her apartment.

Discussion

Formaldehyde solution is a primary skin-sensitizing agent inducing allergic contact dermatitis (Type IV), and may induce immunological contact urticaria (Type I)[1]. Allergic reactions and the induction of asthma-like conditions have been reported following occupational exposure to it[2-6]. However, there are reports that asthma-like symptoms caused by formaldehyde are not due to allergic reaction[7,8]. Krampe et al.[9] mentioned that formaldehyde-specific IgE antibodies could be detected in only one of 86 serum samples from four groups of individuals exposed to formaldehyde by different routes and concentrations, and concluded that exposure to formaldehyde, even in relatively high concentrations, rarely evokes the production of specific IgE antibodies. Moreover, no RAST-positive findings for formaldehyde were observed in a group of subjects, 46% of whom had problems related to formaldehyde[10]. There is also a report that it is possible that clinical IgE-mediated allergy to gaseous formaldehyde does not exist or that if it does exist it is extremely rare[11]. These reports suggest that although formaldehyde can cause IgE-mediated allergy, this condition is very rare.

In the present case, the patient's chief complaint was urticaria, and allergic urticaria typically involves IgE. The methods of determination of the causative agents of allergy include RAST, prick testing, basophil histamine-release testing, among others. There are reports that type I allergy tests sometimes yield false-positive or false-negative findings[8,12]. Therefore, for the present patient we performed a formaldehyde provocation challenge test to determine the causative agent, although no cutaneous symptoms appeared during or after inhalation. Although we were unable to obtain conclusive evidence that her urticaria resulted from formaldehyde, we believe that humoral immunity contributed to her urticaria.

The duration of challenge testing we performed was 15 minutes at around 100μg/m³, and testing was performed twice over a three month period. Since this patient was a housewife and had spent most of her time at home, further investigation is needed to determine whether the protocol for challenge testing we used had been appropriate.

Concerning patch testing, Trattner et al.[5] noted the lack of statistically significant difference in response between 1 and 2% levels with respect to allergic reactions, but reported that 2% yielded significantly more irritant reactions, and thus recommended a 1% patch test concentration for formaldehyde. There is a report that for the general population, dermal exposure to concentrations of formaldehyde in solution in the range of 1–2% is likely to cause skin irritation[3]. In the present case, we used 0.01% formaldehyde solution, and results were positive after both 48 and 72 hours. We therefore believe that cell-mediated immunity also played a role in this patient's urticaria. Based on her lifestyle, we strongly suspect that formaldehyde in her room environment was the cause of sensitization.

There is a report that the hypothalamic-pituitary-adrenal gland axis is affected by low concentrations of formaldehyde[13]. However, in the present case no abnormalities in hormone levels related to it were observed.

In addition, measurement of several factors related to allergic reaction, such as interleukins, lymphocyte surface markers, and substance P, revealed almost no abnormalities.

In the present case, testing for formaldehyde-specific IgE and patch testing with formaldehyde were positive. As noted above, this urticaria appears to have been the result of allergic reaction, and is thus very rare.

Acknowledgement

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室内環境中ホルムアルデヒドによるホルムアルデヒド特異的IgE関連蕁麻疹

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—キーワード—
ホルムアルデヒド，IgE，蕁麻疹

ホルムアルデヒドは，皮膚感作物質であり接触性蕁麻疹を引き起こしうる．しかしながら，IgEに関連したアレルギーに関する報告はわずかしかない．われわれは今回，室内環境中のホルムアルデヒドが原因と考えられるホルムアルデヒド特異的IgE関連蕁麻疹と考えられる症例を経験したので報告する．症例は35歳の女性で，新築マンションに転居後数ヶ月に蕁麻疹を発した．他院での検査にて，ホルムアルデヒド特異的IgEの上昇と非特異的IgEの上昇を認め，ホルムアルデヒドのパッチテストも陽性であった．自宅マンションの室内のホルムアルデヒド濃度は，厚生労働省の室内濃度指針値を超えていた．ホルムアルデヒド吸入負荷テストを行うも環境は整備されず困難であったが，われわれは特異的IgEの上昇，パッチテスト陽性，室内のホルムアルデヒド濃度の結果からこの蕁麻疹がマンション内のホルムアルデヒドに対するIgEに関連したアレルギー反応であると考えた．その後部屋の換気を行い経過観察としたが，症状は生じていない．

（日職災医誌，57：135—139，2009）

1-2) 関西労災病院の受診者では、オフィスの改築によるトルエン(Tol)、キシレン、総揮発性有機化合物曝露によるシックビルディング症候群の集団発生があったので、患者のプロフィール、社員アンケート及び経時的室内環境濃度の推移等を検討して報告した(産衛誌, 53:25-32, 2011)。

産業衛生学雑誌 2011; 53: 25-32

産業衛生学雑誌

報告

シックビル症候群患者の臨床所見並びに環境測定結果について

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抄録：シックビル症候群患者の臨床所見並びに環境測定結果について：吉田辰史ほか．関西労災病院環境医学研究センター―目的：シックハウス症候群の報告は数多くあるが，日本における最新性シックビルディング症候群（SBS）の詳り報告は限られている．われわれは同一会議室において臨床的な観察と環境濃度測定を実施した．対象と方法：オフィス内に新設した学会事務室内部の改装工事後に体調不良を訴え，当科を受診した非喫煙者11名（男性2名と女性9名）に問診，血液一般および生化学検査，免疫学的検査，耳鼻咽喉科的検査および精神心理検査を実施し，事務室内環境測定を改装後27日後，39日後，132日後の3回実施した．非受診者（男性21名と女性1名）においては自記式質問紙調査を実施した．結果：改装後には工事終了9日後に仕上を終えたが，その直後に大半の従業員が異臭を感じ，頭痛，倦怠感，集中力の低下などの異常を訴えた．改装に使用された塗料にアクリル樹脂塗料で，室内空気内のトルエン，キシレン，総揮発性有機化合物（TVOC）の室内濃度は2,972，2,610，7,700 μg/m³であったが，132日後には，78，113，261 μg/m³に低下していた．結論：自覚症状，アレルギー等の他の客観的疾患の検査に特定的なこと，環境測定結果から，受診者11名のうち女性7名をSBSと診断した．非受診者（男性21名と女性1名）における質問紙の回答でも，異臭や体調などの訴えの出現と消滅の時期が室内のトルエンなどの濃度推移と一致していたことから，SBS診断の妥当性が確認された．
（産衛誌 2011; 53: 25–32）

キーワード：Construction and painting, Sick building syndrome, Toluene, Total volatile organic compounds, Xylene

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I．はじめに

欧米では，1970年代後半から1980年代にかけて，熱効率を良くするために外気取り込み量を減らした建築による健康被害が個室型の広い事務所ビルで問題となり，「シックビル症候群」（Sick Building Syndrome, SBS）として社会問題となった[1,2]．日本においては，「建築物における衛生的環境の確保に関する法律」により換気量が規定されていたことから，事務所ビルにおけるいわゆる一般住宅の新築や改築時において健康障害が問題となり，「シックハウス症候群」（Sick House Syndrome, SHS）と呼ばれ社会問題としてマスコミ等に取り上げられた[3]．その原因として建材などから発生するホルムアルデヒド，有機塩素系やフタル酸エステルなど揮発性有機物が問題とされ[4,5]，厚生労働省は13種の化学物質に対しては室内濃度指針値を設定している[6]．さらにこれらの濃度には，カビやダニ等生物学的汚染[7-11]，精神心理的要因の関与も指摘できないと考えられている[7,12,13]．日本で発生された SBS事例のうち，高濃度のホルムアルデヒドが原因と推定された事例では，心理テストで不安傾向が認められていた[14]．疫学調査では，受動喫煙と女性職場をSBSのリスク要因とする報告がある[15,16]．

われわれは，企業内会議室に使用された塗料中の有機溶剤揮発により，体調不良を訴えた事務職員11名を診察する機会を得た．彼らはシックビル症候群の典型例と考えられたので，受診者の臨床所見・臨床検査結果，非受診の従業員に対する質問紙調査および室内環境測定の結果を報告する．

II．対象と方法

1．受診者

従業員数41名の金型業のオフィスである受診者の職場では，某年9月に開設した電動機および耐火金庫室の塗装工事終了8日後に業務を開始したが，その直後から多くの職員が会社室内での異臭を訴えた．また，一部の職員は，頭痛，倦怠感，咽の刺激，集中力や思考力の低下，咳などの症状を訴えた．金庫室は業務の都合上，職員の出入りが多く業務時間内は常に開放状態で使用されていたので，職員は異臭を感じるもそのままの状態で業務を続けていた．職員のうち，体調不良を訴えた11名（男性2名，女性9名，年齢38±11歳，23-57歳）が，業務開始50日後に当院シックハウス外来を受診した．

受診者においては問診，血液一般・血液像および生化学検査（アスパラギン酸アミノトランスフェラーゼ，アラニンアミノトランスフェラーゼ，コリンエステラーゼ，乳酸脱水素酵素，総コレステロール，電解質（Na, Cl, K, Ca），血中尿素窒素，クレアチニン，クレアチニンキナーゼ，アルブミン，総蛋白，[illegible]，C反応性蛋白，甲状腺刺激ホルモン，遊離T_3，遊離T_4），免疫学的検査（総IgE，特異的IgE（環境アレルゲン14項目，食物アレルゲン12項目）），肺機能検査（呼吸機能検査，1秒量，1秒率：スーパースパイロ DS-21 FS[illegible]，チェスト（株），東京），瞳孔反応検査（イリスコーダー C9364，浜松ホトニクス，浜松），眼球追従検査（メディテスター VOG CI2001，松下電工，門真），精神心理学的検査として，不安状態と不安になりやすい性格傾向を測定するState-Trait Anxiety Inventoryの日本語版（日本版STAI，三京房，京都），および気分や感情の変化を測る検査であるProfile of Mood Statesの日本語版（日本版POMS，金子書房，東京）を実施した．

シックビル症候群の診断については，WHO欧州地域事務局の5つのクライテリアおよび米国環境保護庁が示している2つのクライテリア[2]を参考に以下の4項目を満たすものとした：①眼，鼻等の粘膜刺激症状が主であること，②室内居者の多数が症状を訴えること，③その場所を離れると症状が改善すること，④種々の検査によってアレルギー疾患および甲状腺機能などの他疾患が否定できること．

2．室内環境測定

事務室の使用開始直後に臭いに関するクレームが職員から出たことから，室内環境測定を業者に依頼した．測定は換気が行われない条件下で行い，事務室，耐火金庫室，または女子更衣室において塗装工事終了後27日，55日，132日の3回実施した．各部屋の面積は，事務室221 m²，耐火金庫室43 m²，女子更衣室14 m²であった．厚生労働省の標準的な方法[3]に従い，事務室は2ヶ所，他の部屋は中央部とし，各箇所において高さ1.2 mで室内空気を拡散方式により24時間採取した．測定対象物質は，ホルムアルデヒド，トルエン，キシレン，エチルベンゼン，スチレン，パラジクロロベンゼン，および総揮発性有機化合物（T-VOC）であった．ホルムアルデヒドは2,4-ジニトロフェニルヒドラゾン（DNPH）誘導体化固相吸着／溶媒抽出－高速液体クロマトグラフ法により測定した．環境空気中のホルムアルデヒドをDNPH拡散型サンプラー（拡散サンプラーDSD-DNPH，シグマアルドリッチ，東京）に捕集した．サンプリング終了後，DNPH誘導体を5 mlのアセトニトリルで溶出させ，その溶液5 μlをHPLC（HP1100，アジレント社，米国）にて分析した．分析カラムはアジレント社製ZORBAX-Bonus-RP（150 mm×4.6 mm×5 μm）を用い，カラム温度を40℃に設定し，移動相としてアセトニトリル／水（[illegible]）を1 ml/minの流量で流し，360 nmの吸光度により定量した．その他の物質は，固相吸着／溶媒抽出－ガスクロマトグラフ質量分析法により実施した．環境空気中のトルエンなどを拡散型捕集剤サンプラー（パッシブガスチューブ有機溶剤用，柴田科学，東京）に捕集した．サンプリングレートはマニュアル記載値を用いた．サンプリング終了後，トルエン-d8を内部標準とし，2 mlの二硫化炭素で溶出させ，その溶液1 μlをGC-MS（QP2010，島津製作所，京都）にて分析した．分析カラムはJ&W社製DB-1（0.25 mm×60 m×0.5 μm）を用い，SIMにて検出．測定質量数は，厚労省の測定法[4]に従った．得られたピークのうち，ヘキサンからヘキサデカンまでの総検出量をトルエンに換算してT-VOCを求めた．定量下限値（μg/m³）は，ホルムアルデヒド10，トルエン26，キシレン87，エチルベンゼン380，スチレン22，パラジクロロベンゼン24であった．

3．質問紙調査

受診者以外の従業員も含めた職員の室内環境への認知を明らかにする目的で，非受診者を対象に塗装工事終了後3ヶ月において質問紙による調査を実施した．質問内容は塗装工事後の異臭や不快感の有無，それらを感じた期間などで，われわれが独自に作成した．なお，記入は無記名とした．

4．倫理的配慮

受診者および当該企業においては，研究への参加および論文作成の承諾を得るとともに，本研究の実施に当たっては当院倫理委員会の承認を受けた．

III. 結　　果

1. 受診者における結果

受診者のうち4名は受診時に症状は消失していたが，女性7名では症状が続いていた．業務復帰後に起こった主な自覚症状は，頭痛，倦怠感，集中力の低下，咳，眼の刺激などで，それらの発症頻度をTable 1に示した．これらの症状は，帰宅すると消失または軽減していた．全員が非喫煙者であった．血液一般および生化学検査においては，TSHの高値を示すものが女性に1名いたが，遊離T_3および遊離T_4は正常範囲内であった．女性2名の総IgEが高値を示していたが，他の生化学検査項目は全員に異常を認めなかった．スギ花粉のアレルギーを訴えたものは女性3名で，特異的IgEのスギ陽性者が女性5名，ダニ，オオアワガエリおよびヨモギの陽性者が各女性2名で，他は全員陰性であった．呼吸困難，咳，咽頭痛を訴えたものは女性で7名いたが，肺機能検査においては全員に異常を認めなかった．瞳孔反応検査（63%回復時間（T63））および視標追跡検査（0.3 Hz）においては全員が正常範囲[15,16]であった．精神心理学検査のSTAIは不安になりやすい性格傾向を「特性不安」，不安状態を「状態不安」として評価し，5段階にスケール化している[20]．特性不安は「非常に高い」が3名，「高い」が2名，「普通」が5名，「低い」が1名，「非常に低い」は零，状態不安は「非常に高い」が3名，「高い」が1名，「普通」が4名，「低い」が1名，「非常に低い」は零だった．POMSは気分・感情の状態を6つの尺度で表し，判定の基準として全ての得点が40-60点を「正常」，一つでも25点以下や75点以上の場合「専門医の受診を考慮」，それ以外を「他の点数と考えあわせ，専門医の受診を考慮」としている[21]．40-60点以外の受診者は4名でその内訳は，「緊張－不安（T-A）」が3名，「抑うつ－落ち込み（D）」は1名，「怒り－敵意（A-H）」は3名，「活気（V）」は2名，「疲労（F）」は3名，「混乱（C）」は3名であった．そのうち75点以上は2名でT-Aに1名，A-Hに2名認められ，心理的に不安定な状態と判定された．

以上の結果，受診者に該当する症状があり，職場以外では症状が改善され，器質的疾患がみられなかったことから，前述の診断基準に従い，11名中7名をSBSと診断した．

2. 室内環境と環境測定結果

会議室内の天井，壁面，扉などを塗装したアクリル系樹脂塗料の製品安全データシート記載の成分をTable 2に示した．施工業者からの工事内容書によると，事務室の床はナイロン製のタイルカーペットで接着剤としてウレタンアクリル系樹脂エマルジョンが使われ，壁には塩化ビニル製の壁紙クロスが酢酸ビニル系接着剤で貼られた．会議室内の金属扉として，キシレン10-11％，エチルベンゼン89-98％，ホルムアルデヒド0.1-1.0％を含有したアミノアルキド樹脂塗料が使われていた製品を購入した．このアミノアルキド樹脂塗料以外の全ての建材に，ホルムアルデヒドは含有されていないことになっていた．トルエンが含有されていたのは前述のアクリル樹脂塗料のみであった．

Figure 1に示すように，塗装工事終了9日後にオフィスの使用を開始したが，会社側は異臭や体調不良を訴える従業員が多かったため，その工事終了後15日に会議室内に排気ダクトを設置し，業務終了時から翌日の業務開始時まで毎日夜間排気を行った．

事務所内の測定点配置図をFig. 2に，その測定結果をTable 3に示した．事務室内の測定点は2ヶ所とし，異臭の訴えが女子従業員に多かったため，女子更衣室の測定も2および3回目に加えた．

塗装工事終了27日後の第1回の測定では，事務室内のトルエン濃度が室内濃度指針値の約11倍，キシレン濃度が約3倍，T-VOC濃度が約13倍であったが，他の物質の濃度は指針値の半分以下であった．また，トルエン，キシレンおよびエチルベンゼンの合計濃度は6,748 μg/m³となり，T-VOC濃度の95％を占めていた．更衣室では，ホルムアルデヒドとトルエンのみ検出されたが，いずれも指針値の1/4-1/2の濃度であった．

塗装工事終了55日後の第2回の測定においても，会議室内のトルエン濃度は室内濃度指針値の約1.9倍であっ

Table 1. Major subjective symptoms reported by 11 patients after the construction of a fireproof wall in their office

Symptom	N (%)
Headache	8 (73%)
Malaise	8 (73%)
Cough	6 (55%)
Eye irritation	6 (55%)
Poor concentration	6 (55%)

Table 2. Contents of the ingredients of the acryl resin paint

Ingredient	Content (%)
Titanium oxide	20-30
Toluene	13
Xylene	11.5
Ethylbenzene	1.5
n-Butyl alcohol	1-10
iso-Butyl acetate	1-10
Acryl resin	14-43

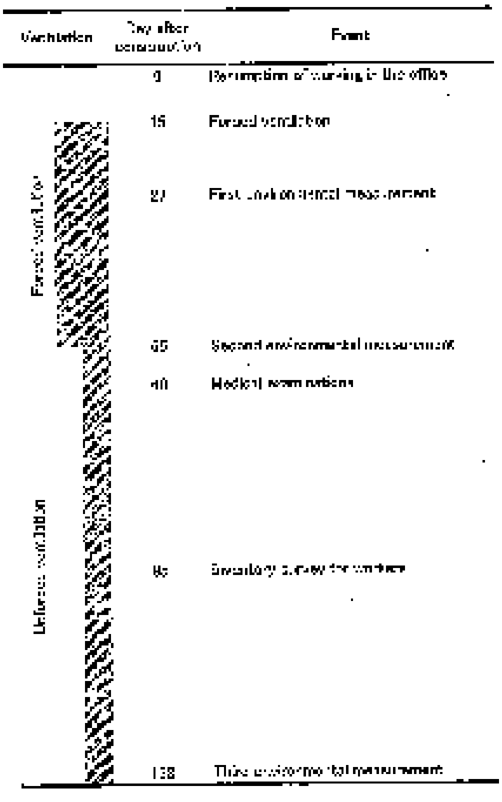

Fig. 1. Time course of events in the office.

た．その後自然換気に変更し，改装工事終了133日後の第3回測定では，トルエン，キシレン，T-VOCの各濃度はすべて指針値以下であり，事務室では低濃度のホルムアルデヒド以外検出されなかった（Table 3）．

3回測定されたトルエンとキシレン濃度は，作業開始時を0時間としたとき，Fig. 3に示すように指数関数的に減衰した．作業開始時におけるそれらの濃度は，各関係式のt=0から算出し，会議室におけるトルエンとキシレンおよび事務室2ヶ所におけるトルエン濃度は，順に3,779，3,641，689，1,243 μg/m^{3}と推定された．また，この関係曲線から，会議室におけるトルエンおよびキシレンの半減期が約25日，事務室のトルエンは約40日と算出された．

3．質問紙調査

非喫煙者33名に質問紙を配布した結果をTable 4に示した．回答者は男性22名，女性1名の合計23名で，回収率は70％であった．回答者の中で1名を除く22名（96％）が業務開始直後に異臭を訴えた．異臭を感じた期間は67.1±28.5日（25-100日）と，室内の有機溶剤濃度が低下した時期と一致していた．体調に悪影響があったと回答したものは7名（30％）で，主な症状としては頭痛，粘膜刺激，悪心が訴えられた．その全員が3ヶ月後の時点では，症状はなくなっていると回答した．総合すると，何も感じなかったと回答した人は1名のみで，異臭を感じた人は34名中33名（97％），症状を呈した人は18名（53％）であった．

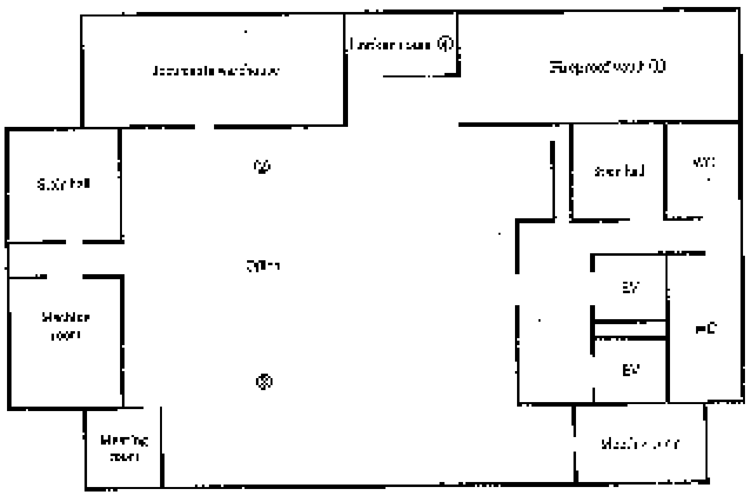

Fig. 2. The layout of the office and the sampling points of the environmental measurements.

Table 8. Changes in formaldehyde and VOC concentrations (μg/m³) after the construction of the fireproof vault

Sampling point	Fireproof vault ①			Office 1 ②			Office 2 ③			Locker room ④	
Days after construction	27	55	132	27	55	132	27	55	132	55	132
Formaldehyde	51		15	75		13	34		14		16
Toluene	3,971	483	78	62	33	ND	137	57	ND	27	27
Xylene	2,610	695	118	ND	ND	ND	ND	ND	ND	ND	ND
Ethylbenzene	1,155		ND	ND		ND	ND		ND		ND
Styrene	ND		ND	ND		ND	ND		ND		ND
p-Dichlorobenzene	ND		ND	ND		ND	ND		ND		ND
T-VOC	7,100		231	140		56	330		47		69

ND: below the detection limit, VOC: volatile organic compounds, T-VOC: total volatile organic compound.

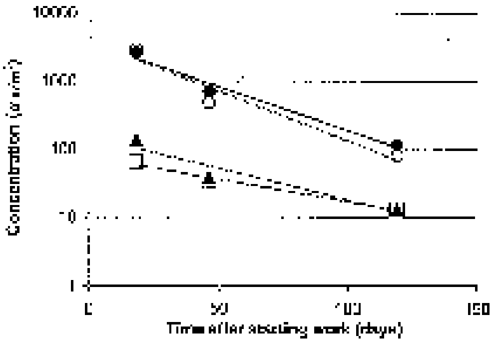

Fig. 3. The decrease of toluene and xylene concentrations in the fireproof vault and the office after the resumption of work. Toluene at ① in the fireproof vault, ○----○, $y=3{,}792e^{-0.0256x}$ ($R^2=0.94$, 1/2T=27d); xylene in the fireproof vault ●——●, $y=3{,}612e^{-0.0262x}$ ($R^2=0.97$, 1/2T=26d); toluene at ② in the office, ..., $y=152e^{...}$ ($R^2=0.9$, 1/2=...d); toluene at ③ in the office, ▲----▲, $y=110e^{-0.0...x}$ ($R^2=0.90$, 1/2T=32d). The concentration of ND is expressed as one-half the value of the detection limit.

Ⅳ．考　察

日本におけるシックビル症候群の症例報告論文は3編あり，それらの報告によると原因物質は2-エチル-1-ヘキサノール[22]またはホルムアルデヒド[16,23]である．本報告では，金庫室塗装工事終了9日後の業務再開後から多くの社員が異臭を訴え，一部の社員は頭痛，倦怠感，集中力や思考力の低下，咳，眼のかゆみや充血などの症状を呈したが，医師の診察では，特に異常は見られなかった．シックビル症候群の主要症状は皮膚・粘膜刺激症状と頭痛，易疲労感，めまい，悪心・嘔吐等の精神・神経症状とされており[24]，彼らの症状はこれらとほぼ一致していた．さらにアレルギー疾患や呼吸器疾患など他疾患が除外されることが必要であるとされているが，今回の受診者においても血液生化学検査，肺機能検査，免疫学的検査，鼻汁好酸球検査および眼科的検査の結果，特に異常は見られず，これらの疾患は除外された．

精神神経症状や眼の刺激症状は，Table 8に示されたようにホルムアルデヒドよりもトルエン，キシレンおよびT-VOC濃度が高値であったことに起因すると考えられた．粘膜の刺激症状の訴えが強くなかったことは，塗装材料のほとんどがホルムアルデヒドの発散が5 μg/m²h以下のF☆☆☆☆を使用しており，環境中のホルムアルデヒド濃度が低かったことと一致した．

住宅室内の化学物質濃度はおおむね経過年数の経過とともに減少する傾向が見られ，瀬戸ら[25]はホルムアルデヒドの減衰は緩慢であるが，トルエン，キシレン，エチルベンゼンなどの塗料に含まれる芳香族類では竣工後間もなく相当量が揮発するので，竣工3ヶ月以降では竣工直後の1/5–1/10の濃度に減衰したと報告しており，星間ら[26]も新築病棟のトルエン，キシレン濃度は3ヶ月で1/10以下に下がったと報告している．今回の測定においても，Fig. 3で明らかなようにトルエンおよびキシレン濃度の指数関数的な減少が見られた．金庫室における減衰の方が早かったが，これは金庫室では初期濃度が高かったことと強制換気が実施されたことによると考えられる．いずれの半減期も瀬戸らの結果と良く一致し，職員が作業を開始した入室時のトルエン濃度は，金庫室では約4,000 μg/m³，事務室では75–150 μg/m³，金庫室のキシレン濃度は約3,000 μg/m³と推定された．トルエンは3,343 μg/m³ (0.9 ppm)，キシレンは435 μg/m³ (0.1 ppm) がヒトの臭覚閾値濃度とされている[26]ので，金庫室の濃度は，いずれも臭覚閾値を超えている．このことは質問紙調査で社員全員が異臭を訴えたことと合致した．

われわれは，使用塗料の成分であるトルエンとキシレンの気中濃度が入室時には臭覚閾値を超えていたと推定され，しかもT-VOC濃度に占めるトルエン，キシレンおよびエチルベンゼンの合計濃度が95%であったため，

Table 4. Responses to the questionnaire (number of effective answers = 23)

Sex	Male	22
	Female	1
Age (yr)	43.7 ± 11.8 yr (24–61 y)	
History of allergy	Yes	5
	No	17
	Not replied	1
Detection of bad odor	Yes	22
	No	1
The period of smelling the bad odor (days)	67.1 ± 28.8 days (25–130 days) (n=17)	
Adverse health effects due to the construction	Yes	7
	No	16
Health condition at 3 mo after the construction	Better	7
	Same	0
	Worse	0

金庫の塗料に含まれる溶剤が原因物質と推定した．塗装工事終了9日後に入室した時点では作業者のほとんどが異臭を感じたこと，受診者の多くが耐火金庫内への出入りが多い女性従業員であったこと，職場から帰宅すると症状は消失または軽減すること，塗装工事後1ヶ月後においても会議室内のトルエンが指針値の約10倍，T-VOC濃度は暫定目標値の約18倍であったこと，および，それらの濃度低下とともに症状が軽快したことにより，金庫室内の塗装が原因によるシックビル症候群と考えた．

非受診者の質問紙調査によると，塗装工事終了1〜2ヶ月後に症状は消失したと回答している．以上のように，室内の揮発性有機物質濃度が高濃度の時，異臭の訴えや症状が出現し，それらの気中濃度が嗅覚閾値濃度を下回るとともに大多数の職員の訴えや症状が消失したことから，嗅覚刺激が症状に及ぼす影響が大きいと考えられた．

石川ら[27]は，シックハウス症候群患者において，神経眼科的検査がその病態診断に必須であるとして，眼球運動性眼球追従運動検査（SPEM）を実施している．当院においても，瞳孔反応検査およびSPEMとほぼ同等な視標追跡検査を行ったが，異常を示す結果は得られなかった．小川ら[28]も，SBS群と健常者群とにおけるSPEM検査および瞳孔検査では有意差はみられなかったが，これらの検査はVDT作業により影響を受けることを報告している．

シックビル症候群の発症や症状の軽減には，精神心理的な要因の関与を指摘する報告があり[2,13,14]，当院では受診者の心理状態の検索として日本版STAIおよび日本版POMSを実施している．不安を示すPOMS検査のT/A値など不安や抑うつの感情が高いヒトにおいては，SBS症状の訴えが多くなることが報告されており[15]，Nakazawaら[16]のシックビル症候群患者は受診時STAI得点が非常に高かったが，症状の改善とともにスコアが下がったと報告している．今回の受診者11名において，SBSと診断された7名はそうでない4名に比べ，両STAI得点，T/A以外のPOMS得点が高値であったことから，自覚症状には，不安を主とする精神心理的な要因も関与していると考えられた．また，長時間のVDT作業はSBS症状を増加させ，女性においては心理社会的苦痛がSBS症状と関連するなど，職場由来の心理社会的ストレスの関与が報告されている[18,29]．

V. 結　語

当該研究所への会議室の新設後に頭痛や倦怠感などを訴えた11名が当科を受診した．発症経過，自覚症状とその推移，各種臨床検査での結果，および環境のトルエン，キシレンなどの室内濃度測定結果から，そのうちの7名をSBSと診断した．非受診者に行った質問紙調査でも，室内使用開始直後には異臭や不快感をほとんどの職員が感じていたが，それらの消失した時期と室内濃度の低下時期とが一致していた．以上のことから，集団発生であるSBSと考えられた．

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2) 特異的診断法の検討

2-1) 神経眼科的検査

SHS と診断した患者と健常者を対照群として滑動性追従眼球運動検査(SPEM)および瞳孔機能検査を実施した。その結果、SHS 群と対照群との間では、SPEM 及び瞳孔検査の結果に有意な差は見られず、神経眼科的検査が診断法として有用であるとは考えられなかった(日職災誌、58:65-69,2010)。

原　著

シックハウス症候群における滑動性追従眼球運動と瞳孔反応の有効性の検討

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要旨：シックハウス症候群（SHS）の診断に様々な検査が補助的に利用されている．SHSの患者を対象とした研究で，約90%以上に滑動性追従眼球運動（SPEM）の異常が見られるとの報告や，自律神経系による種々の調節機能障害が認められるとの報告があることから，SPEM検査や瞳孔反応が補助的検査として用いられることがある．

今回，SHSと診断された患者および健常者を対照群に，SPEM検査と電子瞳孔計を用いた瞳孔機能検査（瞳孔検査）を実施し検査の有用性を検討した．また，眼精疲労の影響を見るため，対照群の中からVDT作業に従事している者（VDT群）と非VDT作業者（非VDT群）を選び，同様に検査の有用性を検討した．さらにSHS群とVDT群，非VDT群との検討も行った．

結果は，SHS群と対照群では，SPEM検査および瞳孔検査で有意差は認められなかった．一方，VDT群と非VDT群別では，SPEM検査において0.3Hzの水平方向でサッケード群に有意差が認められ，瞳孔検査では光刺激後の瞳孔縮小速度および絶対率に有意差が認められた．SHS群とVDT群との比較では，SPEM検査に有意差はなく，瞳孔検査の初期収縮の瞳孔直径，光刺激後の瞳孔最小径，潜伏時で有意差を認めた．非VDT群との比較ではSPEM検査で0.3Hzの水平方向のみに有意差を認め，瞳孔検査ではすべての指標で有意差を認めなかった．

以上の結果から，SPEM検査および瞳孔検査はVDT作業により影響を受けることが示唆された．そのためこれら2つの検査は，SHSの補助検査としては慎重な利用が必要と考えられる．

（日職災医誌，58：65－69，2010）

・キーワード・

シックハウス症候群，滑動性追従眼球運動，瞳孔機能検査，眼精疲労

はじめに

シックハウス症候群（SHS）は，新建材や塗料，接着剤から発生する微量な化学物質による室内空気汚染が原因といわれている．症状は多彩に亘り，自律神経の機能障害をまこすことも知られている[1]が，いまだに発生原因についての説明は不明な点も多い．

SHSを扱う医療機関では，自律神経機能の診断補助的検査として，眼球電位図（EOG）として記録される滑動性追従眼球運動（SPEM）検査や赤外線電子瞳孔計を用いた瞳孔機能検査（瞳孔検査）などを行っている[2]が，決め手となる検査がないのが現状である．今回我々は，これらの検査の有効性および化学物質以外に影響を受けるか否かをみるため，SHS患者及びSHS様症状の既往がないと自己申告した人（対照群）を対象に，可検査を行い比較検討した．また，眼科的検査が眼精疲労の影響を受けるかどうかをみるために，対照群の中からVDT作業者及び非VDT作業者を抽出し，SPEM検査と瞳孔検査を行い検討した．

対象および方法

SHS群は，平成18年6月～平成18年9月に東京労災病院環境医学研究センターを受診し，SHSと診断された男性9名，女性13名の合計22名とし，平均年齢は男性36.7±8.49歳，女性34.3±6.93歳であった．SHSの診断は，明確な定義が存在しないため，WHO欧州事務局，米国環境保護庁が示している定義[3]を参考に，①新築に入居後やリフォーム後に症状が出現する，②当該建物を離れると，症状は軽快する，③症状は眼，鼻，のどの痛みなどの粘膜刺激症状や頭痛が多い，④室内空気調査を実施し

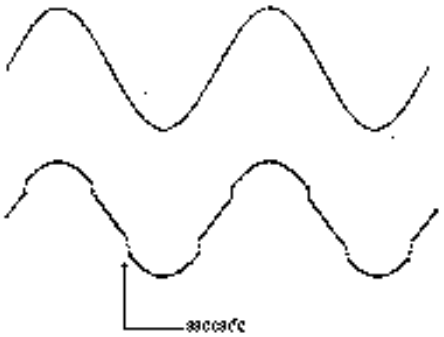

図1　上段：滑動性追従性眼球運動の模式的記録
下段：サッケードを伴う滑動性眼球運動の眼球運動図

ている場合には，室内設定指針値を参考に明らかな上昇がある．⑤空気質調査を実施していない場合には，においなどの化学物質の存在を認め，なお以下の症状の多数が訴える，といった項目を設定し診断した．

対照群は，SHS 群と年齢，性別を調整し，問診により SHS 様症状の既往がない者とした．さらに採血にて糖尿病疾患の有る者，既往症として眼科疾患および精神疾患を有する者は除外した．内訳は男性 27 名，女性 39 名の合計 66 名で，平均年齢は男性 38.0±8.30 歳，女性 34.3±8.00 歳であった．

さらに，日常的設定の眼精疲労による影響をみるため，対照群の中から VDT 作業ガイドライン（厚生労働省労働基準局による VDT 作業における労働衛生管理のためのガイドライン）[7]に基づき，区分 A に当てはまる人々（1 日 4 時間以上 VDT 機器を使用して作業を行う人々）を VDT 群として選出した．また VDT 群と年齢，性別を調整し，区分 A に該当しない人々を非 VDT 群とした．

VDT 群は男性 3 名，女性 9 名の合計 12 名で，平均年齢は男性 35±6.78 歳，女性 30±6.30 歳であった．非 VDT 群は男性 24 名，女性 12 名の合計 36 名で，平均年齢は男性 34.5±8.54 歳，女性 31±5.2 歳であった．

SPEM 検査には，パラレル型赤外線眼球運動モニタリングシステム（JOYTA AB EyeTrace Systems S-851 71 SUNDSVALL SWEDSN）を使用した．0.3Hz，0.5Hz の水平及び垂直の眼球運動を 15 秒計測し，そのうち 2.5 周期を記載した EOC からサッケード値として SPEM 中の潜伏期サッケード（図 1）の挿入率（%）を求めた．サッケード値は，2.5 周期分の振幅に対する追従出来なかった高さの占める割合とし，以下の式で算出した．

サッケード値（%）＝（サッケード予測/振幅×10）×100

また，瞳孔検査の測定に赤外線電子瞳孔計（浜松フォトニクス社製 C7554）を用いた．赤外線電子瞳孔計は，1 秒間の光刺激をゴーグルより発し，約 5 秒後の瞳孔径を赤外線カメラで経時的に計測する装置である[8]．今回，瞳孔検査の測定項目は，暗室で 15 分間の暗順応の後，初期

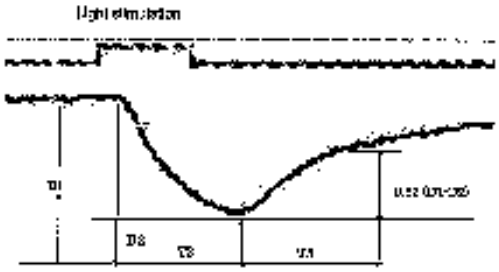

図2　光刺激による瞳孔反応の赤下実孔による記録
D1：初期状態の瞳孔直径（mm）
D2：光刺激後の瞳孔最小径（mm）
CR：縮瞳率（D1-D2）/D1（%）
T3：瞳孔が最小になるまでに要した時間（msec）
T5：最大縮瞳率点から散瞳し 63% まで回復するのに要した時間（msec）

状態の瞳孔直径（D1），光刺激後の瞳孔最小径（D2），縮瞳率（CR），瞳孔が最小になるまでに要した時間（T3），最大縮瞳地点から散瞳し 63% まで回復するのに要した時間（T5）の 5 項目を計測した（図 2）．

統計はサッケード値については Mann・Whitney の U 検定で，瞳孔検査については one-matched t 検定をそれぞれ行った．

結　果

SPEM 検査における サッケード値の結果を表 1 に示す．それぞれの CV 値は 30% を超えており，ばらつきが大きかった．SHS 群と対照群では，0.3Hz，0.5Hz 各々の水平および垂直方向のサッケード量に有意差は認められなかった．一方，VDT 群と非 VDT 群間では，0.3Hz の水平方向のサッケード量において有意差が認められたが，0.3Hz の水平方向および 0.3Hz と 0.5Hz の垂直方向ではともに有意差は認められなかった．

また，SHS 群と VDT 群の比較を行ったところ，0.3Hz，0.5Hz 各々の水平および垂直方向のサッケード値に有意差は認められなかった．一方，SHS 群と非 VDT 群の比較では，0.3Hz の水平方向のサッケード値のみに有意差が認められたが，0.5Hz の水平方向および 0.3Hz と 0.5Hz の垂直方向ではともに有意差は認められなかった．

瞳孔検査の結果を表 2 に示す．それぞれの CV 値は サッケード値の場合に比べ小さく，ばらつきは小さかった．SHS 群と対照群間では，D1，D2，CR，T3，T5 の 5 項目全てで有意差は認められなかった．一方，VDT 群と非 VDT 群間では，D1，T3 および T5 には有意差は認めなかったが，D2 および CR で有意差を認めた．

また，SHS 群と VDT 群の比較を行ったところ，D1，D2，CR で有意差を認めた．一方，SHS 群と非 VDT 群の比較では，5 項目全てで有意差は認められなかった．

表1 眼球追従検査におけるサッケード率の結果

		Horizontal (%) 0.3Hz	Horizontal (%) 0.6Hz	Vertical (%) 0.3Hz	Vertical (%) 0.6Hz
SHS	Average	17.88 *	25.17	26.87	31.31
	SD	12.68	20.19	14.25	16.79
	CV	72%	61%	53%	47%
	Median	15.60	19.40	25.05	30.05
Control	Average	18.33	25.80	23.65	36.55
	SD	17.48	16.40	15.65	17.67
	CV	95%	54%	66%	48%
	Median	11.05	24.40	22.65	37.80
VDT	Average	23.25 **	28.73	18.76	27.45
	SD	20.83	20.18	15.79	18.76
	CV	94%	71%	50%	68%
	Median	15.30	21.25	13.50	23.70
Non-VDT	Average	10.82 *, **	19.36	23.75	31.53
	SD	13.32	10.31	14.75	15.44
	CV	125%	53%	52%	49%
	Median	5.75	20.30	25.85	27.50

*, ** : p < 0.05

表2 瞳孔反応による自律神経検査の結果

		D1 (mm)	D2 (mm)	CR (%)	T3 (msec)	T5 (msec)
SHS	Average	6.31 #	4.05 ##	0.37 ###	1,124.79	1,501.58
	SD	0.56	0.89	0.08	155.16	510.75
	CV	14%	23%	23%	14%	34%
Control	Average	6.47	4.25	0.34	1,121.16	1,649.73
	SD	0.59	0.91	0.07	137.90	435.31
	CV	15%	23%	21%	12%	5%
VDT	Average	7.07 #	5.01 *, ##	0.29 ###, **	1,078.92	1,687.75
	SD	1.07	1.19	0.10	191.15	336.71
	CV	15%	23%	35%	18%	19%
Non-VDT	Average	6.40	4.29 *	0.35 **	1,110.15	1,623.72
	SD	1.01	0.97	0.07	139.25	417.79
	CV	16%	23%	20%	13%	25%

#, ##, ###, *, ** : p < 0.05

考　察

SPEMは，比較的ゆっくり動く対象物を網膜の中心窩で捕らえながら滑らかに追従する運動で，動く視覚対象に注意を保持してそれを認知するために必要である[3]．一方，サッケードは，視覚対象を網膜の中心窩にもたらす速い眼球運動であり，視標と視線の位置のズレの大きさによって，眼球の移動量が決められる[3]．サッケードは統合失調症，感情障害など精神疾患で見られるほか，健常者でも疲労時や注意集中困難時に見られることがある[10]，とされている．

SBSの患者を対象とした研究で，約90%以上にSPEMに異常が見られるといった報告[7]やサッケード値が水平方向で52.5%，垂直方向で60.3%であったという報告[8]がある．しかし，今回のわれわれの検討からは，すべての群でサッケード値は非常に個人差が大きいことが

示された．また SHS 患者において対照群と比較し有意差は認めなかったこと，VDT 群を対象とした検討で非 VDT 群よりも 0.3Hz の水平方向で有意に高値であったこと，SHS 群と非 VDT 群との比較では，0.3Hz の水平方向で SHS 群は非 VDT 群に比べ有意に高値であったものの，SHS 群と VDT 群との比較では有意差を認めなかったこと，これらのことから，サッケードはもともと個人差が大きく，眼精疲労などの影響をより強く受ける可能性が示唆された．つまり特定の疾患の診断補助検査として用いることは困難ではないかと考えられた．

瞳孔の対光反応は本人の意識とは無関係に起きる自律神経系の活動であるため，客観的な評価が可能であり，検査内容の習熟による影響も見られない[11]．また SHS では，自律神経系による種々の身体機能障害が認められ，自律神経機能検査が有用とされている[2]．そのため，電子瞳孔計は，SHS などの患者の多角的診断法として用いられてきた[12]．

われわれの結果を見てみると，SHS 群と対照群との比較では，全てのパラメーターで有意差は認めず，診断の一助とはなり得なかった．VDT 群と非 VDT 群間の比較では D2 および CR で有意差を認めたこと，また SHS 群と非 VDT 群とでは有意差がなかったが，VDT 群とでは D1，D2，CR で有意差を認めたこと，これらのことから眼精疲労などの影響を受ける可能性が示唆された．

西村[13]は，瞳孔検査は，眼精疲労の推定に有効であると報告をしている．さらに CR は副交感神経興奮点眼薬の使用によって低下することが示されている[14]．また交感神経系と抑制系のうち，抑制系が有意になった時に疲労が出現すると言われている[13]．これらのことから眼精疲労により副交感神経系が賦活化され瞳孔検査に影響を与えた可能性がある．

これらの結果から見てみると，瞳孔検査も SPEM 検査と同様，特定の疾患の診断補助検査に用いることは困難ではないかと考える．

今後，これらの検査についての更なる検討及び SHS の診断に特異的な検査の出現が望まれる．

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Evaluation of Effectiveness of Smooth Persist Eye Movement and Pupil Function Tests in Sick-house Syndrome

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Various secondary examinations are used in the diagnosis of sick-house syndrome (SHS). A previous study of SHS patients reported abnormalities in the Smooth Persist Eye Movement (SPEM) and autonomic nervous regulation, which is why SPEM and pupil function tests are carried out in some hospitals. We conducted the SPEM and pupil function test for patients diagnosed with SHS (SHS group) and healthy people (control group) and evaluated the effectiveness of these tests. Moreover, to examine the effect on asthenopia, we divided healthy people into video display terminal VDT workers (VDT) and non-VDT workers (non-VDT) and evaluated VDT vs. non-VDT, SHS group vs. VDT and SHS group vs. non-VDT group in the same way.

There were no significant differences between the SHS group and control group for the SPEM and pupil function tests, whereas there were significant differences between the VDT and non-VDT groups in 0.3Hz horizontal movement, the pupil minimum diameter after light stimulation, and the myosis rate.

Moreover, there were no significant differences for the SPEM test, whereas there were significant differences in the pupil maximum diameter at initial state, the pupil minimum diameter after light stimulation and the myosis rate between SHS and VDT groups. And between SHS and non-VDT groups, there was significant difference only in 0.3Hz horizontal movement for the SPEM test, and there were no significant differences for the pupil function test.

These results demonstrated that the SPEM and pupil function tests are affected by asthenopia and that these tests should be used discreetly for the diagnosis of SHS.

(JJOMT, 58: 65 - 69, 2010)

2-2) 化学物質曝露負荷試験

化学物質過敏症(特発性環境不耐症、IEI)の診断法として、化学物質曝露負荷試験が特異的試験法とされている事から、7 名の IEI 患者に Tol 及び FA の曝露負荷試験を単盲検法で行った。その結果、いずれの曝露によっても何らかの症状やバイタル検査の変動は見られず、IEI 患者の症状等が化学物質曝露によらない事が示唆された(日職災誌、60:11-17, 2012)。

原　著

特発性環境不耐症（いわゆる「化学物質過敏症」）患者に対する単盲検法による化学物質曝露負荷試験

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要旨：【目的】シックハウス症候群と混同されることもある特発性環境不耐症（IEI，所謂「化学物質過敏症」）の患者における化学物質曝露との関係を明らかにする目的で，トルエン（T）とホルムアルデヒド（FA）の曝露負荷試験をおこなった．

【方法】来院したIEI患者のうち，同意を得られた7名（女性5名，男性2名）にFAを，11名（女性9名，内1名はFAにも曝露，男性2名）にTを単盲検法で曝露した．診断指標として，自覚症状，瞳孔反応検査，視標追跡眼球運動検査（ETT），および症状に伴う血圧，脈拍数，SPO_2の変化を観察した．

【結果】FA曝露およびT曝露において，何らかの症状を示した患者は皆無であり，症状に伴うバイタル検査の変動は観察されなかった．また，曝露前後で基準を超えて瞳孔反応検査指標が変動し，あるいはETTにおいてサッケードが25％を超えて増加し，かつ0ppb曝露での有意な増加が観察されないなどの矛盾がない患者は皆無であった．

【結論】IEI患者の症状等が化学物質によらない可能性が示唆された．このことは海外の先行研究と一致していた．国内の先行研究でも用いられた瞳孔反応検査は，測定値が個人間，個人内で大きく変動する検査であり，慎重な解釈が必要である．

（日職災医誌，60：11—17，2012）

—キーワード—
特発性環境不耐症，単盲検負荷試験，化学物質曝露

はじめに

シックハウス症候群（SHSと略）はシックビルディング症候群（SBSと略）の一般住居版と見なされ，省エネを目的とした住宅の高気密化によって，建材に用いられた化学物質などにより発症し，中毒学的にも理解し得るものである．

一方，化学物質過敏症は，1987年に米国のM.R.Cullen[1]が環境暴露による症例から「過去の曝露で急性中毒症状の後に，化学物質に再接触した場合に不快な臨床症状を示す」人達の病態をChemical sensitivity（以下CSと略）として提唱し，日本においては当時北里大学眼科の石川らが「化学物質過敏症」として一般向けに紹介したものである[2,3]．本症は，労働災害の申請によって認定された事例もあり，労災認定基準も設けられるなど，職業医学上の課題ともなっている．

CSの患者は通常の人には影響を及ぼし得ないレベルの化学物質あるいは香料等の臭気によって様々な臓器に関連する症状を呈するが，各種の臨床検査では陽性所見を示さず，病像が多彩である．SHSに続発することもあり，同一視するものもいるなど一部に混乱がある．CSの診断基準は，わが国では石川らの厚生労働科学研究班により示された[4]が，自覚症状に依存しており，検査としては瞳孔反応検査や化学物質への曝露負荷試験を重視しているものの，負荷試験を盲検法とは定めていない．

1996年に世界保健機関（WHO）の化学物質安全性国際プログラム（IPCS）が開催した会議では，化学物質とのかかわりが不明確なためにChemical sensitivityという名称は不適当で，特発性環境不耐症Idiopathic Environmental Intolerance（以下IEIと略）という名称が提案され，定義を明らかにした．本論文では，いわゆる「化学物質過敏症」を以後はIEIと表記して述べる．

先に触れた曝露負荷試験については，二重盲検法によるStaudenmayerら[5]，厚労省研究班[6～10]，宮田・石川

表1 吸着管採気検査結果

患者	性	年齢	曝露物質	負荷試験チャレンジ濃度
A	女性	52	ホルムアルデヒド	3
B	女性	41	ホルムアルデヒド	1
C	男性	59	ホルムアルデヒド	2
D	女性	35	ホルムアルデヒド	1
E	女性	34	ホルムアルデヒド	2
F	男性	47	ホルムアルデヒド	2
G	女性	37	トルエン，ホルムアルデヒド	各1，計2
H	男性	67	トルエン	2
I	女性	51	トルエン	1
J	女性	30	トルエン	2
K	女性	30	トルエン	3
L	女性	49	トルエン	3
M	女性	52	トルエン	3
N	男性	58	トルエン	4
O	女性	55	トルエン	2
P	女性	55	トルエン	2

ら[7]，Bornschein ら[8]，採血検査による報告[9-11]の先行研究がある．その結果は，Staudenmayer ら，Bornschein らは化学物質との関係が認められないとした．環境省研究班は，誘発反応検査については「暴露条件で反応が増悪を示す例があることは，患者の自律神経機能が不安定性を示しているに過ぎない」，症状については「微量化学物質負荷が患者の自覚症状を悪化させているという十分な証拠を提出するには不十分」などとして，化学物質との関係を明確にし得ていない．最近 Hojo ら[12]は，平面検査で11名の患者にホルムアルデヒド 8ppb，40ppb，80ppb を暴露したが，誘発反応を含む各種臨床検査には有意の変化を認めず，陽性は4名に留まったことを示したが，化学物質との関係については触れていない．

一方，吉田ら[13]は，厚労省研究班の2011年度分データ[14]の大部分を用いた論文において，8ppb という「極めて低濃度のホルムアルデヒド曝露で自律神経機能が変動する」結果を得たとし，「多種化学物質過敏症患者は化学的に微量な化学物質に反応することを，客観的に明らかとなし得た」と結論づけている．

著者らが属する診療科は SHS および SBS を診療するのが本来業務であるが，実際にはこれらに加えて IEI も対象としていた．IEI の診断に化学物質の吸着負荷試験が有用と言われていた[15]ため，当科においても IEI 患者について同様な試みをしてきた．同試験においては，平面検法でホルムアルデヒド（以下 FA と略）およびトルエン（以下 T と略）の両方の暴露をおこない，診断指標には一般生理指標以外に，IEI の診断には有用で客観的な方法[16]とされる誘発反応検査と視標追従眼球運動検査を用いてきた．なお，視標追従検査は前検法による吸着負荷試験の先行研究では用いられていず，当科が「おこなってきた吸着負荷試験の特徴である．

本研究の目的は，化学物質吸着負荷試験の結果を示して，仮説「化学物質暴露により，IEI の患者には自覚症状，各種生理検査，特に IEI の診断に有用とされた検査の変化があらわれる」ことを検証し，併せて IEI および本検査について検討することにある．

対象と方法

対象

当科外来患者として来院した IEI 患者のうち，吸着負荷試験の同意を得られた女性13名（42.3 ± 10.7 歳，30〜64 歳），男性4名（48.0 ± 17.0 歳，29〜67 歳）について吸着負荷試験を行った．うち7名（女性5名，男性2名）に FA を，11名（女性9名，内1名は FA にも曝露，男性2名）に T を暴露した．被験者の年齢，性別などを表1に示す．

対象者の診断における IEI の診断は，概ね Cullen[17]あるいは 1999 年の米国での合意事項[18]に従い，複数臓器に関連する自覚症状，複数の化学物質負荷で症状を呈する，原因から離れると症状が消失する，表出に個体差があること，既知の身体疾患・精神疾患によって説明できないこと，を基本にした．

問診により，患者における誘因の聴取，発症経過，各種の自覚症状の出現と消失などを把握するとともに，混入の可能性のある疾患を除外し，血液一般および肝機能や甲状腺機能を含む生化学検査，免疫機能検査，精神生理学検査などにより，既知の身体疾患を除外することにより診断した．

被験者の同意は，医師（当院前環境医学研究センター長の後藤医師）が文書に基づき検査の目的・意義・方法・危険について説明した後，患者自身による同意文書への署名によって得られた．

方法

1．吸着負荷試験

1）基本的なアロケーションは以下の通りである．(1)暴露後1時間にわたりクリーンルームである当科外来の空気に馴染ませた後に，(2)クリーンルーム吸着検査室（以

下，被曝室と略）に入る前に自覚症状を調べ，体温，血圧，脈拍数，パルスオキシメータによる経皮的動脈血酸素飽和度（以下 SPO_2 と略）のバイタル検査の測定，および瞳孔反応検査と視標追跡眼球運動検査を各１回ずつ行う（20分）．(3) その終了後に，空気のみ（プラセボ），FA または T が既に設定濃度に達している曝露室に入室し，曝露を 20 分行い，(4) 曝露終了後退室して曝露前の (2) で行った諸検査及び自覚症状調査を行う（20分）．(5) その終了後休憩時間を 40 分取り，(6) 再度曝露室に入り曝露負荷を 10 分行い，(7) 曝露終了後に曝露室を退室して (2) で行った諸検査及び自覚症状調査を行う．１日に上記の２回の曝露を行うことを１セッションとした．したがって，曝露あるいはプラセボ曝露は，(3) または (6) であった．

2) いずれの曝露においても，患者の主観が関係することが疑われる本症において，患者に化学物質の曝露を知らせるオープン法では客観性が保てないためにオープン法を用いず，患者に化学物質あるいはプラセボ曝露を知らせないが検査者側は知っている単盲検法とした．人員不足により二重盲検法を採用できず，単盲検法でもオープン法の問題点を避けられるという考えで単盲検法を採用した．

１週間以上間隔をおいた２日に，各１回，計２回のセッションを行うことを基本とした．

3) 曝露濃度は，先行研究を参考にして[11,12]，厚労省の室内空気汚染物質の指針値，その半分，および無曝露，すなわちプラセボとした．したがって，FA 濃度は 0・40・80ppb，T 濃度は 0・35・70ppb であった．T のヒトにおける嗅覚閾値濃度は 3,768μg/m³（0.9ppm）[13]，FA の嗅覚や刺激閾値は 0.5ppm[2] と言われていることから，これらの曝露濃度はいずれも臭いや刺激を感じない程度の濃度である．なお，曝露の順は固定せずに適宜変更して曝露を行った．

使用した曝露室は，後藤ら[14]が報告したものを用い，曝露レベルの確認は常時モニタリングシステムによりおこなった．曝露濃度のモニタリングは，曝露室内呼吸域に相当する位置にてアクティブサンプリングして加熱脱着サンプラー（PerkinElmer 製）に吸着させ，自動加熱脱着装置 ATD で脱着させて，ガスクロマトグラフィに導入して測定することによって行われた．

なお，本研究で用いた曝露室（新菱冷熱工業，新宿区）は，換気量が 10m³/分であり，活性炭による化学物質の除去について総揮発性有機化合物を 10μg/m³ 以下にする能力があった．

2. 指標とした検査項目

1) 影響指標として，患者が訴える症状の有無を調べた．また，瞳孔反応試験にはイリスコーダ C7364（浜松ホトニクス，浜松市）を，視標追跡眼球運動検査（Eye Tracking Test，ETT と略）にはメディスター VOG-CD3001（松下電工（現パナソニック電工，門真市））を用いた．また，血圧，脈拍数，SPO_2 の確認も影響指標とした．

2) 陽性の判断は，FA あるいは T 曝露によって，曝露前と異なる症状の発現，これまでに患者が訴えていた自覚症状の出現によった．瞳孔反応検査および ETT が化学物質曝露によりどのように変動するかは統計的研究では明示されていないが，石川ら[5]は，IEI 患者においては瞳孔反応検査における縮瞳および散瞳速度の低下，潜伏時間の延長を，ETT では滑動性追従運動の眼球衝動性変化を認めるとしている．このことから，陽性の基準を次のように設定した．瞳孔反応検査においては，石川[15]が示した正常者のデータ２種のうち多人数のデータの年代毎の平均値＋２標準偏差を超えることとした．例えば，最小縮瞳時間（T3）が 1,400msec，および/または 63％ 回復時間（T5）が 2,600msec を新たに超える場合，曝露前から既に超えている患者では T3，T5 は 200msec 以上延長する場合を陽性とした．ETT においては，衝動性眼球運動（サッケード）が新たに 25％ を超える場合，および曝露前検査で既に超えていて曝露によって 10 ポイント以上の増加する場合を陽性とした．これらの増加が 0ppb 曝露においても生じるなど，矛盾がある場合は陽性とはせず，２回のセッションの検査を行った患者で異なる場合は陽性とはしなかった．血圧，脈拍数，SPO_2 は，症状を伴って前二者は 10％ 以上，SPO_2 は 5％ 以上変化した場合を陽性とすることとした．

倫理委員会：関西労災病院倫理委員会の審査を経て承認された．

結　果

FA 曝露の７人中４人は２セッションの曝露負荷試験を受け，既に T 曝露負荷試験を受けた１人を含む３人は１セッションで終了した．T 曝露の 10 人中８人は２セッションの曝露負荷試験を受け，２人だけ１セッションで終了したが，うち１人は後日 FA 曝露負荷試験を受けた．

FA 曝露および T 曝露において，何らかの症状を示した患者は皆無であり，症状に伴うバイタル徴候の変動は確認されなかった．また，前述の陽性評価基準を超えた瞳孔反応検査指標の変動，あるいは ETT においてサッケードが 25％ を超える増加をきたし，0ppb 曝露での大幅な増加が観察されず矛盾がない患者は皆無であった．表１と図２に，石川によれば「安定している」[15]とされる瞳孔反応検査で T5 遅延時の曝露前と曝露直後における変動を示し，図３に２セッションを実施し得た患者 15 名の２セッションの曝露前の T5 遅延時の変動を示す．

考　察

単盲検法で FA 曝露された７人と T 曝露された 11 人において，自覚症状，一般的生理指標，神経眼科的生理指標を用いて曝露の影響を調べたが，明らかな変化を認

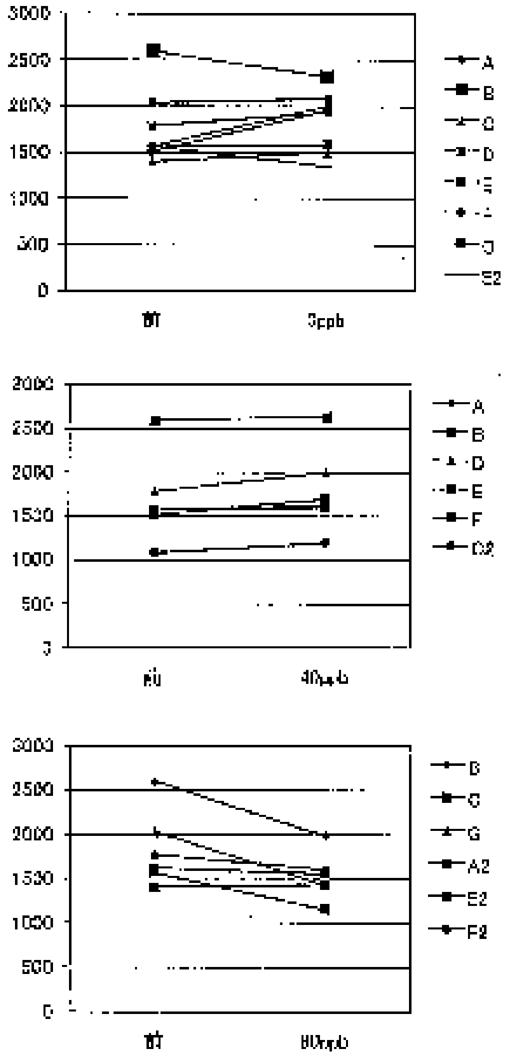

図1　ホルムアルデヒド曝露前後における重心動揺検査の総軌跡長 TS の推移．上段は0ppb曝露，中段は40ppb，下段は80ppb．患者のアルファベットに2が付く場合は第2回セッションの結果を指す．

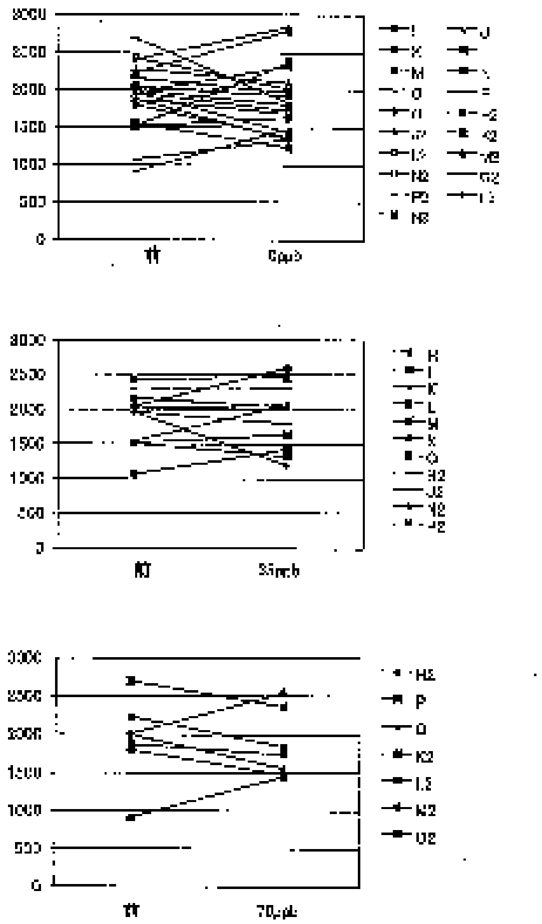

図2　トルエン曝露前後における重心動揺検査の総軌跡長TSの推移．上段は0ppb曝露，中段は35ppb，下段は70ppb．患者のアルファベットに2が付く場合は第2回セッションの結果を指す．

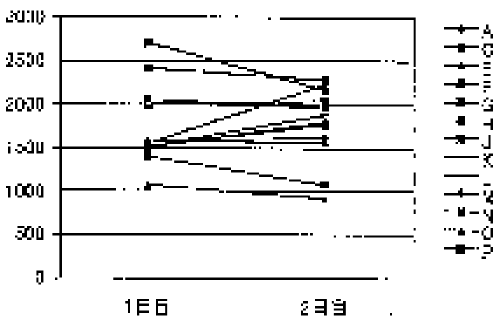

図3　第1回セッションと第2回セッションの曝露前における重心動揺検査の総軌跡長TSの推移

めることはできなかった．このことは，仮説「化学物質曝露により，IEIの患者には自覚症状，精神生理検査，特にIEIの診断に有用とされた検査の変化があらわれる」は棄却され，IEI患者の症状等が化学物質によらない可能性を示唆する．

石川らはIEIの診断検査の考え方や治療法などを米国Environmental Health Center-DallasのW. J. Reaらから導入したが，ReaらのIEIの診断や治療には科学的根拠がないという批判が米国内では以前から存在している．米国のStaudenmayerらは「CS」の身体疾患概念を厳しく批判して精神疾患としての理解を提案している．また，彼は，20人の患者に二重盲検法によって最多

で10回，少なくとも3回曝露し，患者の反応の真陽性，偽陽性，偽陰性，真陰性から，個々の患者における感受性，特異度，有効度と，全体のそれらを算出し，全体で感度33.3%，特異度64.7%，有効度52.4%と著しく低いことを示した[8]．また臭いにより「てんかん」様症状を呈するという患者に香水を曝露して脳波を記録し「てんかん」に関わる電気活動が出ないことを確認して，身体疾患でないことを示している[9]．免疫学的な研究においても関係が認められず[10]，米国では本病は不安障害の一つとされている[11]．

ドイツではBornschein ら[12]がIEI 患者に曝露負荷試験を二重盲検法で行ったが，化学物質曝露による血圧，脈拍数などの生理指標の有意な変化が認められず，「IEI は化学物質と関係がある」という仮説を棄却している．

台湾のLee ら[13]は，トルエンの職業的曝露があった労働者で自動車排気ガスや塗料を嗅いだ時やガソリンスタンドに行った時に動悸や頭痛，めまいを起こすようになった患者にトルエンを曝露した．脈拍数の軽度増加以外には血圧，呼吸数，動脈血二酸化炭素分圧や酸素分圧，酸素飽和度には変化が認められなかったことから，彼らは「CS」を精神疾患との関連があると考えた．

本研究の結果も，前述の先行研究と同様に「化学物質曝露によってIEI 患者に反応が生じる」という仮説を否定する．吉田ら[6]は，瞳孔反応検査を曝露前後で各々4回同時計8回おこない，その各項目の平均値と標準偏差によるt検定をおこなった．本研究では，検査回数とデータ処理法が異なるが，より高濃度曝露にもかかわらず，吉田らと同様な結論を得ることができなかった．なお，宮田・石川らは，従来IEI 患者においては散瞳潜時が延長するとしていたが[3]，8ppb 曝露での患者の反応を「（延長と短縮の双方を含む）変動」[14]とした．

患者に化学物質の種類を教えるオープン法で自覚症状等の変化を示すこと[4]，二重盲検法では本研究同様に曝露の影響が不明確であることから，患者が曝露を認識しないと症状が生じないことを示唆している．これらは，IEI を精神心理的背景で捉えることの重要性を示唆し，ドイツのBailer ら（Central Institute of Mental Health Mannheim）[15-17]やデンマークのSkovbjerg ら[18-20]（Danish Chemical Sensitivity Centre）によって，精神心理学的な視点からの研究が進められていることに留意する必要がある．

曝露負荷試験の評価について，長谷川らはプロトコールが決まってないことを問題にしているが，本研究における曝露濃度，その安定性，影響指標などについての我々の考えを以下に述べる．

本研究における濃度設定は，国内の先行研究とほぼ同等である．当初により高い濃度を企図したが，患者の安全性を優先して，通常では影響が出ないレベルである許容値とそれ以下に設定した経緯がある．この曝露濃度はヒトの嗅覚閾値以下であり，臭いではなくて化学物質曝露によって患者の症状が生じるならば，先行研究の結果を参照すると，症状を生じ得る可能性があるレベルと考えられる．

本研究における曝露濃度の安定性について，後藤らの論文[21]に示したように，安定した濃度が維持されていて問題はないと考えられた．本研究における曝露負荷試験のプロトコールでは，計20分間の曝露に対して休憩を40分入れ，全行程で2時間をかけた．長谷川らの曝露負荷試験の所要時間40～50分に比べて余裕があり，患者の負担によるバイアスを避けることはできたと考えられる．

影響指標については，多くの先行研究は自覚症状や血圧，脈拍などの生理指標を重視しているが，本研究では石川らが重視している神経眼科的な検査を加えた．しかし，瞳孔反応検査については，長谷川ら[1]は有意な差を認めず，小倉ら[2]は一定の傾向を得られないとし，本研究においても先行研究同様に有意な変化を示さなかった．

本研究において影響指標とした瞳孔反応検査は，環境省研究班も用いているが，小川ら[22]は，変動が大きく，定量的な臨床検査としての精度等には疑問があることを述べている．本研究においても，曝露前と0ppb，2セッションの曝露前でT5が200ms を超える大きな変動が見られ（図3），反復測定した際の個人内変動が大きいことが示唆された．

石川[23]は，神経眼科学的に瞳孔反応検査の正常値について述べているが，2種の集団から得られたデータである表1と表2において，縮瞳率CR，縮瞳速度の最高値VC，散瞳速度の最高値VD，T2の数値が著しく異なっている．須藤ら[24]は，同じ機器を用いて，頭痛患者における自律神経機能を調べる目的で瞳孔反応検査をおこなったが，健常人のT5は概ね900～1,000台で外れ値を含めて平均1,157ms であり，石川[23]が述べている1,500ms 前後（表1）とも1,600ms 前後（表2）とも異なる．彼らが示した健常人のT3は600ms 前後で，石川が述べている表1の650ms 前後に近いが，表2の950ms 前後とは大きく異なる．

このように，本法は同じ機器を用いながら個人間，個人内でも大きく変動する検査であることから，検査結果の慎重な取扱が必要である．

結　論

FA 曝露およびT曝露において，何らかの症状あるいは神経眼科的検査で陽性の所見を示した患者は少数であり，仮説「化学物質によりIEI の患者は自覚症状，各種生理検査，特にIEI の診断に有用とされた検査に変化があらわれる」は棄却された．このことはIEI 患者における症状等が化学物質によらない可能性を示唆している．また，瞳孔反応検査は個人間，個人内でも大きく変動する検査

であり，検査結果の慎重な取扱が必要である．

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Single-blind Provocation Test Including Exposure to Formaldehyde and Toluene for Patients with Idiopathic Environmental Intolerance

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In order to clarify the effect of chemicals in patients with idiopathic environmental intolerance (IEI) which is confused with sick house syndrome, we conducted a single-blind provocation test in the patients by using exposure to formaldehyde (FA) and toluene (T).

Of all the outpatients with IEI, 7 (5 women and 2 men) and 11 (9 women and 2 men) patients agreed to be exposed to FA (0, 40, and 80 ppb) and T (0, 35, and 70 ppb) by the single-blind method, respectively. Indicators of the effect due to exposure were subjective symptoms and their associated changes in blood pressure, pulse rate and SPO_2 and the results of neuro-ophthalmologic tests, including pupil function tests (PFT) and eye tracking test (ETT) which are considered to be useful for IEI diagnosis.

In the provocation tests, no patients developed any symptoms and showed significant excess of positive criteria of articles of PFT, and/or increase in the saccade rate exceeding 25% in ETT without contradiction of changes in the 0 ppb exposure.

The results of the present study suggested that symptoms of patients with IEI were not caused by the low level of chemical exposure. This is consistent with the results of foreign studies. Because the PFT data used in previous Japanese studies showed wide intra- and inter-individual variability, we suggest that PFT data should be analyzed very carefully.

(JJOMT, 60: 11—17, 2012)

2-3) SHS と IEI の病像の比較

SHS と IEI が混同されている事から、両疾患の病像を明らかにするために、両患者群を比較した結果、IEI では既往歴として精神疾患とアレルギー疾患率が有意に高く、自覚症状では呼吸困難・息苦しさと関節痛の訴えが有意に高かったが、精神心理検査では、異常率は両群とも高く差はなかった(日職災誌、61:119-124, 2013)。

原　著

特発性環境不耐症の臨床所見—シックハウス症候群との比較—

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要旨：【目的】シックハウス症候群 SHS としばしば混同される特発性環境不耐症（IEI，本論「化学物質過敏症」）の実像を明らかにする目的で，2005年のシックハウス診療科開設後から2008年一時期までに受診した IEI 患者が比較対照としてのシックハウス症候群（SHS）患者と比較した．

【方法】IEI 患者42名とその対照とした SHS 患者88名について，既往疾患，症状，臨床検査結果の比較を行った．

【結果】IEI 群では，数が少ないが消化器疾患とアレルギー疾患を除く自覚の疾患の罹患がある患者の割合が有意に高く，頭痛肩凝・疲労感を訴える患者と少数であるが神経疾患を訴える患者が有意に多かったが，皮膚粘膜症状に有意に少なかった．臨床検査では総コレステロール値，総 IgE，視標追跡検査の 0.5Hz 時サッケード率において，IEI 群の異常者率が高かったが，有意差を示さなかった．

【結論】症状において，IEI と SHS は明瞭に異なることが明らかになり，症状の機序から心理社会ストレスや精神疾患の観点からの検討が今後必要と考えられた．

（日職災医誌，61：119—124，2013）

—キーワード—

特発性環境不耐症，シックハウス症候群との比較，既往歴，症状，臨床検査

はじめに

欧米では，1970年代後半から1980年代にかけて，断熱率を高くするために外気取り込み量を減らした建物における健康障害が発病率の高い「病所ビルで問題となり "シックビル症候群"（Sick Building Syndrome，SBS）と言われた．日本においても建物の高気密化が進んだが，「建築物における衛生的環境の確保に関する法律」により事務所ビルにおいては換気等が守られていたために，一般住宅の新改築時に室内汚染が問題となり，「シックハウス症候群」（Sick House Syndrome，SHS）と呼ばれた[1]．建材などから発生するホルムアルデヒド，有機溶剤やフタル酸エステルなど揮発性有機物が原因とされ，厚生労働省は13種の化学物質に対して室内濃度指針値を設定した．SHS は SBS から派生した日本独自の命名である．その定義からも SBS/SHS は皮膚粘膜などの刺激症状に代表される[2]．しかし，SHS に罹患した患者が，通常の濃度より，数種の化学物質や臭いに対して不定な症状を示すことがあるが，

一方，微量の化学物質に対して症状を来す人達について，1987年に米国の M. R. Cullen が臨床観察による症例から「過去の曝露で急性ないし亜急性の後に，化学物質に再曝露した場合に不快な全身症状を示す」人達の病態を Chemical sensitivity（以下 CS と略）として報告した[3]．米国では CS を一つの疾患と見なすことについて未だ学会が合意していず，不定愁訴の一つとされている[4]．Cullen 自身も，論文で心的外傷後ストレス障害 PTSD と身体表現性障害と DSM の病態を考察している[5]．しかし，世界保健機関 化学物質安全国際プログラム WHO-IPCS の会議では，化学物質によるということは不明確であることから特発性環境不耐症（Idiopathic Chemical Sensitivity，IEI）という名称を用いているが，我々も，ホルムアルデヒド下降負荷およびトルエン暴露による負荷試験によって，何らかの症状あるいは神経眼科的検査で陽性の所見を示した患者は検索できなかったことから，CS 患者における症状などが化学物質によらない可能性を示した[6]．以上から，本論文では，CS および後述のいわゆる「化学物質過敏症」を，原則は IEI と表記して述べる．

日本においては当時北里大学眼科の石川らがIEIを「化学物質過敏症」として一般向けに紹介した。彼らが研究交流を行った米国のW J Rea（Environmental Medicine Center-Dallas）らの見解は米国では否定的であるにもかかわらず，その中でRea らの見解を紹介している。また，石川とその共同研究者達は，前述の遷延したSHS患者にみられる状態をIEIとしつつ，それを「広義のSHS」としてSHSに含めている。

IEIは，化学物質曝露による労働災害の申請によって認定された事例もあり，労災認定基準も設けられている。一方，職場での化学物質曝露あるいはSHS後の労災で認定されなかった患者が数倍をしめすなど，職業医学上の問題ともなっている。

本論文では，IEIの有病を明らかにする目的で，IEIの自覚症状，既往歴及び血液検査などについてSHSを比較対象として検討した。SHSは化学物質による急性影響とみられ，症状以外には有意な発症がない点はIEI患者と共通する状態にあると考え，比較対照とした。なお，本研究の特徴は，罹病期間が短いために修飾が少ないと推察される初診患者でおこなわれたことにある。

対象と方法

1．対象

2005年6月から2008年10月までに当科を受診した患者270名のうち，IEIとSHSと考えられたもの IEI患者42名（以下IEI群，男8名19.0%，女34名81.0%，43.0±12.6歳，22～67歳）とSHS患者89名（以下SHS群，男17名19.3%，女71名80.7%，38.9±13.1歳，22～79歳）とを対象に行った。なお，診断は，熟練した当科医師が行った。いずれも初診患者であり，他のIEIやSHSの診療を行っている医療機関を受診していなかった。

本報告におけるIEIの診断は，概ねCullenあるいは1999年の米国での合意事項に概ね従い，①複数臓器に関連する自覚症状，②微量の化学物質曝露で症状を呈する，③曝露から離れると症状が消失する，④関連がない多種類の化学物質で症状を呈する，⑤慢性に経過することを基準にした。

また，SHSの診断基準は，以下の通りである。①発症のきっかけが，転居，建物の新築・増改築・改装，新しい家具の使用などであること，②複数の症状を訴えていること，③その場所を離れると症状が軽快すること，④頭痛，粘膜の刺激症状が主であること，⑤種々の検査によってアレルギー疾患及び甲状腺疾患などの他の疾患が否定的であること，の全項目を満たし，⑥環境測定により，室内環境汚染が確認され，原因物質が推定されていることが望ましい。

2．方法

カルテに記載された自覚症状，既往歴，心理検査，血液一般・血液生化学検査，免疫系検査，生理検査のデータを用いた。

主な自覚症状は以下のように分類した。気道症状，中枢神経等の症状，精神症状，自律神経症状，眼症状，泌尿生殖器症状，末梢神経と四肢の症状，消化器症状，皮膚症状，その他。

既往歴は以下のように分類した。アトピー性皮膚炎，アレルギー疾患，自己免疫疾患，精神疾患，および前記以外の長期の通院又は入院が必要な疾患として，循環器疾患，消化器疾患，内分泌代謝疾患，感染症，泌尿生殖器疾患，血液・腎臓疾患，眼耳鼻の疾患，皮膚・神経疾患，整形外科疾患・外傷，悪性新生物，呼吸器疾患（喘息を除く），その他。

臨床検査としては，血液一般・血液像，生化学検査（AST，ALT，ChE，LDH，総コレステロール，Na，Cl，K，BUN，クレアチニン，Ca，CPK，アルブミン，総蛋白，血糖値，CRP，TSH，遊離T_3，遊離T_4），免疫学的検査（総IgE，吸入アレルゲン14項目，食物アレルゲン12項目の特異的IgE）であった。

生理検査として，肺機能（努力肺活量，1秒量，1秒率：スーパースパイロ DJ-21 FXIII，チェスト（株），東京），瞳孔反応（イリスコーダー C9364，浜松ホトニクス，浜松市），視標追跡におけるサッケード出現率（メディアスター VOG CD8001，松下電工（現パナソニック），門真市），および血圧，体温，経皮的血液酸素飽和度をおこない，心理検査には不安状態と不安になりやすい性格傾向を測定するState-Trait Anxiety Inventoryの日本語版（STAI），および気分や感情の変化を見る検査であるProfile of Mood Statusの日本語版（日本版POMS，金子書房，東京）をおこなった。

統計検定には統計ソフトSPSS Ver.15（Inc.）を使用し，主な自覚症状や既往歴の有無の割合のIEIとSHSの群間の有意差はχ^2検定によった。

倫理委員会：関西労災病院院内倫理委員会の審査を経て承認された。

結　果

表1，2に主な既往歴と自覚症状のIEI群とSHS群における人数と割合を示す。既往歴がない患者の割合はSHS群に高い傾向を示すのに対して，アレルギー性疾患，中でも花粉症の割合がIEI群に高い傾向が有意差はなく，人数は多くはない精神疾患および耳鼻の疾患（アレルギー性疾患を除く，Ménière病，突発性難聴，鼻内腔，副鼻腔炎，網膜色素変性症など）にIEI群はSHS群に比べ有意に高い割合を示した（各々$p=0.038$，$p=0.035$）。自覚症状において，くしゃみ鼻水，咳嗽などの気道刺激症状は，IEI群においてはSHS群よりも少ない傾向を示したが有意ではなかった。一方，胃腸症状・息苦しさは両群に各々10人余いてIEI群での割合がSHS群に比べ

表1　主な症状を示した患者数とその割合（χ^2検定）

症状	SHS		IEI		p
	人数	%	人数	%	
なし	22/38	57.9	5/42	11.9	0.107
アレルギー性鼻炎	25/38	37.8	18/42	42.9	0.783
下痢症	15/38	39.5	12/42	28.6	0.394
頭痛全般	4/38	10.5	7/42	16.7	0.526
皮・粘膜の症状（アレルギー性症状を除く）	2/38	5.3	5/42	11.9	0.355
その他	2/38	5.3	10/42	23.8	0.025

表2　主な自覚症状を示した患者数とその割合（χ^2検定）

症状	SHS		IEI		p値
	人数	%	人数	%	
くしゃみ鼻水	14/38	36.8	2/42	4.8	0.040
咳・痰	11/38	28.9	5/42	11.9	0.078
呼吸困難・息苦しさ	13/38	34.2	4/42	9.5	0.015
めまい	13/38	34.2	10/42	23.8	0.354
発熱感・倦怠感	3/38	7.9	6/42	14.3	0.357
関節痛	1/38	2.6	7/42	16.7	0.015
動悸	3/38	7.9	6/42	14.3	0.327
皮膚症状	10/38	26.3	1/42	2.4	0.005
皮膚粘膜	27/38	71.1	1/42	2.4	<0.001

表3　主な検査項目において異常値を示した患者数とその割合（χ^2検定）

	SHS		IEI		p値
	人数	%	人数	%	
心理検査					
STAI 特性不安	37/47	83.3	25/42	59.5	0.020
STAI 状態不安	32/47	71.3	34/42	81.0	0.366
POMS 緊張-不安	28/92	30.2	13/41	31.7	0.520
POMS 抑うつ	30/86	34.9	13/40	32.7	0.841
POMS 怒り-敵意	29/86	31.4	13/41	31.7	>0.999
POMS 活気	15/82	18.3	20/42	47.6	0.146
POMS 疲労	34/86	39.5	12/40	45.0	0.701
POMS 混乱	26/86	30.2	13/41	31.7	0.918
血液・尿・生化学					
AST	8/69	9.7	6/35	17.1	0.340
CRE	2/63	3.7	6/32	18.8	0.030
総コレステロール	12/75	16.0	11/37	29.7	0.091
総IgE	11/71	15.5	10/35	28.6	0.070
眼科的検査					
TBUT_OS	2/63	3.1	3/35	8.7	0.353
聴覚検査					
TS値神	8/79	10.1	3/43	6.9	>0.999
視線運動検査					
0.5Hzサッケード PSE	9/77	11.7	6/37	16.2	0.519
0.5Hzアサッケード PSE	13/71	18.3	11/33	34.4	0.074

て有意に高かった（$p=0.015$）．めまいを両群に各々10人程度いてIEI群ではSHS群よりも多い傾向を示したが有意ではなかった．皮膚発症はIEI群においては1名のみであるのに対して，SHS群では30%を超え，有意差を示した（$p<0.01$）．逆に，頭痛症はIEI群では7名に見られたが，SHS群では1名のみで，有意差を示した（$p=0.03$）．

表3に主な検査項目において異常値を示した患者数とその割合を示す．心理検査における異常者の割合は両群とも高いが，群間差が認められなかった．血液・一般・生化学では，総コレステロールの異常値を示す患者が両群で各々10人余いてIEI群で異常値を示す割合が高い傾向が見られたが有意ではなかった．免疫学検査である総IgE量の異常値を示す患者が両群で各々10人程度いてIEI群で異常値を示す割合がSHS群に比べて高い傾向を示したが有意ではなかった．生理学的検査では，0.5Hzで動く視標を追跡する視標追跡検査におけるサッケード出現率の異常値を示す患者が両群で各々10人余いてIEI群に異常値が高い傾向が見られたが，有意ではなかった．

考　察

当科における IEI 群では，頻度は少ないものの気管支喘息およびアレルギー性疾患を除く自律神経の症状が SHS 群に比べて有意に多く，呼吸困難・息苦しさや関節痛の症状が SHS 群に比べて有意に多いが皮膚発疹に有意に少なかった．臨床検査において，IEI 群の総コンステロール値，総 IgE 値，0.5Hz 視標追跡検査におけるサッケード出現率の異常所見者は多い傾向をみすが有意ではなかった．

本研究における既往歴について，IEI 群における精神疾患の既往が SHS 群に比べて多かったが，文献に 42 人

中7人と先行研究[15-17]に比べてその割合は低かった．その理由には，必ずしも精神疾患に着目していなかったために患者の過少申告の可能性の影響が考えられる．一方，国内の研究で心療内科の視点でIEIを検討した江内らは，患者が長期の罹病の間に精神疾患としての診断を受ける機会が増える，生活の制限によるストレスなどの結果，精神疾患と診断されることを述べている[18]．本研究における精神疾患既往の少なさは，対象がすべて初診患者で罹病した患者が少なく，罹病期間が短いために保有が少なかったことによる可能性もある．IEI群の既往歴にアレルギー性疾患を除く耳鼻科の疾患が有意に多かった理由には，慢性な疾患への不安が背景にある可能性も考えられる．

症状については，SHS群では皮膚症状がIEI群に比べ有意に多かったことは化学物質の接触による身体的な自覚症状が現れたものと推測される．これに対して，IEI群では呼吸困難・息苦しさがSHS群に比べ有意に多かったが，頭痛などの異常者の率に差が認められなかった．IEI群に有意に多い関節痛は揮発性化学物質では有機化合物に関わる中毒学的な説明が困難で，化学物質との関係が考え難い．関節痛について，IEIに関する先行研究の記述は少なく，記載されていても列挙に留まり，鑑別診断や病態生理学的検討はなされていない[19]．IEI群における呼吸困難・息苦しさ，関節痛の症状は，化学物質による身体疾患よりも，精神疾患の，例えば過呼吸症候群が見られる不安障害や，身体表現性障害の疼痛性障害などが考慮されるべきかもしれない．

以上のように，IEIとSHSには症状に明確な差がある．その結果，IEIとSHSとでは自ずと対処の方向が異なると推測される．したがって，IEIを「広義のSHS」としてSHSの概念に含めることは治療などに混乱をもたらすと考えられる．

臨床検査について，両群における異常者率の有意差を示した一般的な臨床検査項目は認められなかった．IEIは症状を説明できる臨床検査の異常を示さないことが特徴である[20]．SHSもまた化学物質による粘膜刺激の刺激症状であるために，症状を説明できる臨床検査に異常を示さない[8]とされているため，本研究における臨床検査の比較は正常人との比較に近似すると考えられる．

血液検査におけるIEI群の総IgE値の異常者の割合が高い傾向を示したが，先行研究でもこれらの所見は示されているものの，直接的な関与は否定的である[21]．症状が必ずしもアレルギーと直接関連しないことから，アレルギー性疾患による粘膜の敏感さ，不安によって増幅された感覚，あるいは粘膜刺激への強い注意集中との関係が考慮されるべきかも知れない．

また，神経眼科学検査がIEIの診断に有効と石川らは述べていたが[22]，本研究においては両群における異常値を示した患者の割合に有意差を示さなかった．神経眼科学的検査については感度と特異性が十分とは言えないという批判があり[23,24]，今回の結果もまたそれと合致するものである．

IEIを心療内科の視点から検討した江内らは，IEI患者では対照に比べて発症に先立つ心理社会ストレスに関する1年間のストレス評価点が有意に高いことを見いだし，初診患者においてライフイベントの内訳を検討する必要があること，精神科医による構造化面接では85%がなんらかの精神疾患を合併していることを述べている[18]．また，前報で述べたとおり，米国ではIEIは不安障害と見なされ，欧州でも精神疾患としての研究が進みつつある[25-28]．精神疾患の視点から前述の症状を考えると，「化学物質」＝臭いと関係する不安障害，中でもパニック障害や身体表現性障害の疑い，あるいは心的外傷後ストレス障害PTSDで説明が可能となる．以上から，日本でもIEI患者を心理社会ストレスやそれに関わる精神疾患，それらと関連したIEIの形成過程から検討することが必要と考えられる．

本研究では，対象が外来患者で施行できる検査が限られ，発症に先立つ心理社会ストレスなどの検索が行われていないなどの限界がある．しかし，初診患者を対象とし，比較的多くの患者から得た症状などの結果から，IEIの理解を深める資料を得られたと考えられる．

まとめ

IEIの病像を明らかにする目的で，IEI患者43名とSHS患者38名について，既往疾患，症状，臨床検査の比較を行った．

IEI群では，数は少ないが精神疾患とアレルギー性を除く耳鼻の疾患の既往がある患者の割合が有意に高く，呼吸困難・息苦しさを訴える患者と少数であるが関節痛を訴える患者が有意に多かったが，皮膚発疹は逆に有意に少なく，臨床検査では総コレステロール値，総IgE，視標追跡検査の0.5Hz滑動性サッケード率で異常者率が高い傾向が見られたが，有意差を示さなかった．

症状の検討からは心理社会ストレスや精神疾患の視点からの検討が今後必要と考えられた．

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Clinical Findings in Idiopathic Environmental Intolerance Patients: Comparison with Those in Sick-house Syndrome Patients

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In order to clarify the clinical features of patients with idiopathic environmental intolerance (IEI) which is confused with sick house syndrome (SHS), we compared the clinical findings of IEI and SHS patients visited from 2005 to 2008. We compared the rates of history and subjective symptoms and the abnormal rates of psychological and laboratory tests among 42 IEI patients and 88 SHS patients. The frequencies of past histories of psychiatric diseases and ophthalmologic or oto-rhinologic diseases (except for allergic diseases) in IEI patients were low, but significantly higher than those in SHS patients. The frequency of skin eruptions in IEI patients was significantly less than those in SHS patients, but, that of respiratory distress in IEI patients was significantly higher than that in SHS patients. Compared to the rates of SHS patients, IEI patients showed higher abnormal rates of total cholesterol and total immunoglobulin E values and/or saccade rate of 0.5 Hz. however, this difference was not statistically significant. Evident differences between IEI and SHS were found, and further investigation from the point of view of psychosocial stress and psychiatric diseases is necessary.

(JJOMT, 61: 119—124, 2013)

以上の研究結果から、SHS と IEI の特異的診断法とされていた検査の有用性は確認されなかったが、精神心理検査の異常率が非常に高く、メンタル対応が必要な患者群と考えられた。また、IEI において化学物質曝露との関連性が低い事から、治療としては、継続的な聞き取りやコンサルテーションなど心理面の補佐が必要であることが示唆された。

3) 曝露指標の研究・開発

3-1) ヒ素曝露の評価法

ヒ素曝露の評価法としては、形態別ヒ素分析が必要であり、無機ヒ素とモノメチルアルソン酸の合計量が適切である事を第 1 期において報告し、特化則の特殊健康診断項目として採用された。第 2 期においては、迅速で共存物質の妨害を除去した高速液体クロマトグラフィー―誘導結合プラズマ質量分析法(HPLC-ICP-MS)を開発し報告した(J Occup Health, 51:380-385, 2009)。

J Occup Health 2009; 51: 380–385

Short Communication

Rapid and Effective Speciation Analysis of Arsenic Compounds in Human Urine using Anion-Exchange Columns in HPLC-ICP-MS

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Key words: Arsenic, Biological monitoring, HPLC-ICP-MS, Speciation analysis

Occupational arsenic exposure is mainly in the form of inorganic arsenic (iAs)[1–3]. iAs is methylated to monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) in the human body as shown in Fig. 1[4]. The American Conference of Governmental and Industrial Hygienists (ACGIH)[1] and the Deutsche Forschungsgemeinschaft (DFG)[5] recommend the sum of iAs, MMA, and DMA as the biological exposure value for iAs exposure. However, seafood including seaweeds contains high levels of organoarsenic compounds, such as arsenobetaine (AsBe), DMA, arsenocholine (AsCho), and arsenosugars (AsSugs)[4–9]. AsBe is only minimally metabolized in mammals[10]. AsCho is metabolized extensively to AsBe[7]. AsSugs are extensively metabolized to DMA[6]. These metabolic pathways are shown in Fig. 1. Therefore, large amounts of AsBe and DMA and small amounts of MMA and iAs are observed in the urine of people without occupational iAs exposure who ingest seafood[9].

Urinary speciation analysis of some of the above-mentioned organoarsenic compounds was performed by high performance liquid chromatography (HPLC) with inductively coupled plasma mass spectrometry (ICP-MS). In our previous reports[8, 10], we analyzed arsenic compounds in the urine of Japanese and Bangladeshi people using a cation exchange column and detected some unknown peaks in the urine of Japanese, but not Bangladeshis. When we analyzed extracts from hijiki seaweed using a cation exchange column, arsenosugars were found at the same retention time as sodium arsenate (AsV), suggesting insufficient separation of the metabolites of AsSugs from iAs. Here, we compared speciation analysis of urinary arsenic in Japanese using anion and cation exchange columns.

Materials and Methods

Subjects

The subjects were 172 healthy male workers with a mean age of 46.5 ± 13.6 yr (range 18–74 yr) who were working in Kita Kyushu, Japan. They had no occupational arsenic exposure for at least six months. Urine sampling was performed in the afternoon in a regular medical examination or in a medical checkup conducted by the subjects' employer between August 2008 and October 2008. After urine sampling, the company's health supervisor erased the subjects' names from the samples, and we then analyzed them. This study was approved by the Ethics Committee of the Tokyo Rosai Hospital (approval number: 20–9).

Chemicals

Sodium arsenite (AsIII), sodium arsenate (AsV), MMA, and AsBe were purchased from Wako Pure Chemical Industries (Osaka, Japan). DMA was obtained from Tri Chemical Laboratory (Yamanashi, Japan). Nitric acid (HNO_3; Tamapure AA-100 (68%)), ammonia solution (Tamapure AA-10 (20%)) (Tama Chemicals, Tokyo, Japan), 2,6-pyridinedicarboxylic acid (Tokyo Kasei Industry, Tokyo, Japan), ammonium nitrate (NH_4NO_3; Wako Pure Chemical Industries), and ammonium bicarbonate (NH_4HCO_3; Fulka BioUltra) (Sigma-Aldrich, St. Louis, MO, USA) were used for the HPLC mobile phase. Germanium standard solution for atomic absorption spectrophotometry (Kanto Chemical, Tokyo, Japan) was used as an internal standard for ICP-MS detection. Ultrapure water for analysis was prepared using a Milli-Q-ICP/MS Ultrapure Water Purification System (Millipore, Tokyo, Japan).

Certified reference material, NIES CRM No. 18 (human urine), from the National Institute for Environmental Studies, Japan, was used to validate the analytical procedure.

Analytical conditions for HPLC and ICP-MS

Arsenic speciation analysis was performed using two instrument systems. For the separation of arsenic compounds, we used anion and cation exchange columns as two separation modes.

In the anion mode experiment, an Agilent 1200 HPLC series and an Agilent 7500cs ICP-MS (Agilent Technologies, Santa Clara, CA, USA) were used to separate arsenic species and detect arsenic, respectively. The instrument conditions for ICP-MS were as follows:

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Correspondence to: Y. Suzuki, Research Center for Occupational Poisoning, Tokyo Rosai Hospital, Japan Labour Health and Welfare Organization, 4–13–21 Omori-minami, Ohta-ku, Tokyo 143–0013, Japan (e-mail: suzy@h5.dion.ne.jp)

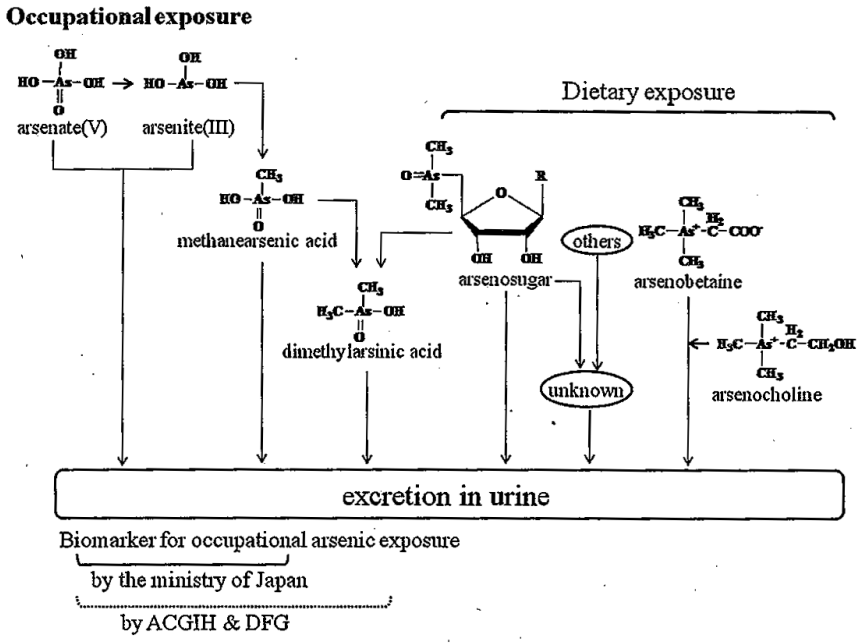

Fig. 1. Metabolic pathways of arsenic compounds from occupational and dietary exposure.

RF power, 1,500 W; plasma argon gas flow rate, 15 *l*/min; carrier argon gas flow rate, 0.9 *l*/min; make-up argon gas flow rate, 0.2 *l*/min; a coaxial-type nebulizer, nickel sampling, and skimmer cones were used. As an anion exchange column, we used a Dionex IonPac AS22 (250 × 4.0 mm i.d., Dionex, Sunnyvale, CA, USA) under the following conditions: mobile phase, 20 mM NH_4HCO_3 at pH 10.0 (adjusted with ammonia solution); flow rate, 1.2 m*l*/min; column temperature, 40°C; and injection volume, 50 *μl*.

In the cation mode experiment, a PU712 pump and a CO631A column oven (GL Science, Tokyo, Japan) and a Midas autosampler (Spark, Emmen, Netherlands) were used to separate the arsenic species, and an Elan DRCII ICP-MS (Perkin Elmer Sciex, Concord, Ontario, Canada) was used for arsenic detection. Instrumental conditions for ICP-MS were as follows: RF power, 1,300 W; plasma argon gas flow rate, 15 *l*/min, auxiliary argon gas flow rate, 1.2 *l*/min, and nebulizer argon gas flow rate, 1.0 *l*/min; a coaxial-type nebulizer was used; skimmer and sample cones were platinum. As a cation exchange column we used a Shodex RSpak NN-614 (150 × 6.0 mm i.d.; Showa Denko, Tokyo, Japan) under the following conditions: mobile phase, 5 mM HNO_3/6 mM NH_4NO_3/1.5 mM 2,6-pyridinedicarboxylic acid; flow rate, 1.0 m*l*/min; column temperature, 40°C; and injection volume, 50 *μl*.

Stock standard solutions of AsIII, AsV, MMA, DMA, and AsBe were prepared by dissolving each compound in ultrapure water at a concentration of 10 mgAs/*l*. Working standard solutions were prepared every month by diluting the stock standard solution to 0.1 mgAs/*l*. The final mixed standard solution (5 *μg*As/*l*) was prepared from the working standard solution just before use. To obtain precise measurements, 0.1 mg/*l* of germanium solution was used as the internal standard for ICP-MS. ICP-MS detection mass was set as *m/z* 75 ($^{75}As^+$), *m/z* 35 ($^{35}Cl^+$), and *m/z* 72 ($^{72}Ge^+$). Limits of detection for AsIII, AsV, MMA, DMA, and AsBe were calculated according to the definition given by Gibbons[11] as 3.14 times the standard deviations of the concentrations corresponding to the peak area ratio obtained for 50 *μl* injection of standard solution containing these compounds at 5.0 *μg*/*l* each.

The accuracy of the analysis was confirmed using reference material, CRM No.18 (human urine), from the National Institute for Environmental Studies (NIES), Tsukuba, Japan.

Urine sample preparation

Urine samples were stored in sealed plastic tubes at −80°C in a refrigerator until analysis. Arsenic species

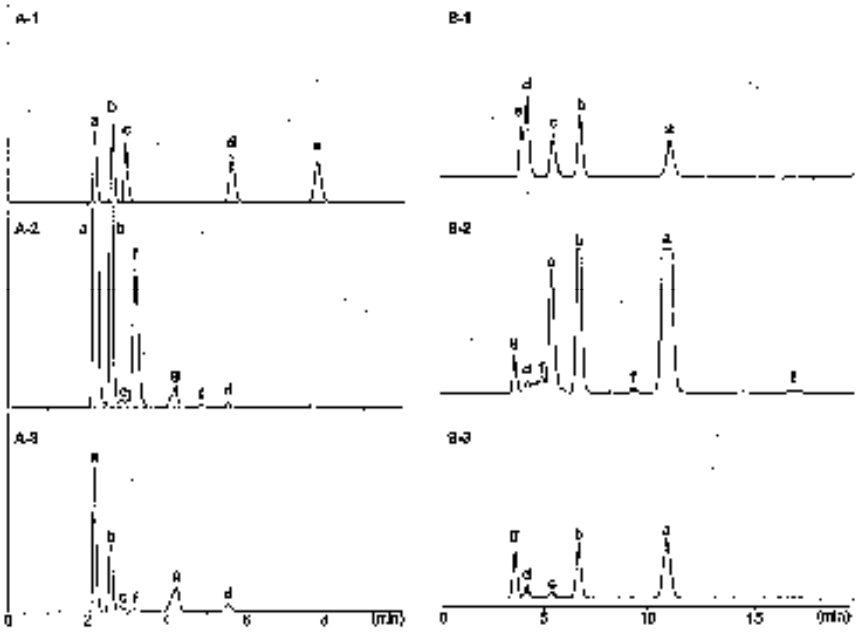

Fig. 2. Chromatograms obtained by HPLC-ICP-MS of a standard solution and two different urine samples. A, analysis with the anion column; B, analysis with the cation column (see Materials and Methods). 1, standard solution; 2, urine sample containing a high UK peak; 3, urine sample containing a very low UK peak. The peaks show a, AsBe; b, DMA; c, AsIII; d, MMA; e, AsV; f, UK; g, ArCl. The total arsenic (the sum of arsenics detected) were 530.1 μg/l for A-2, 475.6 μg/l for B-2, 88.6 μg/l for A-3, and 79.3 for B-3, respectively.

in urine were stable under the conditions described above[14,15]. The defrosted samples were diluted five-fold with ultrapure water, filtered through a 0.45-μm polyvinylidene fluoride membrane filter (Whatman 13 mm GD/X syringe filter; Whatman, Florham Park, NJ, USA), and analyzed by HPLC-ICP-MS as described above.

Creatinine in urine was analyzed photometrically using creatinase and N-(3-sulfopropyl)-3-methoxy-5-methylaniline using a commercial kit (Pure Auto CRE-N; Daiichi Pure Chemicals, Tokyo, Japan).

Statistical analysis

Data collected using a questionnaire and by urinary determinations were analyzed by the means of the SPSS statistical package (SPSS version 11.5J for Windows, SPSS Japan, Tokyo, Japan).

Results and Discussion

Figure 2 shows the chromatograms obtained by HPLC-ICP-MS of a standard solution and two different urine samples. A high unknown (UK) peak (peak *f*) was detected at a retention time (Rt) of 3.7 min in the anion mode (Fig. 2, A-2), but not in the cation mode (Fig. 2, B-2). An argon chloride (ArCl) peak (peak g) was detected in the urine samples at Rt of 4.2 and 3.8 min in the anion (Fig. 2, A-2 and A-3) and cation (Fig. 2, B-2 and B-3) modes, respectively. In our laboratory, cation mode analysis has been used for the speciation analysis of five arsenic species in human urine using HPLC-ICP-MS[16], and we reported the median values (μgAs/l) of urinary arsenics for 210 Japanese male subjects without occupational exposure as 3.5 for AsIII, 0.1 for AsV, 2.1 for MMA, 42.6 for DMA, and 61.3 for $AsBe^{+}$. However, since the separation of AsV and MMA was not sufficient, as shown in Fig. 2, B-1, a chromatographic condition for complete separation of the five arsenics was studied. The anionic condition using an IonPac AS22 column and a mobile phase of ammonium hydrogen carbonate buffer gave successful separation of the five arsenic species

Table 1. Urinary arsenic species concentrations of 172 workers without occupational iAs exposure

a) Analysis by anion column of IonPac AS22								
Item	AsV	AsIII	MMA	DMA	AsBe	Others	T-As	iAs + MMA
25%tile	ND	(ND)	1.2	21.1	31.0	1.9	70.9	2.9
	(ND)	(ND)	(1.2)	(19.4)	(28.8)	(1.5)	(63.9)	(2.6)
Median	ND	1.5	2.3	41.1	72.5	4.1	152.2	4.4
	(ND)	(1.1)	(1.8)	(30.8)	(55.8)	(3.3)	(97.5)	(3.5)
75%tile	0.9	2.7	3.7	62.6	120.7	8.6	300.7	7.1
	(0.8)	(1.7)	(2.4)	(42.8)	(92.2)	(6.5)	(278.2)	(6.9)
95%tile	1.7	5.4	6.2	109.2	243.7	23.7	368.4	12.6
	(2.3)	(2.9)	(3.6)	(87.9)	(217.6)	(21.8)	(314.6)	(8.2)
b) Analysis by cation column of ASpak NN-614								
Item	AsV	AsIII	MMA	DMA	AsBe	Others	T-As	iAs + MMA
25%tile	ND	2.6	2.8	23.3	27.9	ND	72.4	5.6
	(ND)	(2.1)	(2.2)	(22.0)	(26.7)	(ND)	(63.8)	(4.8)
Median	ND	5.3	4.3	44.0	65.0	ND	127.8	10.1
	(ND)	(3.9)	(2.9)	(32.6)	(47.1)	(ND)	(93.8)	(7.0)
75%tile	ND	10.1	6.2	66.1	100.8	2.6	191.0	16.2
	(ND)	(7.0)	(4.1)	(45.7)	(81.9)	(1.9)	(134.9)	(13.8)
95%tile	ND	22.2	10.6	116.4	218.7	8.1	538.4	30.6
	(ND)	(17.7)	(6.8)	(93.7)	(192.7)	(5.9)	(288.4)	(22.9)

Values are expressed as μg As/l and in parentheses as μgAs/g creatinine. T-As: total arsenic. ND: Lower than LOD.

within 10 min (Fig. 2, A-1). When we examined another anion exchange condition using a tandem joint of Gelpack GL-IC A15 and an A13 column (150 × 4.6 mm I.d. and 100 × 4.6 mm I.d.; Hitachi High Technologies, Tokyo, Japan) with a phosphate buffer as a mobile phase, complete separation was obtained within 30 min. However, accumulation of phosphate salts on the sampling cone caused damage to the ICP-MS interface, and thus exchange of the cones was necessary every 60 analyses. The use of volatile ammonium hydrogen carbonate buffer decreased accumulation of salts on the cone to a minimum and resulted in less instrument down time and a lower cost analysis. This analytical condition is suitable for routine analysis.

The limits of detection (LOD; μgAs/l) of the arsenic compounds in the anion and the cation modes were as follows: AsIII, 0.3 and 0.3; AsV, 0.2 and 0.4; MMA, 0.2 and 0.3; DMA, 0.3 and 0.2; AsBe, 0.4 and 0.3, respectively. The UK peak was measured using the DMA standard and the sum of UK was designated "Others." When no peak was detected at the corresponding retention time or the detected peak area was lower than the LOD, the concentration of the component was treated as zero or was presented as the half of the LOD, respectively, to calculate total arsenic.

The AsBe, DMA, and total arsenic concentrations (μgAs/l) in the reference material of CRM No. 18 urine were determined to be 64.0 ± 1.5, 39.3 ± 0.5, and 137.3 ± 4.2 (n=5), respectively, using the anion exchange column, and 70.1 ± 1.0, 37.2 ± 0.5, and 131.5 ± 1.2 (n=5) using the cation exchange column, respectively. These values were within the ranges for the certified values of 69 ± 12, 36 ± 9, and 137 ± 11, respectively.

The results of speciation analysis of arsenic in the urine of 172 subjects are shown in Table 1. For both the anion and cation columns, the highest arsenic concentration in urine was that of AsBe, followed by that of DMA. DMA and AsBe concentrations were almost the same as measured by the two columns, indicating that these two peaks were well separated in both columns. The ratios of these two species increased with total arsenic concentration. AsIII and MMA concentrations measured using the anion column were lower than those measured using the cation column; the reverse was found for the concentration of Others. The sums of iAs and MMA measured using the anion column were much lower than those measured using the cation column. We then examined the relationship between the difference in the sum of iAs and MMA values obtained using the anion column and the cation column, and the difference in the value for Others using the anion column and the cation column. As shown in Fig. 3, the regression line clearly

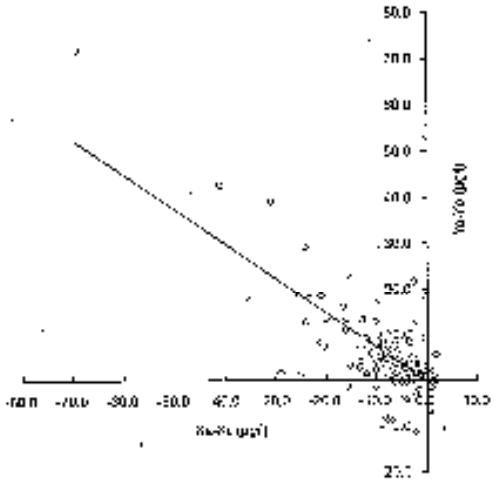

Fig. 3. Relationship between the differences in the sum of iA and MMA values with the anion column (Xa) and the cation column (Xc), and the differences in the Others value with the anion column (Ya) and the cation column (Yc). The horizontal axis shows X = Xa−Xc and the vertical axis Y=Ya−Yc. The regression formula is expressed as follows: y=−0.752x − 3.1541 (R^2=0.5898, p<0.001).

shows a negative linear correlation, indicating that the differences in the sum of iAs and MMA in the two analytical modes may be caused by false recognition of the UK compound; i.e., the UK peak in the anion column may be detected as AsIII or MMA in the cation column.

It is known that seafood and marine samples contain various organic arsenic compounds, such as AsBe, AsChu, DMA, and AsSug[5]. Using liquid chromatography coupled to tandem quadrupole mass spectrometry, we found six oxo-arsenosugars in extracts from hijiki seaweed sold in a Japanese market[16]. After seaweed ingestion, AsSugs are metabolized and their metabolites are excreted in urine[5–7]. Although we did not identify the UK peaks detected using the anion column, they might be the metabolites of AsSugs or other organic arsenic compounds.

Recently, the consumption of seafood has been increasing throughout the world. Seafoods contain DMA; therefore, DFG recommends that only the inorganic arsenic fraction should be determined in future[2]. For biological monitoring of the occupational iAs exposure of workers who habitually consume seafood, the sum of iAs and MMA is expected to be more suitable than the sum of iAs, MMA, and DMA[5]. A Japanese government industry also defines the sum of iAs and MMA in urine as the iAs exposure indicator[18].

Conclusion

We determined urinary arsenic concentrations in 172 subjects living in Japan without occupational exposure to arsenic for at least six months by HPLC-ICP-MS using anion and cation exchange columns. Use of an anion exchange column of Dionex IonPac AS22 with a volatile buffer can completely separate iAs and the metabolites within 10 min and prevents the accumulation of salts on the core, resulting in less instrument downtime and less costly analysis. The ninety-fifth percentiles of AsV, AsIII, MMA, and the sum of iAs and MMA concentrations in the urine of 172 healthy subjects were 1.7, 5.4, 6.2, and 12.6 μgAs/l, respectively. We propose the 95th percentile of the sum of iAs and MMA concentrations measured by the anion-exchange column, 12.6 μgAs/l, as the background value for the biological index of occupational iAs exposure.

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3-2) ガスクロマトグラフィー―質量分析法(GC-MS)による形態別ヒ素分析の開発
更に形態別ヒ素分析を普及させるために、ジメルカプロールを用いて特異的誘導体を作成し、GC-MS による測定法を開発し報告した(J Occup Health, 54:434-440, 2012)。

J Occup Health 2012; 54: 434–440

Journal of Occupational Health

Development of an Analytical Method for the Determination of Arsenic in Urine by Gas Chromatography-mass Spectrometry for Biological Monitoring of Exposure to Inorganic Arsenic

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Abstract: Development of an Analytical Method for the Determination of Arsenic in Urine by Gas Chromatography-mass Spectrometry for Biological Monitoring of Exposure to Inorganic Arsenic: Akito TAKEUCHI, *et al.* Osaka Occupational Health Service Center, Japan Industrial Safety and Health Association—Objectives: The purpose of this study was to develop an analytical method for the simultaneous determination of inorganic arsenic [As(III) and As(V)] and monomethylarsonic acid (MMA) in urine by gas chromatography-mass spectrometry (GC–MS) for the biological monitoring of exposure to inorganic arsenic. **Methods:** Arsenic compounds (after reduction of arsenic to the trivalent state) were derivatized with 2,3-dimercapto-1-propanol and then analyzed using a GC-MS. The proposed method was validated according to the US Food and Drug Administration guidelines. The accuracy of the proposed method was confirmed by analyzing Standard Reference Material (SRM) 2669 (National Institute of Standards and Technology). **Results:** Calibration curves showed linearity in the range 1–100 μg/l for each of the arsenic species, with correlation coefficients of >0.999. For each of the arsenic species, the limits of detection and quantification were 0.2 μg/l and 1 μg/l, respectively. The recoveries were 98–105%, 98–102% and 98–112% for As(III), As(V) and MMA, respectively. Intraday accuracy and precision were 92.7–99.8% and 0.9–7.4%, respectively. Interday accuracy and precision were 91.3–100.0% and 0.8–6.9%, respectively. The analytical values of SRM 2669 obtained by the proposed method were sufficiently accurate. **Conclusions:** The proposed method overcame the disadvantages of high-performance liquid chromatography with inductively coupled plasma mass spectrometry. It was a robust, selective and cost-effective method suitable for routine analyses and could be useful for the biological monitoring of occupational exposure to inorganic arsenic.
(J Occup Health 2012; 54: 434–440)

Key words: 2,3-Dimercapto-1-propanol, Arsenate, Arsenite, Gas chromatography-mass spectrometry, Monomethylarsonic acid, Urine

Arsenic is widely distributed in the environment. Pollution due to arsenic worldwide has become a serious problem and has caused various adverse effects on human health [1]. Significant occupational exposure to arsenic exists in several industries, such as non-ferrous smelting, electronics and wood preservation[2–4]. Such exposure is mainly in the form of inorganic arsenic (iAs), including arsenite [As(III)] and arsenate [As(V)][5,6]. Arsenic and inorganic arsenic compounds have been classified as Group 1 (carcinogenic to humans) compounds by the International Agency for Research on Cancer (IARC) because they cause cancers of the skin, bladder and lungs, and there is limited evidence that they may also cause cancers of the kidney, liver and prostate[4].

iAs is methylated to monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) in the human body[7–9]. The American Conference of Governmental

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and Industrial Hygienists (ACGIH)[8] and the Deutsche Forschongsgemeinschaft (DFG)[9] recommend the sum of iAs, MMA and DMA concentrations as the biological exposure value for iAs exposure. However, large amounts of DMA and arsenobetaine (AsBe) and small amounts of MMA and iAs have been observed in the urine of Japanese individuals who habitually ingest seafood without occupational exposure to iAs because seafood (including seaweeds) contains high levels of organoarsenic compounds, such as DMA, AsBe, arsenosugars and arsenocholine[10]. Recently, Hata et al. reported that the sum of iAs and MMA concentrations in urine was more suitable than that of iAs, MMA and DMA concentrations in urine for the biological monitoring of occupational iAs exposure of workers who habitually consume seafood[5]. The Ministry of Health, Labour and Welfare of Japan also defined the sum of iAs and MMA concentrations in urine as the iAs exposure indicator[6]. Therefore, a rapid and simple analysis for only iAs and MMA is more effective than speciation analysis for the biological monitoring of occupational exposure to iAs.

Currently, high-performance liquid chromatography with inductively coupled plasma mass spectrometry (HPLC-ICP-MS) is the mainstay of speciation analyses of arsenic in biological samples because of its sensitivity and effectiveness[7-11]. However, it is a remarkably expensive and complicated system, and hence it is not commonly used in many laboratories. Moreover, its operation requires skilled techniques and high running costs.

The aim of the present study was to develop and validate a gas chromatography-mass spectrometry (GC-MS) method for the simultaneous determination of iAs and MMA in urine. To obtain the sensitivity required for biological monitoring, we modified the previous GC-flame photometric detection method using 2,3-dimercapto-1-propanol (also known as British Anti-Lewisite: BAL) as the derivatizing reagent[6]. We used GC-MS equipped with a capillary column, because it has a high capability of separation, high sensitivity and high efficiency for confirmatory identification of compounds. Another required modification was optimization of the procedure for sample preparation, including optimization of reaction conditions, scale-down of the sample volume and change of extraction solvent. The proposed method overcame the disadvantages of HPLC-ICP-MS. It was a robust, selective and cost-effective method suitable for routine analyses.

Materials and Methods

Materials

Sodium metaarsenite was purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Disodium hydrogenarsenate heptahydrate and BAL were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Methylarsonic acid/water solution (1,050 μg/ml) was procured from Tri Chemical Laboratories Inc. (Yamanashi, Japan). Hydrochloric acid (HCl), tin (II) chloride dihydrate ($SnCl_2.2H_2O$), potassium iodide (KI), dichloromethane (DCM) and hexadecane (C16) were of analytical grade or better (for atomic adsorption spectrometry or for analysis of poisonous metal). Water was purified with an Elix 5 system (Millipore, Bedford, MA, USA). Standard Reference Material (SRM) 2669 (arsenic species in frozen human urine) was purchased from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA).

$SnCl_2$ solution was prepared by dissolving $SnCl_2.2H_2O$ in HCl (50 mg/ml). KI solution [20% (w/v)] and BAL solution [0.2% (v/v)] were prepared by dissolving KI and BAL, respectively, in water. The extraction solution was DCM containing C16 (10 μg/ml) as an internal standard (IS). Standard stock solutions of As(III), As(V) and MMA were prepared in water (100 mg/l as As) and stored in PFA bottles at 4°C.

Urine samples were collected from healthy adult volunteers. These volunteers were not occupationally exposed to arsenic and were under dietary restriction of seafood. Informed consent had been obtained from the volunteers before collection of urine. This study was approved by the Ethics Committees of the Graduate School of Medicine, Osaka City University (approval number 2367).

Instruments

The GC-MS system used was a 7890A gas chromatograph equipped with a 5975C inert XL mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). The column was a 30 m × 0.25 mm ID InertCap 5MS/NP capillary column with a 0.25-μm film thickness (GL Sciences Inc., Tokyo, Japan). Helium was used as the carrier gas at a flow rate of 1.0 ml/min. The temperatures of the injection port and the transfer line were set at 250 and 280°C, respectively. The oven temperature was set at 70°C for 1 minute and then increased to 280°C at a rate of 10°C/min. Samples (2 μl) were injected in the pulsed splitless mode (pulse pressure, 25 psi; pulse time, 1 minute; purge activation time, 0.9 minutes). The mass spectrometer was operated in the electron impact (EI) mode at an electron energy of 70 eV. The ion source and quadrupole analyzer were maintained at 230 and 150°C, respectively. Data were obtained in the selected ion monitoring (SIM)/scan mode. The ions selected for SIM were m/z 197 and 212 for BAL-MMA, m/z 226 and 183 for C16 and m/z 212 and 165 for

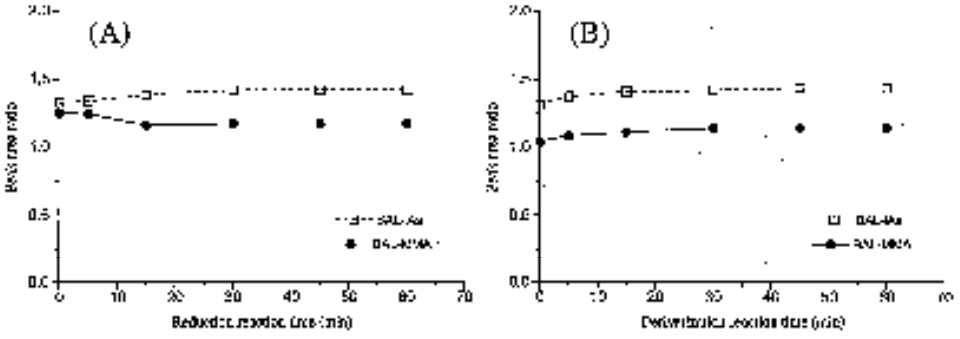

Fig. 1. Effects of the (A) reduction reaction time and (B) derivatization reaction time on the production of the derivatives of inorganic arsenic (BAL-iAs) and monomethylarsonic acid (BAL-MMA).

BAL-iAs, in which the former was selected as the quantifier ion and the latter as the qualifier ion. To confirm the mass fragmentation of the derivatives, data were obtained in scan mode with a scan range from m/z 40 to 250.

Sample preparation

Urine (2 ml) was placed in a glass test tube. SnCl$_2$ (0.4 ml) and KI (0.2 ml) solutions were added to the tube, which was vortex-mixed (10 seconds) and then allowed to stand for 30 minutes at room temperature. BAL solution (0.2 ml) was added to the tube, which was vortex-mixed (10 seconds) and then allowed to stand for 30 minutes at room temperature. Extraction solution (0.5 ml) was added to the tube and shaken gently for 1 minute to avoid emulsion formation. The mixture was centrifuged at 3,000 rpm for 10 minutes, and a 3-μl aliquot of the extraction solution layer was injected into the GC-MS system.

Method validation

Method validation was conducted according to the US Food and Drug Administration (FDA) guidelines[9]. To plot calibration curves, urine samples spiked with As(III) or As(V) and MMA at six concentrations (matrix-matched standard) ranging from 1 to 100 μg/l were prepared in triplicate, and the prepared samples were analyzed using the procedure for sample preparation described above. Calibration curves were obtained by plotting the peak area ratio of BAL derivatives of As(III), As(V) or MMA to IS against their respective concentrations. The reproducibility, which was defined as precision, of the developed method was evaluated by analyzing urine samples containing three concentrations (1, 25 and 100 μg/l) of As(III) or As(V) and MMA on the same day (five replicates; intraday reproducibility) and over three consecutive days (five replicates; interday reproducibility). Recovery was determined by comparing the responses of the BAL derivative of As(III), As(V) or MMA in spiked urine samples with those of water standards subjected to the same procedure. The limit of detection (LOD) and limit of quantification (LOQ) were defined as the amounts of As(III), As(V) or MMA in urine that corresponded to 3 and 10 times the baseline noise, respectively. Finally, the accuracy of the proposed method was confirmed using SRM 2669. The obtained results and certified values were compared according to application note 1 of the Institute for Reference Materials and Measurements (IRMM)[10].

Results

Optimization of reaction conditions

To determine the optimal reaction conditions, we examined the influences of the reduction reaction time and the derivatization reaction time using urine samples spiked with 100 μg/l each of As(V) and MMA. When the reduction reaction time ranged from 0 to 60 minutes with a derivatization reaction time of 30 minutes, the reduction reaction was complete within a short time, and the BAL-iAs/IS and BAL-MMA/IS peak area ratios remained at a plateau until 60 minutes [Fig. 1(A)]. When the derivatization reaction time ranged from 0 to 60 minutes with a reduction reaction time of 30 minutes, the BAL-As/IS and BAL-MMA/IS peak area ratios attained maximum area ratios after 30 minutes and remained constant for 60 minutes [Fig. 1(B)].

Validation

Calibration curves showed linearity in the range 1–100 μg/l for each of the arsenic species, with correlation coefficients of >0.999. For each of the inorganic

Table 1. Range of linearity and correlation coefficient of the proposed method

	Detection limit (μg/l)	Range of linearity (μg/l)	Linearity		Correlation coefficient
			Slope	Intercept	
As(III)	0.2	1–100	0.0149	0.009	1.000
As(V)	0.2	1–100	0.0146	0.004	1.000
MMA	0.2	1–100	0.0122	−0.005	0.999

As(III): arsenite. As(V): arsenate. MMA: monomethylarsonic acid.

Table 2. Intra- and interday coefficients of variation of the proposed method

Spiked concentration (μg/l)	Recovery (n = 5)		Intraday (n = 5)			Interday (n = 15)		
	Mean ± SD (%)	RSD (%)	Mean ± SD (μg/l)	RSD (%)	Accuracy (%)	Mean ± SD (μg/l)	RSD (%)	Accuracy (%)
As(III)								
1	100 ± 7.4	7.4	0.90 ± 0.07	7.4	90.3	0.90 ± 0.04	4.8	89.5
25	97 ± 0.9	0.9	24.34 ± 0.23	0.9	96.5	24.28 ± 0.20	0.8	97.1
100	95 ± 1.9	2.0	99.79 ± 2.03	2.0	99.8	99.95 ± 1.53	1.5	100.0
As(V)								
1	102 ± 5.7	5.6	0.91 ± 0.05	5.6	91.1	0.91 ± 0.09	9.9	90.9
25	99 ± 1.2	1.2	24.21 ± 0.30	1.2	96.8	24.33 ± 0.44	1.8	97.3
100	99 ± 3.1	3.1	99.66 ± 3.09	3.1	99.7	99.97 ± 2.18	2.2	100.0
MMA								
1	107 ± 6.9	6.4	0.83 ± 0.05	6.4	82.7	0.91 ± 0.05	6.2	81.4
25	99 ± 1.1	1.1	23.17 ± 0.25	1.1	92.7	23.47 ± 0.56	2.4	93.9
100	103 ± 3.0	2.7	99.36 ± 2.71	2.7	99.4	99.78 ± 2.80	2.8	99.8

As(III): arsenite. As(V): arsenate. MMA: monomethylarsonic acid. RSD: relative standard deviation.

Table 3. Results for arsenic species in NIST SRM 2669 (Levels I and II)[a]

Species	Level I		Level II	
	This method[b]	Certified[c]	This method[b]	Certified[c]
As(III)	—	0.47 ± 0.10	—	5.03 ± 0.31
As(V)	—	3.41 ± 0.30	—	6.15 ± 0.95
iAs	3.9 ± 0.0	—	11.4 ± 0.1	—
MMA	2.4 ± 0.4	1.87 ± 0.39	6.9 ± 0.4	7.18 ± 0.56

[a] Concentrations in μg/l as As. [b] Values are expressed as the mean ± SD (n=3). [c] Values are expressed as the certified value ± the expanded uncertainty (95% confidence interval). As(III): arsenite. As(V): arsenate. MMA: monomethylarsonic acid. iAs: inorganic arsenic. NIST: National Institute of Standards and Technology. SRM: Standard Reference Material.

species, LOD and LOQ were 0.2 μg/l and 1 μg/l, respectively (Table 1). The recoveries were 96–103%, 99–102% and 99–112% for As(III), As(V) and MMA, respectively. Intraday accuracy, expressed as the deviation from the nominal value, was 82.7–99.8%, and intraday precision, expressed as the relative standard deviation (RSD), was 0.9–7.4%. Interday accuracy and precision were 81.3–100.0% and 0.8–9.9%, respectively (Table 2). The analytical values of SRM 2669 obtained by the proposed method are shown in Table 3.

Discussion

Our goal in the present study was to develop and validate a GC-MS method for the simultaneous determination of iAs and MMA in urine. Several GC methods have been reported for the determination of iAs, MMA or DMA[a] [11–20]. In these methods, derivatizations using various thiols, such as BAL[8,21], thioglycol methylate (TGM)[12–17] and 1,3-propanedithiol (PDT)[18–20], were used to enable GC analyses because these arsenic species are essentially non-volatile. For the choice of an optimal derivatizing reagent, we

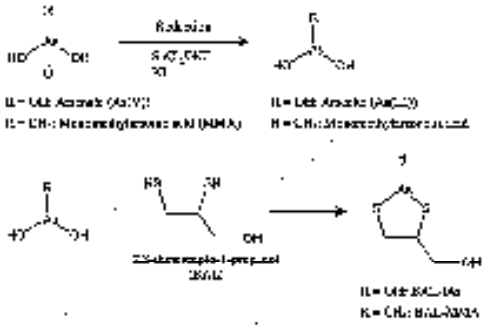

Fig. 2. Reaction scheme of inorganic arsenic and monomethylarsonic acid with 2,3-dimercapto-1-propanol as reported by Fukui *et al.*[9]

initially examined the use of TGM, but this choice was unsuccessful for iAs. Claessen *et al.* suggested that an incomplete reaction of iAs with TGM or decomposition of the derivative after injection into the GC system could be the possible reasons for this failure[14]. Szostek *et al.* also reported decomposition of the derivative in the GC injector[28]. We next investigated the use of PDT but did not observe any signal for iAs. In contrast to these derivatizing reagents, BAL showed the best results for iAs and MMA in the derivatization reaction and chromatographic separation. Therefore, we adopted BAL as the derivatizing reagent.

The reaction scheme of iAs and MMA with BAL reported by Fukui *et al.* is presented in Fig. 2[9]. BAL selectively reacts only with arsenic compounds in the trivalent state to give organic solvent extractable volatile complexes. Therefore, As(V) and MMA in their pentavalent state must be reduced to a trivalent state before derivatization. As(III) and As(V) form the same derivative upon reaction with BAL and are determined as the sum of these derivatives. Typical mass chromatograms of blank urine, standard spiked urine and SRM 2669 level II, as well as the mass spectra of BAL-iAs and BAL-MMA, are shown in Fig. 3. The common major fragment ion in both spectra corresponds to $[AsS]^+$ at m/z 107. In the mass spectrum of BAL-MMA, the molecular ion $[M]^+$ of the structure reported by Fukui *et al.* was observed at m/z 212[9], and the base peak was observed at m/z 197, corresponding to the fragment ion of a demethyl derivative ($[M-CH_3]^+$). Other minor fragment ions were at m/z 179 $[C_3H_4S_2As]^+$ and 165 $[C_2H_2S_2As]^+$. Although the mass spectrum of BAL-MMA agreed with the data of Fukui *et al.* and the peak of BAL-MMA was assigned on the chromatogram, the peak of BAL-iAs with the mass spectrum reported by Fukui *et al.* was not observed. However, we found a quantitatively detected peak with a different mass spectrum from that found by Fukui *et al.* and denoted it as the peak of BAL-iAs. The fragment ions of BAL-iAs reported by Fukui *et al.* were at m/z 214 $[M]^+$), 212, 196, 166 and 107[9], and Shi *et al.* observed fragment ions of BAL-iAs at m/z 190, 166 and 107[15]. Our mass spectrum showed major fragment ions at m/z 212, 165 and 107, and the $[M]^+$ of the expected structure was not observed, even when GC-MS conditions (temperature of the injection port and ion source and electron energy) were changed. From the difference of these mass spectra, the peak that we regarded to be BAL-iAs may be different from the presented structure in previous studies. Therefore, further studies (including nuclear magnetic resonance analyses) are in progress to determine the exact structure of BAL-iAs.

The derivatization and extraction procedure was performed according to the method of Fukui *et al.* with some modifications, including scale-down of the sample volume and change of extraction solvent[9]. Benzene was used as the extraction solvent in the method of Fukui *et al.* However, benzene is highly toxic. Therefore, we examined if DCM, toluene, hexane, ethyl acetate or ethyl ether could be used as suitable alternate solvents, as they are less toxic than benzene. DCM and toluene showed almost the same extraction efficiency as that of benzene. Hexane and diethyl ether resulted in poor extraction of BAL-MMA. Ethyl acetate was hardly able to extract BAL-MMA and BAL-iAs. We finally chose DCM as the extraction solvent, because it is less toxic than toluene and give higher peaks. Moreover, we changed the volume ratio of the sample-extraction solvent from 2 to 4 to obtain higher sensitivity. Details of optimal reaction conditions have not been reported previously. Therefore, we confirmed the influence of the reduction time and the derivatization reaction time. From the obtained results, the optimal reaction conditions were found to be a reduction reaction for 30 minutes and a derivatization reaction for 30 minutes as shown in Fig. 1.

The proposed method was validated according to the US FDA guidelines[24]. The accuracy and precision values of the proposed method met the FDA criteria. The proposed method was further validated by analyzing SRM 2669. The obtained results showed that the proposed method had sufficient accuracy, since the results were not significantly different from the certified values according to application note 1 of the IRMM[26] (Table 3). Under the constant GC-MS condition, the robustness of the proposed method was evaluated by using three different columns with the same column size (InertCap 5MS/NP, HP-5MS

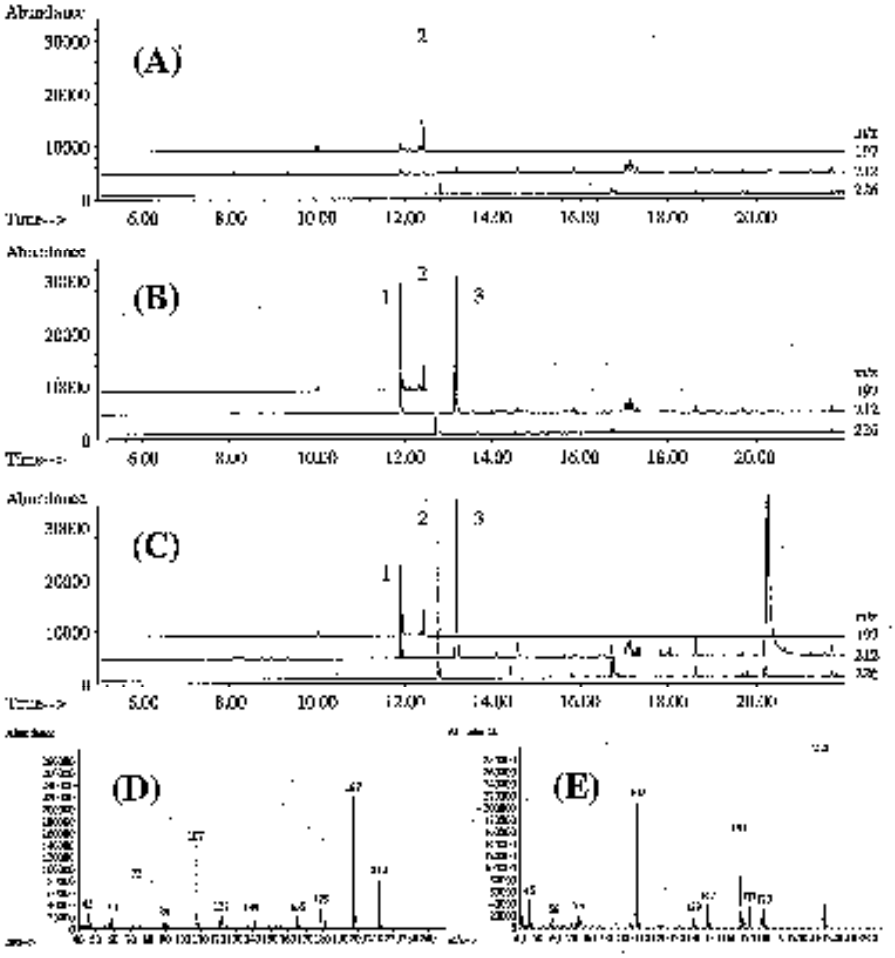

Fig. 3. Typical reconstructed mass chromatograms of a (A) blank urine (MMA, <LOQ; iAs, <LOQ), (B) standard spiked urine (MMA, 10.0 μg/l; iAs, 10.0 μg/l) and (C) Standard Reference Material (SRM) 2669 level II (National Institute of Standards and Technology) (MMA, 5.8 μg/l; iAs, 11.3 μg/l). Typical mass spectra of the derivatives of (D) monomethylarsonic acid (MAL-MMA) and (E) inorganic arsenic (BAL-iAs). Peak 1, BAL-MMA; peak 2, internal standard (IS); peak 3, BAL-iAs.

and HP-IMS; Agilent Technologies). There were no observed discrepancies in the chromatographic data. Therefore, the proposed method could be considered to be robust.

In comparison with a previously reported HPLC-ICP-MS method[3,4], the proposed GC-MS method not only showed a similar LOD (HPLC-ICP-MS: 0.2–0.3 μg/l) but also had several advantages. The proposed GC-MS method had a higher capability of separation for the use of a capillary column and had a higher selectivity and higher capability of identification and confirmation of compounds because mass spectra were obtained. Moreover, it does not require expensive reagents, an expensive and complicated instrument system and high running costs. One of the disadvantages of this method was that it could not distinguish As(III) and As(V). Although a speciation analysis was not required in this study, if necessary, it may be achieved by using a sequential procedure for sample preparation. Another disadvantage was that

It required a derivatization procedure and a relatively large volume of urine (2 mL). However, requesting a large volume of urine is not a serious problem in routine analyses at periodic health checkups.

In conclusion, we developed and validated a GC-MS method to simultaneously determine iAs and MMA in urine. The proposed method was a robust, selective and cost effective method suitable for routine analyses and could be useful for the biological monitoring of occupational exposure to iAs.

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3-3) GC-MS)による形態別ヒ素分析の応用

この GC-MS 法はギリアンバレー症候群様の患者が無機ヒ素含有健康食品によるヒ素中毒か否かを判断するのに適用され、無機ヒ素による神経障害例として報告した(J Occup Health, 54:344-347, 2012)。

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Case Study

A Guillain-Barré Syndrome-like Neuropathy Associated with Arsenic Exposure

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Abstract: A Guillain-Barré Syndrome-like Neuropathy Associated with Arsenic Exposure: Sunyoung Kim, *et al*. Department of Neurology, Ulsan University Hospital, University of Ulsan College of Medicine, Republic of Korea—Objectives: We report on a patient presenting with an isolated polyneuropathy mimicking Guillain-Barré syndrome (GBS) associated with arsenic exposure. **Case:** A 43-year-old man visited our emergency room complaining of progressive quadriparesis over the prior 5 days. His clinical course with laboratory data was typical of GBS. However, because of his recent use of herbal medication, we screened for the presence of several heavy metals. Serial analyses of urinary inorganic arsenic concentrations confirmed exposure to arsenic. He was diagnosed as arsenic neuropathy mimicking GBS without any systemic manifestation of arsenic intoxication. **Conclusions:** The present case study emphasizes the need to consider arsenic intoxication in patients presenting with acute demyelinating neuropathies and histories of herbal medication use.
(J Occup Health 2012; 54: 344–347)

Key words: Arsenic, Guillain-Barré syndrome, Peripheral neuropathy, Species

Acute arsenic intoxication is a rare cause of acute demyelinating polyneuropathy[1]. The earliest clinical features of acute, high-dose arsenic poisoning reflect multiorgan involvement and often include acute gastroenteritis widely associated with encephalopathy, pancytopenia, hepatitis, cardiomyopathy and dermatitis[2,3]. However, chronic low-level arsenic exposure may cause distal axonopathy, predominantly sensory polyneuropathy, that is not preceded by multiorgan involvement[2,4]. The acute neuropathy is usually initially misdiagnosed as Guillain-Barré syndrome (GBS); the electrophysiological and spinal fluid examination data support such a diagnosis[1]. Several cases of acute arsenic neuropathy mimicking GBS have been reported, with all patients exhibiting severe motor-sensory polyneuropathy and various systemic ramifications. Herein, we report an isolated GBS-like neuropathy associated with arsenic exposure.

Case presentation

A 43-year-old man visited our emergency room complaining of progressive quadriparesis over the prior 5 days. Previously, he had been healthy. For 25 days prior to admission he had consumed a herbal medication for treating psoriasis. Five days after ceasing to take this medication, he experienced bilateral arm weakness and hand paresthesia. Weakness in both legs developed 2 days later, and finally, he found it difficult to raise either arm, to walk and to climb steps. His blood pressure was 130/77 mmHg, and his pulse was 59 beats per minute and regular. No cutaneous abnormality, such as maculopapular scaly rash or Mee's line, was evident. Neurological examination revealed moderate proximal dominant symmetric arm and leg weakness. No cranial nerve sign was present, and his mentality was not altered. Reflexes were brisk in the arms but reduced in the knees and ankles.

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He complained of mild paresthesia in his feet and hands, but no impairment in distal vibration or joint position sense was evident. Laboratory data were as follows: Hemoglobin 15.9 g/dl, white blood cell (WBC) 12,800/mm^3 with 1% eosinophils, aspartate amino transferase (SGOT) 23 IU/l and alanine aminotransferase (SGPT) 39 IU/l. Test results for hepatitis-associated antigen, antinuclear antibody and a serum Venereal Disease Research Laboratory test (VDRL) were all negative. IgG and IgM antibodies to Herpes simplex, *Mycoplasma pneumoniae* and human T-cell lymphoma virus type I and II were negative. Polymerase chain reaction for tuberculosis and Herpes simplex virus type I and type II produced normal results.

Vitamin B-12 and folate serum levels were normal. No anti-ganglioside M1 (anti-GM1) antibodies or anti-ganglioside D1 (anti-GD1) antibodies were detected. Spinal fluid examination revealed no WBCs; the protein level was 56.2 mg/dl. Electrodiagnostic studies revealed the presence of an acquired demyelinating polyradiculoneuropathy. The median peroneal nerve revealed no F-wave and increased terminal latency was evident. The median and ulnar nerves showed only mild reductions in distal motor amplitudes, and conduction blocks were evident. Electromyography showed that the recruitment patterns were discrete. Spontaneous activity was not noted, and no evidence of reinnervation was found. We diagnosed GBS, possibly associated with a recent viral infection of uncertain etiology. He was treated with 0.4 g/kg/day of intravenous immunoglobulin (IVIG) for 5 days and responded with a clear improvement in limb strength.

He was an office worker and had no recent work experience in which he could have been exposed to arsenic. He used a tap water and had not used groundwater recently. Detailed history taking in regard to his job and daily life failed to show any other possibility of arsenic exposure. Because of his recent use of a herbal preparation, we screened for the presence of several heavy metals. The levels of blood mercury and lead were normal. The urinary total arsenic concentration measured with an atomic absorption spectrometer graphite furnace in a commercial lab in Korea was increased (240.7 μg As/l). On suspicion of arsenic-induced neuropathy, speciation analyses of urinary arsenic concentrations were performed at the Occupational Health Research and Development Center, Japan Industrial Safety and Health Association, in Japan. However, a sample of the relevant herbal medicine was not available. Informed consent was obtained from the patient.

Determination of arsenic compounds in urine

The arsenic compounds in urine were analyzed using an Agilent 1260 HPLC (Agilent Technologies, Santa Clara, CA, USA) and an Agilent 7700x ICP-MS (Agilent Technologies, Santa Clara, CA, USA) to separate arsenic species and to detect such species, respectively. Urine was diluted five-fold with ultrapure water and filtered through a cellulose filter 0.45 μm in pore size (Minisart C15; Sartorius Stedim, Germany). Arsenite (AsIII), arsenate (AsV), monomethylarsonic acid (MMA), dimethylarsinic acid (DMA) and arsenobetaine (AsBe) were separated by HPLC using an anion-exchange column (IonPac AS22, 250 mm × 4.0 mm internal diameter; Dionex, Sunnyvale, CA, USA). The mobile phase was 20 mM NH_4HCO_3 (pH 10.0), and the flow rate 1.0 ml/min. The ICP-MS detector was set to *m/z* 75 for $^{75}As^+$ and *m/z* 77 for argon chloride ($^{40}Ar^{37}Cl^+$). The detection limits for AsIII, AsV, MMA, DMA and AsBe were 0.2, 0.2, 0.2, 0.3 and 0.3 μg As/l, respectively.

The urinary arsenic concentrations measured on the second day of hospital stay (HD 2) are shown in Table 1. These are values obtained 1 wk after cessation of herbal medicine treatment. The total concentrations of inorganic arsenic and methylated metabolites thereof (MMA plus DMA) were above 35 μg As/l of the ACGIH BEI®. In addition, the sum of the concentrations of arsenic and MMA was high; this value on HD 7 and HD 12 fell compared to that on HD 2.

Discussion

Humans may be exposed to both organic and inorganic arsenic compounds. Contaminated drinking water and food are the main sources of inorganic arsenic, such as arsenite and arsenate[9]. Organic arsenic compounds including arsenobetaine and arsenosugars are derived mainly from seafood[10]. Inorganic arsenic compounds are metabolized to MMA and DMA and are excreted in the urine together with unchanged

Table 1. Arsenic species in urine (μg As/l) after cessation of herbal medicine treatment

Sample	AsIII	AsV	MMA	DMA	Arsenobetaine	Others	Total
HD 2	6.4	ND	8.9	77.5	402.6	12.5	507.9
HD 7	3.2	ND	3.7	26.5	57.6	9.1	100.1
HD 12	3.7	ND	3.9	32.1	57.6	ND	97.3

The totals are the sums of the levels of all detected arsenic compounds.

inorganic arsenic[3]. Thus, 35 μg As/l, the sum of the levels of inorganic arsenic, MMA and DMA in urine, has commonly been used as a biomarker of inorganic arsenic exposure by the ACGIH[3]. Most fish and shellfish are rich in arsenobetaine, which is rapidly excreted (unchanged) in the urine and is a principal contributor to the total urinary arsenic level. Seaweed and some seafood, including scallops and mussels, are also rich in arsenosugars, which are metabolized to several compounds (mainly DMA) that also contribute to total urinary arsenic level[3]. Consequently, the total urinary arsenic concentration, and that of arsenobetaine, may differ greatly when levels in Western countries, and Japan or Korea, are compared. Arsenic concentrations in general populations (the sums of the levels of inorganic arsenic, MMA and DMA in the urine) are approximately 10 $\mu g/l$ in European countries and the USA but about 50 $\mu g/l$ in Japan[4]. Some of the difference between the levels of Western countries and those of Japan or Korea may be attributable to among-country variation in the levels of consumption of seaweeds and some types of seafood, including scallops and mussels. These foods are rich in arsenosugars that are metabolized to DMA[16]. Therefore, the concentration of inorganic arsenic or MMA may be more useful in assessment of exposure to inorganic arsenic than the sum of the levels of inorganic arsenic, MMA and DMA[6].

On HD 2, the total concentration of inorganic arsenic and methylated metabolites therein (92.8 μg As/l) was above the 35 μg As/l set as the ACGIH BEI. In addition, the sum of the concentration of inorganic arsenic and MMA is considered to be a good biological indicator of inorganic exposure among people generally taking seafoods[17]. The sum (15.3 μg As/l) of the AsIII and MMA concentration of HD 2 was higher than 95th percentile of the general population (12.6 $\mu g/l$), but those on HD7 and HD12 were almost the 75th percentile[18]. In particular, an MMA level higher than that of inorganic arsenic indicates inorganic exposure based on inorganic arsenic metabolism[3]. The concentrations of arsenic compounds were lower on HD7 and HD12 compared with those on HD 2. Further, the peak concentrations may have been eight-fold higher than the concentrations measured 1 week after cessation of herbal medication, when it is considered that the biological half-life of urinary total arsenic is approximately 60 h[19]. Therefore, our patient may have been exposed to very high levels of inorganic arsenic.

The source of arsenic in the present instance was presumed to be a herbal medicine; such Korean medicines have been previously reported to contain arsenic[9,14]. We could not directly confirm this hypothesis because we could not obtain a sample of the relevant medicine.

The most frequent neurological complication induced by arsenic poisoning is symmetrical sensorymotor polyneuropathy featuring more distal impairment[20]. The most prominent electrophysiological findings are marked abnormalities in both sensory and mixed nerve conduction and moderate abnormalities in motor conduction[20]. In addition to the classic presentation of chronic axonal polyneuropathy, acute or subacute demyelinating polyneuropathy commencing 1–3 wk following arsenic exposure has been described[9]. Such acute neuropathy is usually initially misdiagnosed as GBS; the electrophysiological and spinal fluid data support such a diagnosis. This was true of the present case; we first diagnosed GBS because the clinical course and the laboratory data were typical of GBS. The patient presented with acute progressive motor weakness, demyelinating polyneuropathy and albuminocytologic dissociation. However, because of his recent use of a herbal preparation, we screened for the presence of arsenic, and thus serial analyses of inorganic arsenic concentrations confirmed exposure to arsenic. In particular, the temporal relationship between use of the herbal medication and development of motor weakness caused us to suspect arsenic intoxication. We also ruled out GBS-related viral diseases such as *Herpes simplex*, *Mycoplasma pneumoniae* and human T-cell lymphoma virus type I and II.

Notably and unlike what was found in previously reported instances of arsenic neuropathy mimicking GBS[9], our present patient showed mild neuropathy in the absence of any systemic manifestation of arsenic intoxication. We presume that the severity of neuropathy and the existence of systemic symptoms depend on both the level of toxic material ingested and the duration of exposure. In addition, host factors, such as individual variability in the hepatic P450 system, may play a role in determining the severity of neuropathy and clinical manifestations. The exact pathophysiology of arsenic toxicity is not known, but it has been suggested that arsenic is primarily toxic to the cell body; segmental demyelination occurs prior to axonal degeneration[9]. Taken together, this neuropathy is possibly due to arsenic poisoning, although a causal relationship between arsenic exposure and a GBS-like neuropathy was not demonstrated. Any history of arsenic exposure such as a history of herbal medication use in a peripheral neuropathy should raise a suspicion of arsenic poisoning.

However, the present work has some limitations. First, we could not identify arsenic in a sample of the herbal medicine, although our serial analyses of the inorganic arsenic concentration (and the levels of its methylated metabolites) strongly suggest evidence of expo-

sure to arsenic. Second, we could not completely rule out a real causal pathogen that might have existed in the herbal medicine or from some unknown origin. One example would be a plant-derived phorbol ester that can reactivate latent virus in the body, which could be the real cause of GBS in this case.

In summary, we presented a GBS-like neuropathy associated with arsenic exposure. Serial species analyses of urinary inorganic arsenic concentrations confirmed exposure to arsenic. The temporal relationship between use of the herbal medication and development of motor weakness was also shown. This peripheral neuropathy was possibly due to arsenic poisoning, although a causal relationship between arsenic exposure and GBS was not determined.

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3-4) 食品中の無機および有機ヒ素化合物の同定と定量

日本人においては、食品からの無機および有機ヒ素の摂取が問題となる事から、海藻（ひじき）中のヒ素化合物の同定と定量を HPLC-ICP-MS と HPLC-タンデム質量分析法(HPLC-MS/MS)により実施した結果、2 種の無機ヒ素と 4 種のアルセノ糖が検出されたことを報告した(J Health Sci, 56:47-56, 2010)。

Speciation Analysis of Arsenics in Commercial Hijiki by High Performance Liquid Chromatography-tandem-mass Spectrometry and High Performance Liquid Chromatography-inductively Coupled Plasma Mass Spectrometry

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Edible brown alga (hijiki in Japanese, *Hijikia fusiformis*) contains not only a high content of inorganic arsenic (iAs) but also various arsenosugars (AsSugs) which are metabolized to dimethylarsinic acid (DMA) in mammals. Since DMA is considered to be carcinogenic in rodents, it is necessary to accurately measure the contents of AsSugs as well as iAs for the risk assessment of seaweed consumption. Seven commercially available dried-hijiki products and two raw hijiki products were analyzed. Total-As was measured by inductively coupled plasma mass spectrometry (ICP-MS) with the Dynamic Reaction Cell (DRC) mode after acid-digestion. After water extraction, AsSugs were detected by HPLC-MS/MS with multiple reaction monitoring in the positive ion mode and speciation analysis of arsenics was performed by HPLC-ICP-MS. The ranges of total-As obtained by acid digestion (A-TAs) in dried hijiki samples were 37.1–118.6 μg As/g dry weight (dw), and those of water extracted total-As (W-TAs) were 18.4–81.0 μg As/g dw. The ratios of water extracted iAs (W-iAs) to A-TAs ranged from 24.6 to 60.1%. The major compound detected was arsenate in all samples (8.9–70.5 μg As/g dw). Dimethylarsenosugar sulfate, AsSug 408, showed the highest peak among AsSugs detected. The content ratio of water extracted AsSugs (W-AsS) to A-TAs was estimated to be from 3.7 to 27.6%. The contents of A-TAs, W-iAs and W-AsS varied depending on the hijiki product. HPLC-MS/MS detected AsSugs more sensitively than HPLC-ICP-MS. Since iAs could not be detected by HPLC-MS/MS, combined analysis consisting of HPLC-MS/MS and HPLC-ICP-MS is necessary for accurate determination of arsenic species in seaweed products and also for the toxicological evaluation of AsSugs.

Key words——hijiki, arsenosugar, HPLC-MS/MS, inorganic arsenic, dimethylarsinic acid

INTRODUCTION

The consumption of seaweed as a diet food has been increasing in recent years in Western countries.[1–3] The edible brown alga *Hijikia fusiformis*, called hijiki in Japanese, has a high content of inorganic arsenic (iAs). Therefore, in 2004, the Food Standards Agency (FSA) of the United Kingdom[4] advised consumers to avoid eating hijiki.[5] The iAs concentration in hijiki depends on the producing district and manufacturing method.[6,7] Five oxo-arsenosugars (oxo-AsSugs) have been detected in hijiki as well as other seaweeds.[8–10] In humans, oxo-AsSug is metabolized and excreted into urine as dimethylarsinic acid (DMA) and several oxo- or thio-dimethylarsenic compounds.[11,12] In 2004, the International Agency for Research on Cancer (IARC)[13] declared that there is sufficient evidence indicating that DMA, a major metabolite of iAs in mammals, is carcinogenic in animal experimental models. Therefore, in order to assess the risk from

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hijiki ingestion, both AsSugs and iAs should be determined. Arsenic speciation analysis in biological samples is generally performed using HPLC inductively coupled plasma mass spectrometry (HPLC-ICP-MS).[14–15] Recent publications have reported the usefulness of HPLC with tandem mass spectrometry (HPLC-MS/MS) for the identification and determination of organoarsenic compounds in biological samples.[17–19] These analytical apparatuses have made it possible to detect known arsenic compounds in samples without standard compounds and to estimate As concentration using appropriate standards.[20,21]

In this study, we determined total arsenic and water-extracted iAs, and estimated the AsSugs contents of 7 commercially available dried hijiki products and two raw hijiki products using HPLC ICP-MS and HPLC-MS/MS for the speciation analysis of arsenics in hijiki products from various regions of Japan and elsewhere in east Asia produced using several different production systems.

MATERIALS AND METHODS

Chemicals —— Sodium arsenite (AsIII), sodium arsenate (AsV), methanearsonic acid (MMA), and arsenobetaine (AsBe) were purchased from Wako Pure Chemical (Osaka, Japan). DMA, trimethylarsine oxide (TMAO), and arsenocholine (AsCho) were obtained from Tri Chemical Laboratory (Yamanashi, Japan). Germanium standard solution (Kanto Chemical, Tokyo, Japan) was used as an internal standard for HPLC-ICP-MS analysis. Nitric acid (HNO_3, TAMAPURE AA-100, 68%, Tama Chemicals, Kawasaki, Japan) and H_2SO_4 [for Ultratrace Analysis (Ultrapure Anal.), 97%, Wako Pure Chemical] were used for sample treatment. Ammonium bicarbonate (NH_4HCO_3, Wako Pure Chemical) was used for the HPLC mobile phase. Ultrapure water was purified by Milli-Q Element A-10 with Quantum TOP cartridges (Millipore, Tokyo, Japan). A certified reference material, CRM No. 18 (human urine), obtained from the National Institute for Environmental Studies (NIES), Tsukuba, Japan, was used to validate the procedure used for analysis.

Samples of Commercial Dried-hijiki and Raw-hijiki —— As shown in Table 1, nine hijiki samples derived from six different producing districts were collected from six stores in each producing district. Two of the commercial samples were imported from China (Hong Kong) and South Korea and packed by a Japanese provider. Two raw hijiki samples were harvested on the seashores of Aburatsubo and Kominato, respectively, which are located on the Boso peninsula of Chiba prefecture. Each raw sample was immediately washed with fresh water, sealed in an airtight plastic bag, and then stored by freezing at −80°C until analysis. The manufacturing methods for commercial dried-hijiki can be broadly divided into two systems, one that involves a boiling system in a large vessel, and the other a closed or open steam-system.[22] In Japan, major manufacturing methods for commercial dried-hijiki basically use a steam system.[23] A system involving boiling has been used traditionally in South Korea and China.[22] Moreover, there are two types of commercial dried-hijiki, naga-hijiki, which consists of the entire seaweed plant, and me-hijiki, which is the sprouts of the seaweed.[22]

Preparation of the Hijiki Samples —— The commercial dried-hijiki samples and the lyophilized raw hijiki samples were finely ground to powder using a food processor. For total-As analysis, twenty milligrams of the powdered hijiki samples was digested by adding 0.5 ml of HNO_3 and 0.5 ml of H_2SO_4 and then kept at 330°C for 2 hr using a dry block bath. After cooling, the solutions were adjusted to 50 ml with 0.1 M HNO_3 and their total arsenic concentrations were determined by ICP-MS using the Dynamic Reaction Cell (DRC) mode.

For speciation analysis, powdered hijiki samples (0.5 g) swollen by the addition of 20 ml of ultrapure water were treated with an ultrasonic cell disruptor (Misonix, Inc., Farmingdale, NY, U.S.A.) for 1 min, and centrifuged at 3000 rpm for 15 min. The supernatant collected was adjusted to 50 ml by adding ultra pure water. The supernatant was applied to speciation analysis of arsenic compounds by HPLC-ICP-MS and by HPLC-MS/MS.

Determination of Total Arsenic Concentration —— The acid digested solution was diluted to 50 times with buffer containing 0.5% aqueous ammonia, 2 mM EDTA, and 0.8 mM TritonX-100. The determination was performed by the standard addition method. The diluted solution was introduced into an Elan DRCII ICP-MS (PerkinElmer SCIEX, Concord, Ontario, Canada) using the DRC mode. The instrument settings were as follows: radio-frequency (RF) power 1300 W, argon plasma gas flow 15 l/min, auxiliary flow 1.2 l/min, and nebulizer flow 1.0 l/min. A coaxial-type nebulizer was used; skimmer and sample cones were platinum, and elemental As was measured at *m/z*

Table 1. Hijiki Producing Areas and Production Systems Used for Their Dried products

Sample	Producing area	Production system
1 mo-hijiki (sprouts of weed)	China (Hong Kong)	unknown
2 mo-hijiki (sprouts of weed)	South Korea	boiling system[a]
3 mo-hijiki (sprouts of weed)	Mie prefecture (Japan)	boiling system[a]
4 nagu-hijiki (whole of weed)	Oita prefecture (Japan)	closed-steam system
5 me-hijiki (sprouts of weed)	Oita prefecture (Japan)	closed-steam system
6 nagu-hijiki (whole of weed)	Aomori (Japan)	open-steam system
7 nagu-hijiki (whole of weed)	Kumamoto (Japan)	open-steam system
8 raw hijiki (whole of weed)	Aomori (Japan)	
9 raw hijiki (whole of weed)	Kumamoto (Japan)	

a) The production systems could be printed on their packages.

Table 2. Fragmentation Parameters of MRM for Arsenic Compounds[24]

Compounds	Precursor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (eV)
DMA	138.9	90.8	30	24
MMA	140.9	90.8	30	20
AsBe	179.0	119.9	30	24
AsSug 254	255.0	96.9	25	30
AsSug 328	329.0	96.9	25	32
AsSug 391	392.0	96.9	25	30
AsSug 392	393.0	96.9	25	32
AsSug 408	409.0	96.9	20	38
AsSug 482	483.0	96.9	30	35

of 75 and 77. The DRC mode operation settings were as follows: ammonia (NH_3, > 99.999%) was used as reaction gas, and the rejection parameter q (RPq) of the DRC, the axial field voltage, and the flow rate of NH_3 were optimized and set at 0.5, 275 V, and 0.3 ml/min, respectively. No interference from argon-chloride at m/z = 75 and 77 was detected with NaCl solutions ranging from 10 mM to 500 mM. The instrument limit of detection (LOD) of As aqueous standard solution and the method limit of quantitation (LOQ) were calculated according to the definition stipulated by Japanese Industrial Standards (JIS).[25] The counts per second (CPS) of a blank solution at m/z 75 and 77 were almost equal to the background levels of 13–17 CPS and 3–7 CPS of ICP-MS, respectively. The LOD and the LOQ were calculated as 0.2 μg/l and 1.1 μg/l, respectively. The total As concentration in the reference material, NIES CRM No 18 urine, was determined to be 137.5 ± 4.2 μg/l (n = 5) and the value was within the range for the certified value of 137 ± 11 μg/l.

Detection of AsSugs in Hijiki Samples —— Detection of AsSugs was performed using an HPLC system (Alliance 2695, Waters, Milford, MA, U.S.A.) connected to a Quattro micro API tandem mass spectrometer (Waters) with electrospray ionization (ESI) positive ion mode by setting the capillary voltage to 0.5 kV, ion source temperature to 120°C, desolvation nitrogen gas temperature to 400°C, desolvation gas flow to 600 l/h, and cone gas flow to 50 l/h. Collision induced dissociation (CID) was performed with argon gas introduced into the collision cell placed between the quadrupoles. Multiple reaction monitoring (MRM) was optimized to detect CID fragmentations (mass transition) of selected AsSugs, AsBe, DMA, and MMA. The fragmentation parameters of MRM[24] are shown in Table 2. The molecular structures of the selected AsSugs are shown in Fig. 1. The divert valve was set to introduce only the HPLC effluent from 2 to 50 min to the mass analyzer. An anion exchange column PRP-X100 (250 × 2.0 mm i.d., Hamilton, Reno, NV, U.S.A.) was used under the following conditions: mobile phase of 20 mM NH_4HCO_3 (pH 9.0), flow rate of 0.2 ml/min, and column temperature of 40°C. Ten microliters of supernatant extracted from hijiki was injected into the HPLC.

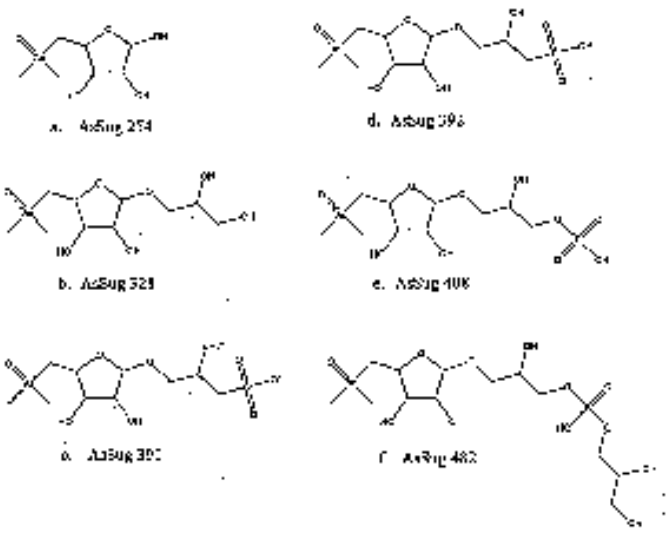

Fig. 1. Chemical Structures of Six AsSugs

For the assignment of ^{75}As, an HPLC system (GL Science, Tokyo, Japan) connected to an Elan DRCII ICP-MS was used under the same conditions except a thicker diameter column [PRP-X100, 250 × 4.6 mm inner diameter (i.d.)] at a flow rate of 1.0 ml/min was used. The injection volume of the supernatants was 50 µL.

Determination of Arsenic Compounds in Hijiki by HPLC-ICP-MS — For the estimation of AsSug content, the same HPLC-ICP-MS system and analytical conditions as in "Analysis of AsSugs in hijiki samples" was used. Since we do not have AsSug standards, we used a DMA standard calibration curve to estimate the As concentration in each peak of AsSug. For AsIII, AsV, MMA, and DMA determination, an HPLC-ICP-MS system (consisting of a Model HP1200 and a Model HP7500cx, Agilent, Santa Clara, CA, U.S.A.) was used. The same column (PRP-X100, 250 × 4.6 mm i.d.) was used under the following conditions: mobile phase 20 mM NH_4HCO_3 (pH 9.5), flow rate 1.2 ml/min, temperature 40°C, and injection volume 50 µL. Stock standard solutions of AsIII, AsV, MMA, DMA, and AsBe were prepared by dissolving each compound in ultra-pure water at a concentration of 100 mgAs/l. The final diluted aqueous standard solutions (5 µg/l and 50 µg/l) were prepared from stock standard solution before use. To obtain precise measurements, 100 µg/l of germanium solution was used as the internal standard for ICP-MS by a post-column addition method. The ICP-MS detection mass was set to m/z 75 ($^{75}As^+$), m/z 72 ($^{72}Ge^+$), and m/z 35 (^{35}Cl) to monitor the chloride ion source of m/z 75 of ArCl. The instrument settings were as follows: RF power 1500 W, argon gas flows of plasma 15 l/min, and carrier gas flow 1.1 l/min. A coaxial-type nebulizer, nickel sample, and skimmer cones were used. LOD for AsIII, AsV, MMA, DMA, and AsBe were calculated according to the definition given by Gibbons.[23] The LOD of AsIII, AsV, MMA, DMA, and AsBe measured by HPLC-ICP-MS were calculated as 0.8, 1.1, 0.2, 0.3, and 0.5 µg As/l, respectively. When a measurement was below the LOD, it was calculated to be half the value of these limits. The AsBe and DMA concentrations in the reference material of CRM No.18 urine were determined to be 7.5.3 ± 0.7 µg/l and 30.4 ± 0.4 µg/l ($n = 5$) and the values were within the ranges for the certified values of 60 ± 12 µg/l and 36 ± 9 µg/l, respectively.

RESULTS

Detection of AsSugs in Hijiki Samples by HPLC-MS/MS

Using MRM techniques, we detected 9 organoarsenics in water extracts of hijiki samples (Table 2). The chromatograms of ^{75}As detection by HPLC-ICP-MS and MRM by HPLC-MS/MS of a raw hijiki sample harvested in Kominato (sample

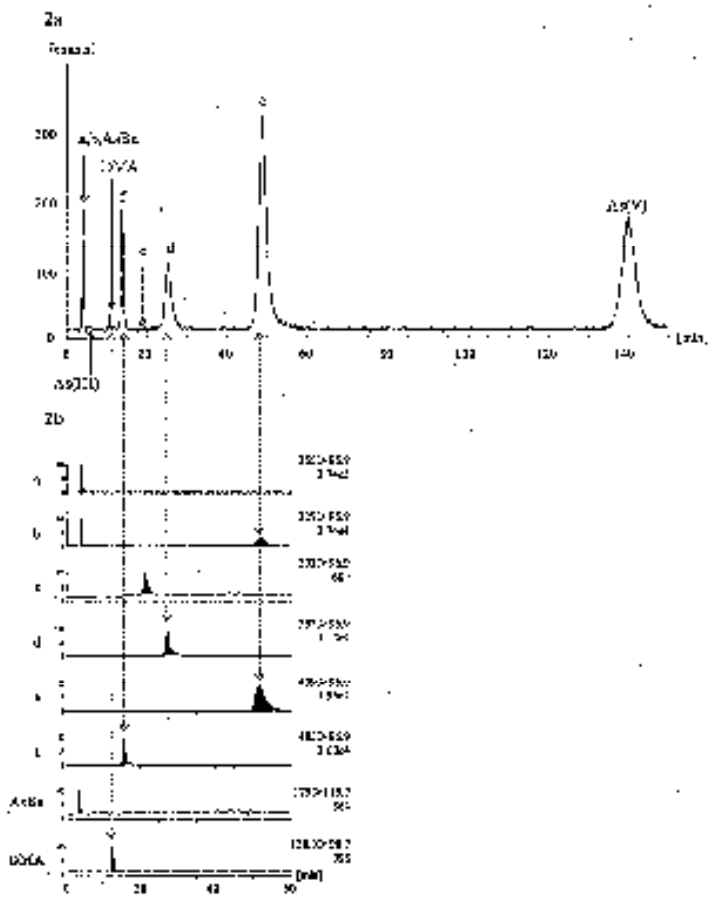

Fig. 2. HPLC-ICP-MS Chromatogram (2a), and HPLC-MS/MS (MRM) Chromatogram (2b) of a Raw Hijiki Sample Harvested in Kumamoto (Sample #9) with Mobile Phase at pH 8.0

Analytical conditions are described in text. The columns were normalized to 100% for the base peak of each MRM chromatogram. (a), AsSug 254; (b), AsSug 328; (c), AsSug 361; (d), AsSug 392; (e), AsSug 408; (f), AsSug 482.

#9), are shown in Fig. 2a and 2b, respectively. Seven peaks of arsenic compounds including two peaks of inorganic arsenic compounds (AsIII and AsV) were detected by HPLC-ICP-MS (Fig. 2a). However, with the exception of MMA, eight of the nine organoarsenics monitored were detected by HPLC-MS/MS conducted simultaneously (Fig. 2b). In the HPLC-ICP-MS chromatograms, compounds with molecular ion $[M+H]^+$, and m/z of 255 (AsSug 254), 329 (AsSug 328), and 179 (AsBe) were detected as unresolved peaks because of the close retention times, while that with $[M+H]^+$ and m/z of 392 (AsSug 391) was not detected because of the low concentration. In HPLC-MS/MS analysis, a molecular ion peak of m/z 141 (MMA) was not detected. Low sensitivity for MMA in analysis using MS/MS has been reported.[20] The MS/MS spectra of four major AsSugs, 328, 392, 408, and 482, are shown in Fig. 3. AsSug 408 had a daughter ion with m/z of 328.6, and further fragmentation of the m/z 328.6 ion gave rise to an ion with m/z of 96.8 (Fig. 3e). The other three AsSugs (328, 392, and 482) showed fragmentations to their product ion with m/z of 96.9 (Fig. 3b, 3d, and 3f). The MS/MS spectra of AsSug 254, AsSug 391, and AsBe were not obtained because of their low intensity; however, AsBe was confirmed by spiking standard compounds. AsSug 391 and AsBe were not detected in samples #1 and #6, respectively.

Table 3. Concentrations (μg As/g dw) of Water-extracted Arsenic Compounds in Hijiki Samples by Speciation Analysis Using HPLC-ICP-MS

Sample	As(III)	DMA	As(V)	AsSugs					
				328[a]	391	295	408	482	Total
1	0.10	2.6	29.61	0.95	ND[b]	0.12	1.79	0.08	2.96
2	0.44	2.72	55.35	1.48	ND	0.20	4.77	0.09	6.04
3	0.08	1.52	51.10	1.25	ND	0.22	3.85	0.05	5.39
4	0.13	2.72	50.93	0.85	ND	0.46	2.99	0.06	4.36
5	0.79	1.68	30.52	1.50	ND	0.48	5.76	0.05	7.79
6	0.26	0.74	8.05	0.39	ND	0.07	7.77	0.04	8.40
7	0.09	0.85	43.55	0.42	ND	0.07	10.15	0.05	10.98
8	0.25	0.16	17.15	1.41	ND	0.50	12.81	ND	14.78
9	0.16	0.25	17.21	1.39	ND	0.56	18.10	0.04	20.09

Values represent means of concentration of arsenic compounds as As ($n = 3$). a) The values are estimated from a peak of AsSug 328 containing AsBe and AsSug 254. b) ND, not detected by HPLC-ICP-MS.

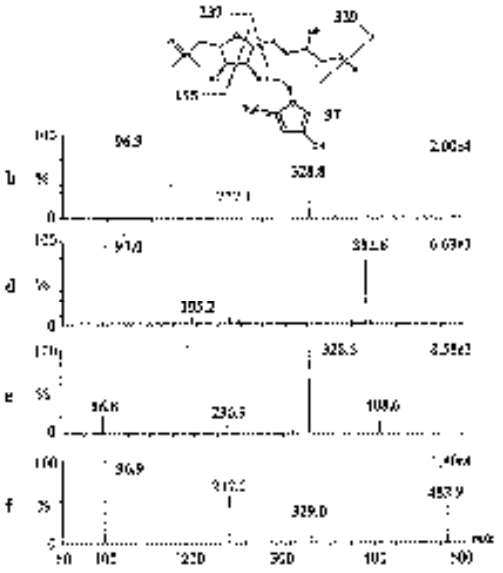

Fig. 3. The MS/MS Ion Spectra
(b) AsSug 328, (d) AsSug 392, (e) AsSug 408, and (f) AsSug 482.

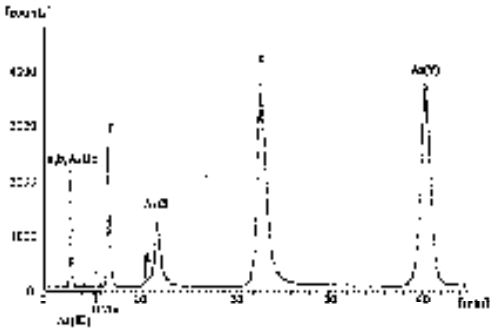

Fig. 4. HPLC-ICP-MS Chromatogram of a Raw Hijiki Sample Produced in Kominato (Sample #9) with a Mobile Phase at pH 9.5
(a), AsSug 254; (b), AsSug 328; (d), AsSug 392; (e), AsSug 408; (f), AsSug 482; AsBe.

Determination of Arsenic Compounds in Hijiki Samples by HPCE-ICP-MS

For the speciation analysis, a mobile phase with a pH of 9.5 was used to improve the detection limit of AsV. The HPLC-ICP-MS chromatogram of the speciation analysis of arsenic compounds in a water extract of a raw hijiki sample from Kominato (sample #9) is shown in Fig. 4. In all nine hijiki samples, AsSug 391 was not detected, and AsSug 328, AsSug 254, and AsBe were unresolved and detected as one peak, therefore, their As concentration was estimated as a mixture.

The arsenic concentrations in water extracts of the nine hijiki samples are shown in Table 3. Arsenic concentrations of peaks of unresolved and AsSugs were estimated using the nearest-neighbor standard arsenic species (DMA). In all nine samples, the major compound detected was AsV. The concentrations of the AsV produced by the open steam system (samples #6 and #7) were much lower than other manufacturing methods (samples #1–5). The DMA concentrations in raw hijiki (samples #8 and #9) were very low, and the DMA concentrations in the open steam system products (samples #6 and #7) were 3–4 times higher than those in raw samples. The major AsSug in water extracts was AsSug 408. The concentrations of AsSug 408 in hijiki samples harvested in the Boso area (samples #6–9) were higher than in those produced in other districts. The concentrations of AsSug 392 in raw hijiki samples

Table 4. Total As and Water-extracted iAs, AsSugs, and Total As in Hijiki Samples and Their Ratios to Total As

Sample Number (#)	Acid digested Total-As (μg As/g dw) (A-TAs)	Water-extracted As (μg As/g dw) Total-As (W-TAs)[a]	Inorg-As (W-iAs)[b]	Total-AsSugs (W-AsS)[b]	W-TAs/A-TAs (%)	W-iAs/A-TAs (%)	W-AsS/A-TAs (%)
1	80.9 ± 9.4	33.4 ± 2.6	24.7 ± 1.7	3.0 ± 0.0	41.3	34.5	3.7
2	112.6 ± 1.5	69.1 ± 2.7	55.8 ± 2.8	6.6 ± 0.6	37.8	49.5	5.9
3	86.7 ± 6.7	59.0 ± 5.3	51.5 ± 2.9	5.4 ± 0.3	68.0	59.0	6.9
4	86.3 ± 5.7	58.1 ± 1.8	51.1 ± 1.5	4.4 ± 0.3	67.4	59.2	5.1
5	118.5 ± 11.5	81.0 ± 2.5	71.2 ± 2.3	7.8 ± 0.7	68.4	60.1	6.6
6	37.1 ± 1.2	18.4 ± 0.7	9.2 ± 0.4	8.4 ± 0.4	49.5	24.8	22.7
7	48.7 ± 2.4	25.4 ± 1.3	13.6 ± 0.5	10.9 ± 0.7	52.1	28.0	22.4
8	59.3 ± 8.4	35.4 ± 1.2	17.4 ± 0.1	14.8 ± 1.2	54.6	29.5	24.9
9	72.8 ± 1.8	37.7 ± 1.7	17.4 ± 0.3	20.1 ± 1.5	51.8	23.9	27.6

Each sample solution was measured in triplicate. Each value represents the mean ± S.D. ($n = 3$). a) Sum of the concentrations (μg/g dw) of water-extracted inorganic As, arsenosugars and DMA indicated in Table 3. b) Sum of the concentrations of inorganic arsenic compounds As(III) and As(V) indicated in Table 3. c) Sum of the concentrations of arsenosugars indicated in Table 3.

(#8 and #9) were higher than those in other samples. AsSug 482 was not detected in sample #8.

The concentrations of total As and water-extracted As in hijiki samples are summarized in Table 4. Total-As concentrations obtained by acid-digestion of nine hijiki samples ranged from 37 to 119 μg As/g dw. The highest level of total As was found in the Ohita product (sample #5), which was almost three-fold higher than that of the lowest of the Amatsu product (sample #6). The concentration of total water-extracted arsenic compounds obtained by speciation analysis ranged from 18.4 to 81.0 μg As/g dw and their content ratios to the total As were about 60%. The lowest concentration of iAs was observed in the hijiki produced in Amatsu (sample #6). The ratios of iAs to total As ranged from 25 to 60%. Hijiki products produced by the Boso-method (samples #6 and #7) showed relatively low iAs levels compared with the other methods.

DISCUSSION

The results of the present study suggest the existence of six oxo-AsSugs in commercial and raw hijiki analyzed by MRM of HPLC-MS/MS. The four major AsSugs in the present study, 328, 392, 408 and 482, were identified by a high-resolution MS of HPLC-Quadrupole time of flight (QTOF)-MS (unpublished data). However, we could not clearly identify AsSug 254 and 391 or AsBe with the MS/MS ion spectra because of the overlapping peaks and the very low concentrations. The existence of AsSug 391 in *Hijiki fusiforme* was reported by Edmonds *et al.*[19] Formation of AsSug 254 from AsSugs 328, 392, 408 and 482 in acidic environments has been reported.[20] Even though AsBe was recently detected in six kinds of marine algae,[27] its contamination from epifauna should be examined more carefully. Therefore, further study is necessary to confirm the existence of AsSug 254 and AsBe in hijiki seaweed.

It has been pointed that ICP-MS response for arsenic is essentially uniform for all (known) arsenic species allows this detector, in most cases, to quantify the various arsenicals by comparison with simple standards such as As(V) or arsenobetaine.[26] Since we could not obtained AsSug standards, we estimated the quantity of AsSugs using DMA having near-retention time to AsSugs in the present study. We, however, had paid much attention to matrix effects *etc.* because the result will always be subject to them. Among the AsSugs determined, the content of AsSug 408 was the highest and it corresponded to 5–48% of the total water-extracted As. The speciation analysis of arsenic in seaweeds has made progress recently,[27,28] and consequently some AsSugs (dimethylarsinoyl ribose derivatives) have been identified as major organic arsenics in seaweeds.[29–31] Shibata *et al.*[29] reported that AsSug 408 was the major, AsSug 392 was the minor, and AsSug 482 and 391 were the trace constituents in *Hijiki fusiforme*. AsSug 482 was not determined in our samples of #8. Almela[9] and Rubio *et al.*[9] reported the concentrations of AsSug 328, 392, 408 and 482 in water extracts of hijiki products. Schmeisser *et al.*[20] reported the detection of the four AsSugs in hijiki and that the content of As-

AsSug 408 was the highest at 11.3 mg As/kg. This value is very similar to our samples (samples #6–9) produced in the Boso area. Castlehouse *et al.*[31] reported that the four AsSug were detected in both *Laminaria digitata* and *Fucus vesiculosus*, with the major species being AsSug 392, which was found in only slight amounts in our hijiki samples.

In our HPLC-ICP-MS analysis of raw hijiki samples (samples #8 and #9), the early co-eluted As peaks at the positions of AsSug 254, AsSug 228 and AsBe were four-fold of those in processed samples (samples #6 and #7). Gamble *et al.*[32] reported that AsSug 408 and AsSug 482 were degraded to DMA via AsSug 328 in a basic environment. In acidic conditions, the formation of AsSug 254[26] and an increase in DMA with heating[23] were also reported. Both of our Boso hijiki products (#6 and #7) contained DMA at the concentration of 0.9 μg/kg dw hijiki, while the DMA levels in two raw hijiki samples (#8 and #9) were much lower (0.2 μg/kg dw hijiki), and these changes in DMA amount may be enhanced by heating.

Our results confirmed the previous findings that the total-As concentrations obtained by acid digestion in hijiki products depend on the producing district.[6,7] Previous studies on arsenic in seaweed have shown that the majority of arsenic can easily be extracted by water or water/methanol extraction.[33] Our values of the total-As obtained by water extraction using ultrasonication are consistent with the values already reported.[8,9,12,21]

The present study indicates that AsSugs in hijiki account for 3.7–27.6% of total-As (Table 4). To take into consideration that the major metabolite of AsSugs is DMA, which is carcinogenic in rodents,[13] for the safe consumption of hijiki by humans, the levels of not only total-As and/or inorganic arsenic but also those of each AsSug species should be determined. Six oxo-AsSug were detected in this study by HPLC-MS/MS, which provides more information about the chemical structure than HPLC-ICP-MS, although HPLC-MS/MS cannot detect inorganic arsenics. Therefore, analysis performed using combined HPLC-MS/MS and HPLC-ICP-MS is necessary for the estimation of arsenic species in seafood as well as for the toxicological evaluation of AsSugs.

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3-5) N-メチル-2-ピロリドン（NMP）の生物学的モニタリング法の開発

樹脂系の溶剤として使用量が増加している NMP の生物学的モニタリング法については、第 1 期の炎光光度-ガスクロマトグラフィー法(FPD-GC)ではできなかった全ての代謝物であるメチルスクシンイミド、NMP、2－水酸化メチルスクシンイミド、および 5―水酸化メチルピロリドンを含めた一斉分析法を LC-MS/MS により開発して報告した(J.Chromatogr. B, 877:3743-3747, 2009)。

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Short communication

Direct determination of N-methyl-2-pyrrolidone metabolites in urine by HPLC-electrospray ionization-MS/MS using deuterium-labeled compounds as internal standard

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ABSTRACT

N-Methyl-2-pyrrolidone (NMP) has been used in many industries and biological monitoring of NMP exposure is preferred to atmospheric monitoring in occupational health. We developed an analytical method that did not include solid phase extraction (SPE) but utilized deuterium-labeled compounds as internal standard for high-performance liquid chromatography-electrospray ionization mass spectrometry using a C30 column. Urinary concentrations of NMP and its known metabolites 5-hydroxy-N-methyl-2-pyrrolidone (5-HNMP), N-methyl-succinimide (MSI), and 2-hydroxy-N-methylsuccinimide (2-HMSI) were determined in a single run. The method provided baseline separation of these compounds. Their limits of detection in 10-fold diluted urine were 0.0001, 0.006, 0.008, and 0.03 mg/L, respectively. Linear calibration covered a biological exposure index (BEI) for urinary concentration. The within-run and total precisions (CV, %) were 5.0% and 9.2% for NMP, 3.4% and 4.2% for 5-HNMP, 3.7% and 6.0% for MSI, and 6.5% and 6.8% for 2-HMSI. The method was evaluated using international external quality assessment samples, and urine samples from workers exposed to NMP in an occupational area.

1. Introduction

N-Methyl-2-pyrrolidone (NMP) has been used in many industries as an organic solvent for the dissolution of various resins [1,2]. Experiments show that NMP is absorbed by inhalation and ingestion [3,4]. Vaporous NMP is rapidly absorbed through the skin [5,6] and dermal absorption plays an important role [7,8]. Therefore, for exposure assessment, biological monitoring of individual workers is preferable to atmospheric monitoring in workplace environments.

In the human body, NMP is metabolized successively to 5-hydroxy-NMP (5-HNMP), N-methylsuccinimide (MSI), and 2-hydroxy-MSI (2-HMSI), and these compounds are excreted in urine. Recently, 2-pyrrolidone was also reported as a metabolite of NMP in human urine [9]. As major metabolites, 5-HNMP and 2-HMSI are present in urine in high concentrations [4]. In 2007, the American Conference of Governmental Industrial Hygienists (ACGIH) proposed 100 mg/L of 5-HNMP in urine as a biological exposure index (BEI) for NMP [10]. The Deutsche Forschungsgemeinschaft (DFG) proposed 150 mg/L of 5-HNMP in urine as a biological tolerance value (BAT; biologische arbeitsstoff-toleranzwerte) [11]. 2-HMSI is the second major metabolite present in the same magnitude of concentration as 5-HNMP, but has a longer biological half-life, which is favorable for its use as a biomarker for monitoring exposure [12].

Analytical methods for NMP and its metabolites have been developed using solid phase extraction (SPE) as an essential sample preparation step [3,13,14]. Because of its thermolability, 5-HNMP requires trimethylsilyl derivatization of its OH group before GC measurement [15,16]. We previously reported a GC method using SPE and a flame thermionic detector (SPE-GC/FTD) without the derivatization process, but excluded the determination of 5-HNMP because of its thermolability [17]. HPLC–MS/MS methods incorporating electrospray ionization (ESI) or atmospheric pressure ionization (APCI) have been developed for the analysis of 5-HNMP and 2-HMSI in urine and plasma, though these methods were combined with sample preparation using SPE [9,14].

However, from our experience with SPE using poly(divinylbenzene/hydroxymethacrylate), the recovery of an analyte of interest was severely limited by the variations of the operations, and all of these procedures were time consuming [17]. Therefore,

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deuterium-labeled compounds have been used for urine sample preparation with SPE in order to correct for recovery variations. In addition, the metabolites of interest are not conjugated with glucuronic acid or sulfate [5], which encourages us to omit SPE use.

This work aimed to develop an HPLC–ESI–MS/MS method that excluded using SPE, but included the use of deuterium-labeled compounds to directly correct the remaining matrix effect for ESI detection. The method was evaluated with international external quality assessment samples for the determination of 2-HMSI and 5-HNMP in urine and tested using urine samples obtained from workers exposed to NMP in an occupational area.

2. Materials and methods

2.1. Chemicals

NMP was purchased from Wako Pure Chemicals (Osaka, Japan), 5-HNMP and 2-HMSI from SynChelec (Lund, Sweden), and MSI from Avocado Research Chemicals (Heysham, UK). Each deuterium-labeled compound (2-HMSI-d_3, 5-HNMP-d_4, and MSI-d_4) was synthesized by Kemidos AB (Lund, Sweden). NMP-d_9 was obtained from Cambridge Isotope Laboratories (Andover, MA, USA). HPLC-grade acetonitrile and formic acid were purchased from Wako Pure Chemicals. Tap water was purified through a Milli-Q Element A10 (Millipore Japan, Tokyo, Japan) and used as pure water.

2.2. Sample preparation

Calibration standards were prepared in both water and 10-fold diluted urine (free from the compounds of interest) from 3 volunteers. These were spiked with NMP, 5-HNMP, MSI, and 2-HMSI at 0.1, 0.5, 1, 5, 10, and 20 mg/L, and also with NMP-d_9, 5-HNMP-d_4, MSI-d_4, and 2-HMSI-d_3 at 1 mg/L as internal standards. All analytical samples were prepared by mixing 100 μL of urine, 100 μL of a 10 mg/L mixture of NMP-d_9, 5-HNMP-d_4, MSI-d_4, and 2-HMSI-d_3, and 800 μL of pure water as 1 mL of final volume in a vial. These solutions were stable during storage at −20 °C for 3 months.

2.3. HPLC–ESI–MS/MS measurement

A highly hydrophobic C30 stationary column (Develosil C30-UG-5; 250 mm long × 2.0 mm I.d., 5 μm particle size; Nomura Chemical, Seto, Japan) and a guard column (10 mm × 1.5 mm I.d.) were used. The mobile phase was a linear gradient between solvent A (water containing 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid), and programmed for solvent B as follows: 0–5 min, 0 → 30%; 5–9.5 min, 30 → 100%; 9.5–10.5 min, hold at 100%; 10.5–11 min, 100 → 0%; and 11–25 min, 0% for the equilibration. Thus, a measurement cycle took 35 min. The flow rate was 0.2 mL/min, column temperature was 40 °C, and sample injection volume was set to 5 μL. An HPLC system (Alliance 2695; Waters, MA, USA) was used. Detection used a Quattro micro API tandem quadrupole mass spectrometer (Waters) with ESI-positive ion mode by setting the capillary voltage to 3.0 kV, ion source temperature to 120 °C, desolvation nitrogen gas temperature to 450 °C, desolvation gas flow to 700 L/h, and cone gas flow to 50 L/h. The divert valve was set to introduce only the HPLC effluent to the mass analyzer from 5 to 13 min. Collision-induced dissociation (CID) was performed with argon gas. Multiple reaction monitoring (MRM) optimized by the infusion of each aqueous standard solution was applied to a fragment combination of each compound, as listed in Table 1.

3. Results and discussion

3.1. Chromatographic conditions and their influence on ESI efficiency

Co-eluting compounds very readily reduce the ionization efficiency of ESI and result in a reduced sensitivity, known as *ion suppression* [16]. Therefore, an appropriate sample preparation and/or an improved chromatographic separation are required [18–20]. The inorganic ions of urine constituents and highly hydrophilic organic compounds in urine emerge in the solvent front of reversed-phase (RP) chromatography. Among the metabolites of interest, 2-HMSI, which elutes earlier than other compounds, is significantly influenced by these interferences. Thus, in this work, a pre-cut method with valve switching was incorporated in order to waste the column effluent before the emergence of 2-HMSI.

Fig. 1(A) shows baseline separations of a urine sample from German External Quality Assessment Scheme (G-EQUAS), which was diluted 10-fold and spiked with the deuterium-labeled compounds at 1 mg/L. The separation in Fig. 1(B) was performed with a C18 column (Genesis C18; 50 mm × 2.1 mm I.d., 4 μm particle size; Grace Davison Discovery Sciences, IL, USA) following Carrasco et al. [14]. There is a peak adjacent to 2-HMSI and 2-HMSI-d_3 in the C18 column separation, which is not seen in the C30 column separation. The common noisy baselines for NMP are due to the expansion at very low concentrations.

The C30 column stationary phase used in this work is denser than the C18 column, and a highly hydrophobic stationary phase [21], which retarded the elution of hydrophobic urinary constituents. Without the pre-cut, the total UV detector trace (not shown) reported large peaks starting at 3 min, which is close to the void volume (3 min = 0.6 mL of column void volume/0.2 mL/min of flow rate), as is common for urine samples using RP separations.

Next, the urine samples from 10 individuals were examined using the present method. The analytical sample was prepared from 100 μL blank urine samples obtained from 10 volunteers who were not exposed to NMP and were confirmed not to have 2-HMSI, 5-HNMP, MSI, and NMP in their urine. These were mixed with 100 μL of a 10 mg/L aqueous mixture of the standards, 300 μL of the deuterium-labeled compounds at the same concentrations, and 700 μL of pure water. Thus, a sample solution contained (as final concentrations) 1 mg/L of each analyte and its internal standard, along with a 10-fold dilution of the urine sample. The ion suppression, which is expressed as peak area ratio, similar to the recovery (i.e., between spiked urine sample and aqueous standard solution),

Table 1
Parameters of multiple reaction monitoring (MRM).

Compounds	Precursor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (eV)	Acquisition period (min)
2-HMSI	130.0	57.8	20	20	5.5–7.5
2-HMSI-d_3	133.0	60.8	20	22	5.5–7.5
5-HNMP	116.0	84.8	20	14	6.5–8.5
5-HNMP-d_4	120.0	88.8	20	14	6.5–8.5
MSI	114.0	56.8	25	14	9.0–11.0
MSI-d_4	118.0	58.8	25	14	9.0–11.0
NMP	99.8	57.8	35	23	10.5–12.5
NMP-d_9	108.9	51.8	35	26	10.5–12.5

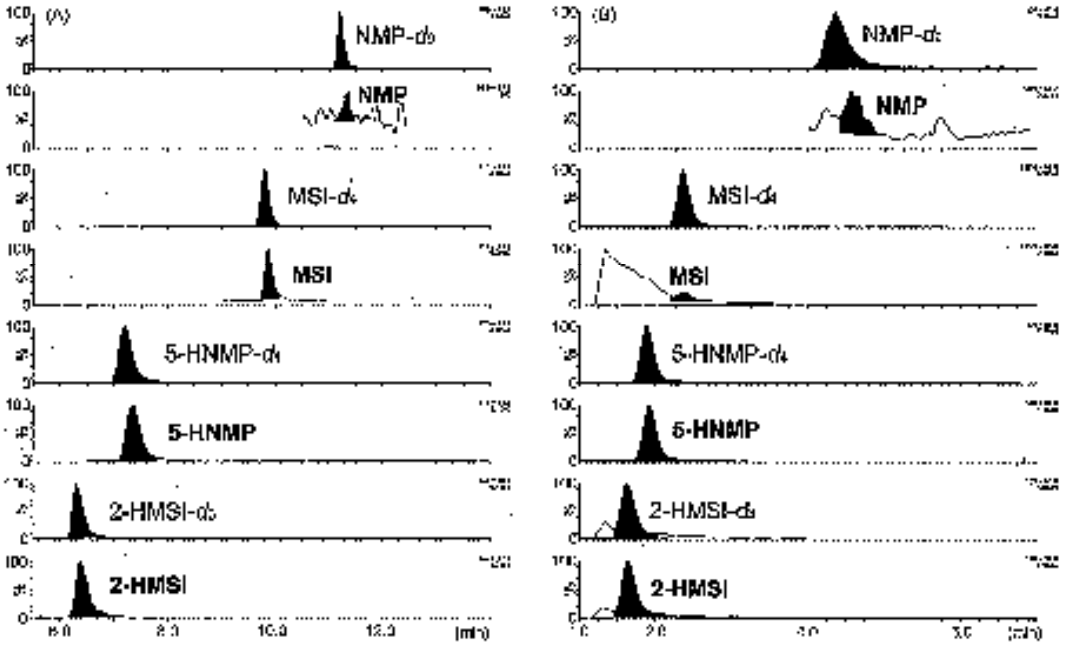

Fig. 1. XRM chromatograms of 2-HMSI, 5-HNMP, MSI, and NMP in 10-fold diluted urine sample of G.SQUAS 43cc 3A, to which 2-HMSI-d_5, 5-HNMP-d_4, MSI-d_4, and NMP-d_6 were spiked, each at 1 mg/L, and separated with a C30 column (A) and a C18 column (B).

found 65% recovery for 2-HMSI and higher for others. However, the deuterium-labeled compounds used to correct for the ionization gave 93.8 ± 1.0% for 2-HMSI, 107.4 ± 1.7% for 5-HNMP, 105.0 ± 3.5% for MSI, and 108.7 ± 2.4% for NMP. These results suggested that the C30 column separation and the use of each deuterium-labeled internal standard made this method workable when allowing for a 10% variation range, similar to the variation using SPE–GC/FPD [17].

3.2. Calibration curves, limits of detection, and precision

Linear calibrations were examined by preparing aqueous solutions and 10-fold diluted blank urine samples (from 3 volunteers). These were spiked with NMP and its metabolites at 0.1, 0.5, 1, 5, 10, and 20 mg/L and also with deuterium-labeled compounds at 1 mg/L. A calibration curve was prepared by plotting the peak area relative to each internal standard (y) against the known concentration of each component (x). Slopes (a) and intercepts (b) were calculated by linear regression, $y = ax + b$, after weighting the ordinate values by $1/x^2$.

The results (summarized in Table 2) indicate that the calibration equations obtained with 10-fold diluted spiked urine samples were, irrespective of the urine sources, very similar to each other as well as to those obtained with aqueous solutions (i.e., similar slopes, small intercepts, and high correlation coefficients). Linear concentration responses ranged up to 20 mg/L for 2-HMSI, 5-HNMP, and MSI, but the range was only up to 5 mg/L for NMP. These limits were

Table 2
Calibration equations for 2-HMSI, 5-HNMP, MSI, and NMP in aqueous solution and 10-fold diluted urine samples spiked with standards

Compounds	Matrix	Concentration range (mg/L)	Slope (a)	Intercept (b)	Correlation coefficient
2-HMSI	Water	0.1–20	1.58	0.010	0.999
	1/10 Urine A[a]	0.1–20	1.59	−0.004	0.997
	1/10 Urine B[a]	0.1–20	1.57	−0.002	0.999
	1/10 Urine C[a]	0.1–20	1.59	−0.010	0.994
5-HNMP	Water	0.1–20	2.45	0.036	0.995
	1/10 Urine A[a]	0.1–20	2.48	0.019	0.993
	1/10 Urine B[a]	0.1–20	2.46	0.012	0.995
	1/10 Urine C[a]	0.1–20	2.41	0.017	0.997
MSI	Water	0.1–20	1.53	−0.012	0.998
	1/10 Urine A[a]	0.1–20	1.49	−0.111	0.988
	1/10 Urine B[a]	0.1–20	1.55	0.147	0.998
	1/10 Urine C[a]	0.1–20	1.53	0.010	0.999
NMP	Water	0.1–5	19.7	0.535	0.994
	1/10 Urine A[a]	0.1–5	18.1	0.094	0.996
	1/10 Urine B[a]	0.1–5	20.0	0.290	0.987
	1/10 Urine C	0.1–5	19.0	0.572	0.992

[a] Urine samples A, B, and C were from NMP non-exposed individuals.

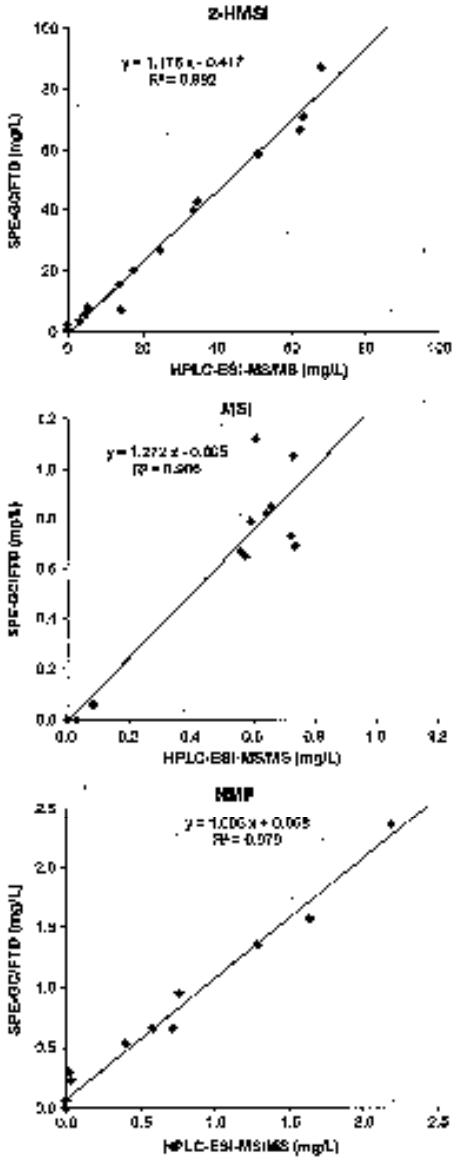

Fig. 2. Correlations of urinary metabolites between HPLC-ESI-MS/MS and SPE-GC/FTD for urine samples from workers exposed to NMP in an occupational environment.

due to the specific ionization characteristics of the compounds (i.e., 2-HMSI at the lowest detection and NMP at the highest, as found in the infusion experiments with aqueous solutions). In practice, the urinary concentration of NMP is one-digit lesser than the others, as seen in Fig. 2, and the upper concentration linearity limits match the ACGIH BEI value of 100 mg/L [16] and the DFG HAT value of 150 mg/L [17] for 5-HNMP in raw urine.

The limits of detection (LOD) for NMP and its metabolites were calculated according to the definition given by Gibbons [22]. The samples used for LOD determinations were aqueous solutions of standards and the 10-fold diluted urine samples from 3 volunteers, but spiked at concentrations of 0.2 mg/L 2-HMSI, 0.02 mg/L 5-HNMP, 0.01 mg/L MSI, and 0.01 mg/L NMP, which gave chromatographic peaks with S/N ratios of 10–15 (peak to peak) for each peak. The calculated LODs were 0.02 mg/L for 2-HMSI, 0.005 mg/L for 5-HNMP, 0.01 mg/L for MSI, and 0.0003 mg/L for NMP in aqueous solutions, and 0.03 mg/L for 2-HMSI, 0.006 mg/L for 5-HNMP, 0.008 mg/L for MSI, and 0.0001 mg/L for NMP in 10-fold diluted spiked urine samples. There was little difference in LODs between aqueous solutions and the 10-fold diluted spiked urine samples.

The precision of the HPLC–MS/MS measurements was determined using 9 diluted urine samples from 3 volunteers, spiked at arbitrary concentrations of 5, 10, and 100 mg/L each of 2-HMSI, 5-HNMP, MSI, and NMP. These were subjected to 10 replicate measurements for the within-run precision, or 3 replicates on 4 different runs for the total precision. Within-run precision (CV, %) was 6.5% for 2-HMSI, 3.4% for 5-HNMP, 3.7% for MSI, and 5.1% for NMP. Total precision (CV, %) was 6.9% for 2-HMSI, 4.2% for 5-HNMP, 6.0% for MSI, and 9.2% for NMP. These results seem to be reasonable by comparison with the reported within-day and between-day precisions obtained with SPE-HPLC-MS/MS [9,14].

3.3. Method evaluation with test urine samples

First, the present method was applied to urine samples of G-EQUAS 41st and 42nd intercomparison programs held in 2008 for 2-HMSI and 5-HNMP [23]. These urine samples were diluted 10-fold with pure water, the deuterium-labeled internal standards were added as concentrations of 1 mg/L, then measured (*n* = 5). All of the measured values were found in the tolerance range, as seen in Table 3. Thus, the accuracy of this method was reasonable.

Next, to compare the present method with the SPE-GC/FTD method [17], urine samples from 5 workers exposed to NMP, which had been stored frozen at −20°C, were subjected to the present HPLC-ESI-MS/MS measurements. The relationships of measured values between HPLC-ESI-MS/MS and SPE-GC/FTD are shown in Fig. 2. Correlations between the two methods are significant: $r^2 = 0.892$ for 2-HMSI, $r^2 = 0.979$ for NMP and $r^2 = 0.986$ for MSI with several scattered data points.

Comparable data by SPE-GC/FTD were not available for 5-HNMP because of its thermal instability for GC measurements, as previously mentioned. However, the concentration of 5-HNMP found in these 5 workers' urine samples (collected at the end of a shift) was 37.5 ± 17.2 mg/L (mean ± SD). These workers were exposed to 6.2 ± 1.1 ppm NMP vapor in the occupational atmosphere for 8 h

Table 3
Measured values, reference values, and tolerance ranges for 2-HMSI and 5-HNMP in G-EQUAS urine samples.

G-EQUAS urine samples	2-HMSI (mg/L)			5-HNMP (mg/L)		
	Measured value	Reference value	Tolerance range	Measured value	Reference value	Tolerance range
41st-5A	17.7 ± 0.5	21.2	16.3–26.1	30.1 ± 0.4	30.4	24.4–36.3
41st-5Q	36.6 ± 0.6	42.4	36.0–48.8	101.7 ± 1.5	103.4	94.3–112.5
42nd-5A	20.9 ± 0.5	25.1	19.3–31.2	85.9 ± 2.4	85.7	82.3–110.6
42nd-5Q	37.6 ± 2.1	43.2	34.7–51.6	150.4 ± 4.6	151.7	131.2–172.1

Data values are expressed as mean ± SD (*n* = 5).

[17]. This urinary concentration of 5-HNMP seems to be reasonable based on the proportionality of NMP exposure level and the concentration of 5-HNMP excreted in urine [12]. It is also in line with the values reported by Workplace Environmental Exposure Level [24] of 10 ppm (40 mg/m^3) NMP and its excretion of 100 mg/L 5-HNMP (BEI) [10], and of 19 ppm (80 mg/m^3) NMP for Maximum Concentrations at the Workplace (MAK) and its excretion of 150 mg/L 5-HNMP [11].

4. Conclusion

The present analytical method using HPLC–ESI-MS/MS, which comprises highly hydrophobic chromatographic separation and the use of deuterium-labeled compounds as internal standards, allows the direct determination of NMP and its urinary metabolites by simple aqueous dilution of urine samples and without laborious SPE preparations. This method is useful for high-throughput biological monitoring of NMP exposure in the workplace.

Acknowledgments

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3-6) コーヒー摂取と尿中馬尿酸濃度

トルエン曝露の代謝物測定として、尿中馬尿酸が規定されているが、非曝露者における高値が問題となっている事から、その原因がコーヒー摂取による可能性を検討し報告した(Ind Health, 49:195-202, 2011)。

Industrial Health 2011, 49, 195–202 Original Article

Influence of Coffee Intake on Urinary Hippuric Acid Concentration

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Abstract: Intake of foods and drinks containing benzoic acid influences the urinary hippuric acid (HA) concentration, which is used to monitor toluene exposure in Japan. Therefore, it is necessary to control the intake of benzoic acid before urine collection. Recently, some reports have suggested that components of coffee, such as chlorogenic, caffeic, and quinic acids are metabolized to HA. In this study, we evaluated the influence of coffee intake on the urinary HA concentration in toluene-nonexposed workers who had controlled their benzoic acid intake, and investigated which components of coffee influenced the urinary HA concentration. We collected urine from 15 healthy men who did not handle toluene during working hours, after they had consumed coffee, and we measured their urinary HA concentrations; the benzoic acid intake was controlled in these participants during the study period. The levels of chlorogenic, caffeic, and quinic acids in coffee were analyzed by LC-MS/MS. Urinary HA concentration increased significantly with increasing coffee consumption. Spectrophotometric LC-MS/MS analysis of coffee indicated that it contained chlorogenic and quinic acids at relatively high concentrations but did not contain benzoic acid. Our findings suggest that toluene exposure in coffee-consuming workers may be overestimated.

Key words: Biological monitoring, Hippuric acid, Toluene, Coffee intake, Chlorogenic acid

Introduction

In Japan, health control of workers is accomplished by controlling work environment conditions and improving work practice management and health care. Urinary hippuric acid (HA) levels are measured through periodic monitoring of workers exposed to toluene during medical examinations, as mandated by the Ministry of Health, Labour and Welfare[1].

However, toluene is metabolized to HA via benzoic acid in the human body. Therefore, the dietary intake of benzoic acid, which is present in certain acidic foods (e.g., beans, plums, cranberries, and prunes) and used in food preservatives, also influences urinary HA concentration. Hence, it is necessary to develop methods that eliminate the effect of dietary HA and other environmental influences that contribute to the background concentration of HA[2].

The Ministry of Health, Labour and Welfare of Japan has established a 3-tier classification system (distributions 1, 2, and 3) based on urinary HA level in workers (Table 1). "Distribution 3" consists of workers exposed to concentrations that are more than the 1988–1989

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Table 1. Classification of urinary hippuric acid for biological monitoring in Japan[2]

Chemical determination	Assay Material	Unit	Category		
			1	2	3
Toluene→hippuric acid	Urine	g/l	<1	1–2.5	>2.5

In Japan, a category is called distinction.

biological exposure indices released at the American Conference of Industrial Hygienists (ACGIH)[3]. The HA concentration in most workers is usually within "Distinction 1" under appropriate control of toluene exposure, especially when the intake of benzoic acid is controlled for at least 1 day before urine collection. Nevertheless, elevated urinary HA concentrations are sometimes observed even when the intake of benzoic acid is strictly controlled[4].

It was recently reported that the consumption of coffee results in the elevation of urinary hippuric acid concentration[5–7], and it was indicated that the components of coffee (in particular, chlorogenic, caffeic, and quinic acids) are metabolized to HA[8]. Muraki *et al.*[9] reported that the consumption of green tea and/or coffee can result in an overestimation of urinary HA concentrations and cause false-positive results during the biological monitoring of workers exposed to low doses of toluene.

In this study, we evaluated the influence of coffee intake on urinary HA concentration when benzoic acid intake was controlled in toluene-nonexposed workers, and analyzed the components in coffee that influenced urinary HA concentration.

Materials and Methods

Participants and urine collection

The participants in this study were 15 healthy male medical staff (age range, 29–56 yr) who had not handled toluene. This study was approved by the Ethics Committee of Tokyo Rosai Hospital (approval number 20-6). All subjects gave informed consent.

Urine was collected on days when the participants did not drink coffee or had 1, 2, or 3 cups of coffee (per day). Samples were obtained every 3–4 d, and in some cases, every other day. The participants consumed the drip coffee that is readily available in Japan. They were not permitted to consume alcohol or foods/drinks containing benzoic acid for at least 1 d before urine collection. Moreover, they were instructed to drink only coffee or water during working hours.

On the urine collection day, they could drink coffee at any time during working hours. Similar to the procedure followed in the biological monitoring of toluene, we collected urine at the end of the working day. The urine samples were preserved at 4°C in a refrigerator before further analysis.

Measurement of urinary HA and creatinine concentrations

Urinary HA was measured by high-performance liquid chromatography (HPLC) within 1 wk after collection in the laboratory of Mitsubishi Chemical Medience Corporation (Tokyo, Japan) following the procedure reported by Ogata *et al*[2]. The analytical quality of HA analysis in this laboratory was certificated by National Federation of Industrial Health Organization in Japan[10].

We preserved urine samples at 4°C until HA analysis. Although we did not examine the stability of HA in these samples, the National Institute for Occupational Safety and Health (NIOSH) indicates that hippuric acid in urine is stable at 4°C for 1 wk[11]. Therefore, we assumed that the urinary HA concentration did not change during preservation.

Urinary creatinine was measured by an enzymatic method in the same laboratory, following the procedure reported by Yoshioka *et al*[12]. In order to adjust urinary HA concentration, urinary HA divided by urinary creatinine was used as corrected HA.

Chemicals

We purchased chlorogenic acid, quinic acid, HA, caffeic acid, benzoic acid, HPLC-grade acetonitrile, and formic acid from Wako Pure Chemicals (Osaka, Japan). Tap water was purified using Milli-Q Element A10 (Millipore Japan, Tokyo, Japan) and used as pure water.

HPLC-electrospray ionization-tandem mass spectrometry measurement

Standard solutions of chlorogenic, caffeic, quinic, HA, and benzoic acids were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Alliance 2695-Quattro Micro API; Waters, Milford, MA, USA). The levels of chlorogenic, caffeic, and quinic acids, and the absence of benzoic acid in the coffee were also analyzed using the same device.

A highly hydrophobic C30 stationary column (Develosil C30 UG-3 column; 150 mm long × 2.0 mm i.d., Nomura Chemical Corporation, Aichi, Japan) was used to analyze 500-μg/l standard solution and ten-fold

diluted coffee. The mobile phase was a linear gradient of solvent A (water containing 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid). The program for solvent B was as follows: 0–8 min, 2→50%; 8–8.5 min, 50→100%; 8.5–9 min, held at 100%; 9–9.5 min, 100→2%; 9.5–20 min, held at 2%. The flow rate was 0.2 ml/min and the injection volume was 5 μl.

The coffee components were detected by positive electrospray ionization (ESI+); capillary voltage, 1 kV; ion source temperature, 120°C; desolvation gas temperature, 350°C; desolvation gas flow rate, 800 l/h; and cone gas flow rate, 50 l/h.

Multiple reaction monitoring (MRM) was used to confirm the detected substances. The MRM parameters used in these analyses are shown in Table 2.

This method employed the same device we used to determineN-methyl-2-pyrrolidone metabolites in urine as reported previously[13].

Statistical methods

Statistical analyses were performed using StatView 5.0 (SAS Institute Inc., Cary, NC, USA). Spearman's rank correlation test was used to evaluate the relationship between urinary HA concentration and the amount of coffee consumed.

Results

HA concentration

Uncorrected urinary HA concentrations and urinary HA concentrations corrected for urinary creatinine concentration are summarized in Table 3.

Both uncorrected and corrected urinary HA concentrations increased with increasing coffee consumption (Figs. 1 and 2), and Spearman's rank correlation test indicated a significant relationship between the HA concentration and the amount of coffee consumed (uncorrected, $p<0.05$; corrected, $p<0.01$).

Table 2. Parameters of multiple reaction monitoring

Compounds	Precursor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (eV)
Chlorogenic acid	355.1	163.0	20	12
Quinic acid	191.1	147.0	20	10
Hippuric acid	180.1	76.0	20	26
Caffeic acid	181.1	88.9	20	24
Benzoic acid	121.0	78.8	25	15

Table 3. Urinary hippuric acid concentration after coffee intake

Case	Without coffee			After 1 cup of coffee			After 2 cups of coffee			After 3 cups of coffee		
	HA (g/l)	Cre (g/l)	Corrected HA (g/g Cre)	HA (g/l)	Cre (g/l)	Corrected HA (g/g Cre)	HA (g/l)	Cre (g/l)	Corrected HA (g/g Cre)	HA (g/l)	Cre (g/l)	Corrected HA (g/g Cre)
1	0.12	1.12	0.11	0.14	1.23	0.11	0.27	1.12	0.24	0.25	0.62	0.4
2	0.26	1.19	0.22	0.78	0.57	0.13	0.21	0.48	0.44	0.19	0.38	0.5
3	0.12	0.64	0.19	0.78	1.22	0.1	0.37	0.82	0.45	0.58	1.56	0.46
4	0.1	1.47	0.07	0.09	0.6	0.05	0.07	0.72	0.1	1.45	1.56	0.77
5	0.13	1.06	0.14	0.01	0.49	0.01	0.12	1.34	0.09	0.28	0.58	0.1
6	0.04	1.12	0.04	0.01	0.55	0.02	0.01	0.51	0.09	0.03	0.8	0.04
7	0.24	1.03	0.23	0.27	0.99	0.22	0.6	1.57	0.39	0.65	1.22	0.54
8	0.06	1.2	0.05	0.07	1.52	0.05	0.05	1.01	0.06	0.05	1.37	0.04
9	0.02	1.24	0.02	0.13	0.89	0.15	0.05	0.96	0.06	0.13	1.09	0.08
10	0.25	1.38	0.20	0.23	1.09	0.21	0.31	1.39	0.29	0.18	0.79	0.23
11	0..	1.27	0.06	0.14	1.45	0.1	0.2	2.22	0.09	0.25	1.11	0.21
12	0.08	0.94	0.09	0.08	1.62	0.05	0.38	1.17	0.33	1.26	2.89	0.44
13	0.04	0.5	0.08	0.11	1.07	0.2	0.08	0.71	0.21	0.02	1.82	0.03
14	0.1	1.02	0.10	0.42	1.83	0.23	0.09	0.56	0.25	0.17	0.57	0.25
15	0.09	0.66	0.05	0.41	1.17	0.35	0.35	1.16	0.3	0.11	0.25	0.44
Median	0..0		0.09	0.13		0.10	0.20		0.24	0.23		0.40

HA, hippuric acid; Cre, creatinine. Corrected HA, urinary HA adjusted by urinary creatinine.

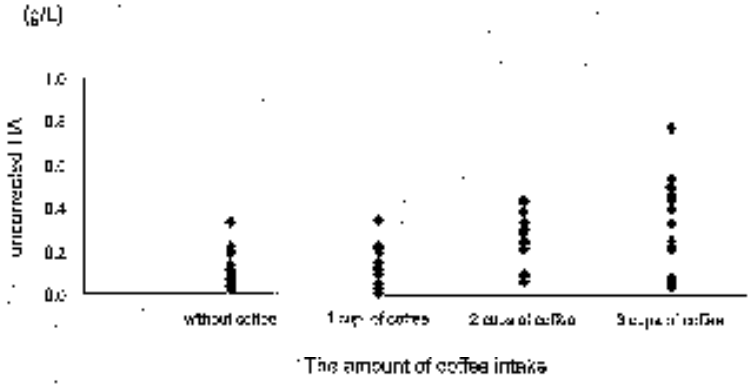

Fig. 1. Uncorrected concentrations of urinary hippuric acid (HA) after coffee intake.
Spearman's rank correlation test showed a significant relationship between the uncorrected HA concentration and the amount of coffee consumed ($p<0.05$).

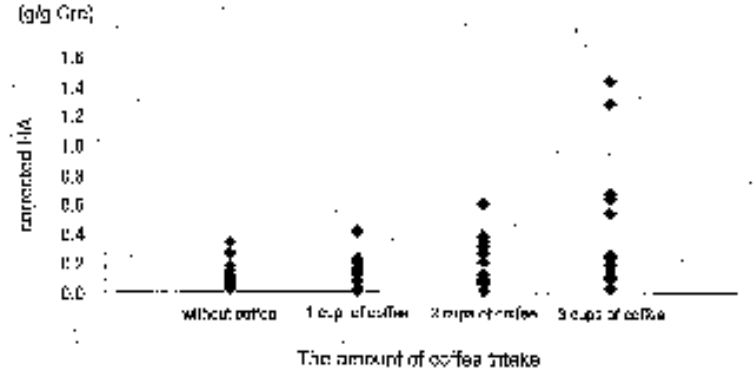

Fig. 2. Concentrations of urinary hippuric acid (HA) corrected for creatinine (Cre) after coffee intake.
Spearman's rank correlation test showed a significant relationship between the corrected HA concentration and the amount of coffee consumed ($p<0.01$).

HPLC-ESI-MS/MS measurements

MRM chromatograms of 5-μl aliquots of 500-μg/l standard solutions of chlorogenic acid, quinic acid, HA, caffeic acid, and benzoic acid are shown in Fig. 3, and those of a 5-μl aliquot of ten-fold diluted coffee are shown in Fig. 4.

The chromatograms indicated that the coffee consumed by the participants in this experiment did not contain benzoic acid, and that the concentrations of chlorogenic, quinic, and caffeic acids, and HA were 906.3 mg/l, 482.8 mg/l, 10.3 mg/l, and 2.2 mg/l, respectively.

Discussion

We allowed the participants to drink coffee at their convenient time during working hours because the resting times of and the ways adopted by workers who were coffee drinkers were generally different. Hence, we evaluated the influence of coffee intake on the urinary HA concentration under conditions similar to those observed in daily work. Moreover, we did not restrict the intake of any foods, except foods/drinks containing benzoic acid, because we sought to evaluate the influence of coffee intake on urinary HA concentration while maintaining the usual restrictions described in the regulations in Japan. In terms of the study design, not allowing the participants to take coffee at any time may be considered a limitation; our results may have been stronger had we performed a prior experiment in which the participants took coffee at a specified time prior to urine collection.

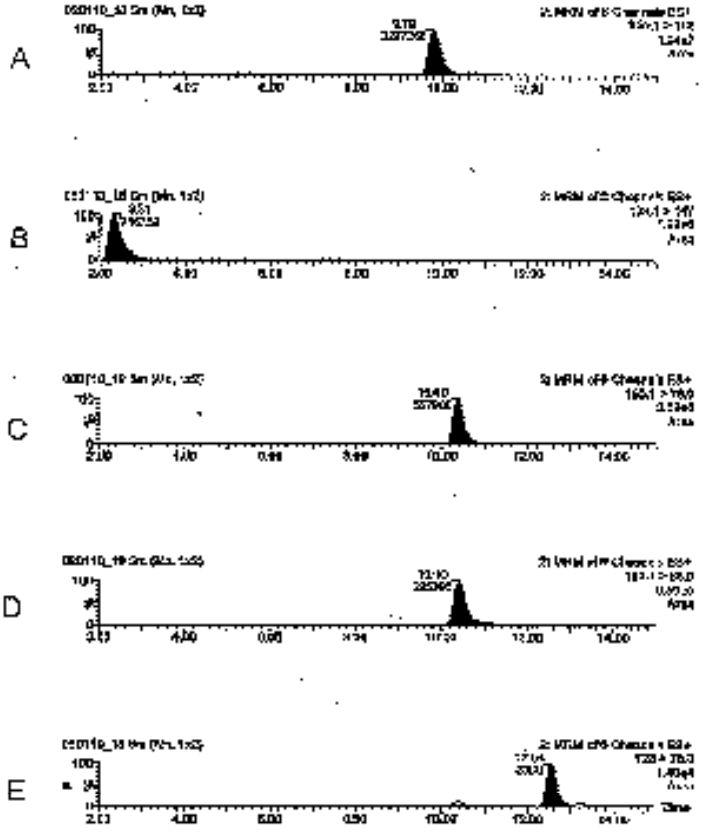

Fig. 3. Multiple reaction monitoring chromatograms of standard solutions. A 5-μl aliquot of the 500 μg/l standard solution was analyzed.
A: chlorogenic acid, B: quinic acid, C: hippuric acid, D: caffeic acid, E: benzoic acid

Our study showed that coffee intake influenced urinary HA concentration in toluene-nonexposed workers, even though the intake of food and drinks containing benzoic acid was controlled. It has been shown previously that green tea and/or coffee elevated the urinary HA levels in toluene-exposed workers[8]. Taken together, these results indicate that the consumption of coffee does not accelerate toluene metabolism but rather that a component of coffee is itself metabolized to HA.

Spectrophotometric analysis revealed that chlorogenic and quinic acids were present at relatively high concentrations in coffee. These 2 components could be the main source of elevated HA levels in the body. Olthof et al.[9] described a pathway in which chlorogenic acid is metabolized to HA as follows. Ingested chlorogenic acid reaches the colon and is hydrolyzed to caffeic and quinic acids by the colonic microflora. Subsequently, caffeic acid is dehydroxylated by the bacteria in the colon; after absorption, it is β oxidized into benzoic acid in the liver. Quinic acid is dehydroxylated into cyclohexane carboxylic acid and aromatized into benzoic acid by the colonic microflora. Benzoic acid is conjugated with glycine and excreted in the urine as HA (Fig. 5). Our results show that urinary HA concentration in some participants was not elevated even after drinking 3 cups of coffee as compared to the HA concentration in those who did not drink coffee or had 1 or 2 cups of coffee. We suspect that this difference was mainly due to individual variations of microflora activity in the colon and the degree of absorption of the components into the circulation.

Our results showed that there was a significant relationship between HA concentration and coffee consumption in toluene-nonexposed workers; i.e., when a worker consumes coffee during working hours, he may have a high background level of HA that would influence the

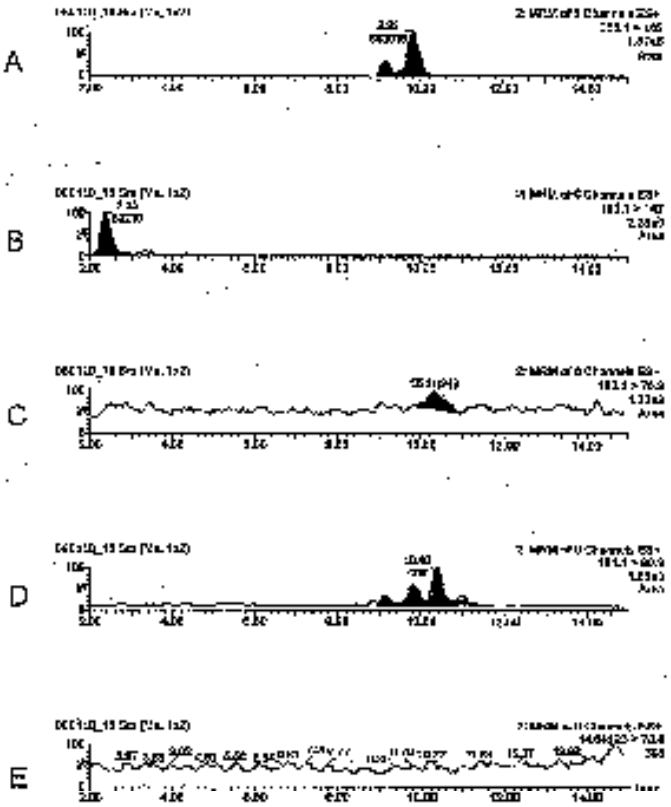

Fig. 4. Multiple reaction monitoring chromatograms of coffee. A 5-μl aliquot of ten-fold dilution of coffee was analyzed.
A: chlorogenic acid, B: quinic acid, C: hippuric acid, D: caffeic acid, E: benzoic acid.

toluene exposure estimates. Therefore, the toluene exposure of coffee-consuming workers may have been overestimated.

The chlorogenic acid content in the coffee used in this study was about 996 mg/l. Some studies have reported the chlorogenic acid contents in coffee. Ohta *et al.*[6] reported that 1 l of coffee contains 500–800 mg of chlorogenic acid. Monteiro *et al.*[14] reported a chlorogenic acid content of 1,068 ± 49 μmol/100 ml (1,991.6 ± 91.6 mg/l) (mean ± SD) in coffee. We analyzed the chlorogenic acid content in another coffee source, and found it to be 1,074 mg/l (chromatogram not shown). Thus, the coffee used in this study contained chlorogenic acid in the normal concentration range.

It has been reported that in humans, 75–100% of the ingested benzoic acid (up to 160 mg/kg body weight) is metabolized to HA within 6 h, and the remaining is metabolized within 2–3 d[15–17]. We therefore suggest that as long as HA is used as a biomarker of toluene exposure, coffee intake should be restricted at least 2–3 d before biological monitoring in addition to the restriction on foods and drinks containing benzoic acid.

The measurement of urinary HA concentration is mandatory as per occupational health regulations in Japan; however, the occupational exposure limits set by the Japanese Society for Occupational Health and the Biological tolerance values set by the Deutsche Forschungsgemeinschaft do not use urinary HA to monitor toluene exposure[18, 19]. Moreover, in 2009, ACGIH introduced a change in the biological monitoring of toluene and proposed that BEI should not include urinary HA[20]. It has been reported that urinary HA cannot be used to assess the exposure to low concentrations of toluene owing to the effect of its background concentration[9]. Therefore, it is recommended that the Japanese authorities reconsider the use of HA in the assessment of toluene exposure.

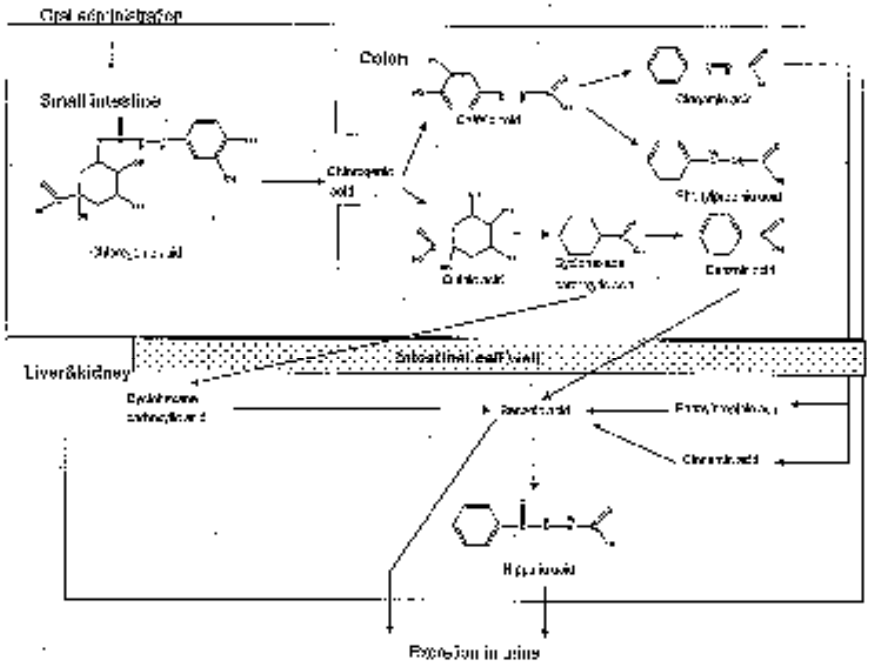

Fig. 5. The metabolic pathway of ingested chlorogenic acid.

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3-7) 作業環境中オルトフタルアルデヒド(OPA)の測定法の開発

医療器具の殺菌消毒剤グルタルアルデヒド(GA)の代替品であるオルトフタルアルデヒド(OPA)については第 1 期において医療従事者における世界初症例を報告したが、その環境中濃度の測定法を開発したので報告した（J Occup Health, 51:386-390、2009)。

J Occup Health 2009; 51: [illegible]

Short Communication

Optimization of the Determination of Ortho-phthalaldehyde in Air by Derivatization with 2,4-dinitorophenylhyrazine (DNPH)

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Key words: Derivatization, 2,4-dinitorophenylhydrazine, Ortho-phthalaldehyde

Endoscopic equipment and other medical devices are currently sterilized with three types of sterilizing agent, glutaraldehyde (GA), ortho-phthalaldehyde (OPA) and peracetic acid. OPA is considered a powerful, high-level disinfectant because it is more effective against GA-resistant mycobacteria, is less irritating and has a shorter disinfection time than GA, and does not require any activation step[1].

Due to the development of allergic dermatitis and asthma among medical workers exposed to GA[2-4], the Japanese Ministry of Health, Labour, and Welfare[5] recommended in February 2005 that GA exposure in sterilization units should be kept below 0.05 ppm. OPA as a safer alternative to GA has therefore been more frequently utilized for sterilization in an increasing number of Japanese hospitals. However, allergic disorders have also been reported among medical workers using OPA[6].

OPA concentrations in work environments have been reported to be lower than those of GA[7, 8], indicating a need for reliable methods for measuring OPA. Moreover, the safety of medical workers requires strict control of OPA, maintaining low concentrations in air.

Measurements of OPA in the air of work environments have been quantitatively determined by HPLC after pre-column derivatization with 2,4-dinitrophenylhydrazine (DNPH)[illegible]. We recently[illegible] found that the hydrazone derivatives of OPA with DNPH are comprised of several different chemical forms, the major one being bis-DNPhydrazone. We also found that the rate of formation and the relative abundance of the hydrazone derivatives of OPA with DNPH depend on the eluting conditions, such as phosphoric acid concentration or reaction time, during the extraction of OPA from DNPH-silica cartridges. In this report we describe the characteristic profiles of the hydrazone derivatives of OPA with DNPH, as well as the optimal analytical conditions to measure OPA in the air of work environments.

Materials and Methods

Materials

Ortho-phthalaldehyde (OPA), 2,4-dinitrophenylhydrazine (50% aqueous solution, DNPH) and phosphoric acid of guaranteed reagent grade were purchased from Tokyo Chemical Industry (Tokyo, Japan), Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Sigma Aldrich Japan (Tokyo, Japan), respectively. HPLC grade acetonitrile was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Water was purified with a Milli-Q water system (Millipore, Bedford, MA, USA). DISOPA solution (0.55% OPA) was purchased from Johnson and Johnson (Tokyo, Japan).

A series of phosphoric acid/acetonitrile solutions was prepared by dissolving calculated amounts of phosphoric acid in acetonitrile. A standard solution of OPA was prepared by dissolving a calculated amount of OPA in acetonitrile (2 µg/ml). DNPH solution was prepared by dissolving a calculated amount of DNPH, which recrystallized with acetonitrile, in acetonitrile (0.2 mg/ml).

DNPH-silica cartridges of LpDNPH S10 and GL-Pak mini AERO DNPH for OPA sampling were purchased from Supelco Inc. (Bellefonte, PA, USA) and GL Sciences Inc. (Tokyo, Japan), respectively. Sampling was performed by introducing air into the DNPH-silica cartridge with a SKC Air Check 2000 (SKC Inc., Eighty Four, PA, USA).

Characterization of the hydrazone derivatives of OPA with DNPH by HPLC-MS/MS

Ten microliters of a reaction mixture of OPA with DNPH was injected into an HPLC system (HP1100, Agilent, USA) connected to an Inertsil CN-3 column (250 × 4.6 mm i.d., GL Science, Tokyo, Japan) under the following conditions: mobile phase of 70% acetonitrile and 30% water; flow rate, 1.0 ml/min; column temperature, 26°C. The hydrazone derivatives of OPA with DNPH were detected using an ion trap mass analyzer

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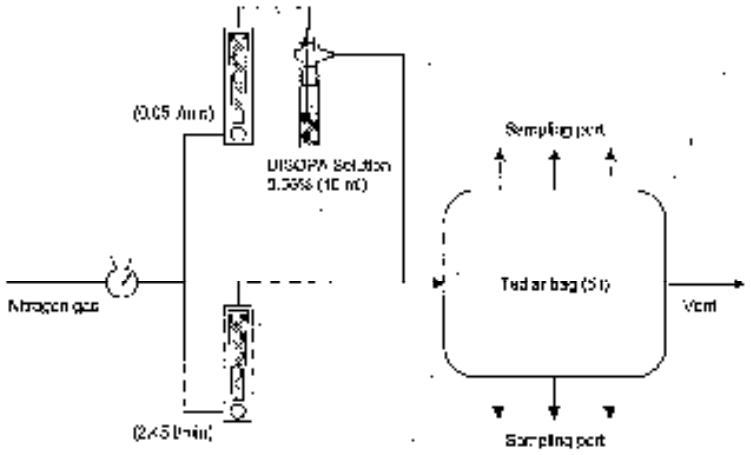

Fig. 1. Schematic illustration of the test gas generator.

type of LC-MS/MS (LCQ, Thermoquest, Japan). The mass-spectra of the derivatives was obtained by atmospheric pressure chemical ionization (APCI) in negative ion mode. Collision induced dissociation (CID) was used for MS^n spectrometry.

Derivatization of OPA with 2,4 dinitrophenylhydrazine in phosphoric acid solutions

The hydrazone derivative of OPA with DNPH were analyzed with a TOSHO 8020 Series HPLC system (TOSHO, Tokyo, Japan), and were monitored with a UV spectrophotometric detector at 383 nm (SPD-6A, Shimadzu, Kyoto, Japan). The analytical column and the mobile phase were the same as those used for the LC-MS/MS analysis. The column temperature was kept at 40°C and a 20-μl aliquot of the sample solution was injected.

To determine the optimal reaction conditions for derivatization of OPA with DNPH, we assessed the effect of phosphoric acid concentration and the transformation of OPA mono-DNPhydrazone to OPA bis-DNPhydrazone using a standard solution of OPA and a UV detector.

1. Effect of phosphoric acid concentration

To examine the effect of phosphoric acid concentration on the derivatization reaction, a 50-μl aliquot of OPA standard solution (2 μg/ml) was added to each test tube containing 0.9 ml of DNPH solution (0.2 mg/ml) and 50 μl of phosphoric acid/acetonitrile solution, containing different concentrations of phosphoric acid, ranging from 0.0005% to 20% (v/v). Each tube was vortexed for 30 s and allowed to stand for 30 min at room temperature. The final concentration of phosphoric acid in the test tubes ranged from 0.00025 to 1.0% (v/v). The amounts of hydrazone derivatives of OPA with DNPH in each test tube were determined by HPLC analysis.

2. Transformation of OPA mono-DNPhydrazone to OPA bis-DNPhydrazone

Fifty microliters of OPA standard solution (2 μg/ml) was added to a test tube containing 0.9 ml of DNPH solution (0.2 mg/ml) and 50 μl of phosphoric acid (0.1%, v/v)/acetonitrile solution. The tube was vortexed for 30 s and allowed to stand at room temperature. At various time points, ranging from 0 to 10.5 h, aliquots were withdrawn, and the amounts of OPA mono- and OPA bis-DNPhydrazone were determined by HPLC analyses.

Generation of OPA test gas and collection with DNPH-silica cartridge

OPA test gas was generated with a gas generator, as shown in Fig. 1. Nitrogen gas, at a flow rate of 0.05 l/min, was introduced to the impinger containing 10 ml of DISOPA solution, and the dynamically generated OPA gas was diluted with nitrogen gas, at a flow rate of 2.45 l/min, into a Tedlar® bag (5 l), which was used as an exposure chamber, at ambient temperature. Due to the relatively low vapor pressure of OPA, 30 min was necessary to stabilize the OPA gas concentration in the Tedlar bag. The relative standard deviation of OPA gas concentration was 16% for intra-day reproducibility.

The OPA test gas was passed through the DNPH-silica cartridge for 10 min at a flow rate of 1.0 l/min. The OPA-DNPH derivatives collected in the DNPH-silica cartridge were extracted immediately with 5 ml of acetonitrile. A 25-μl aliquot of phosphoric acid/

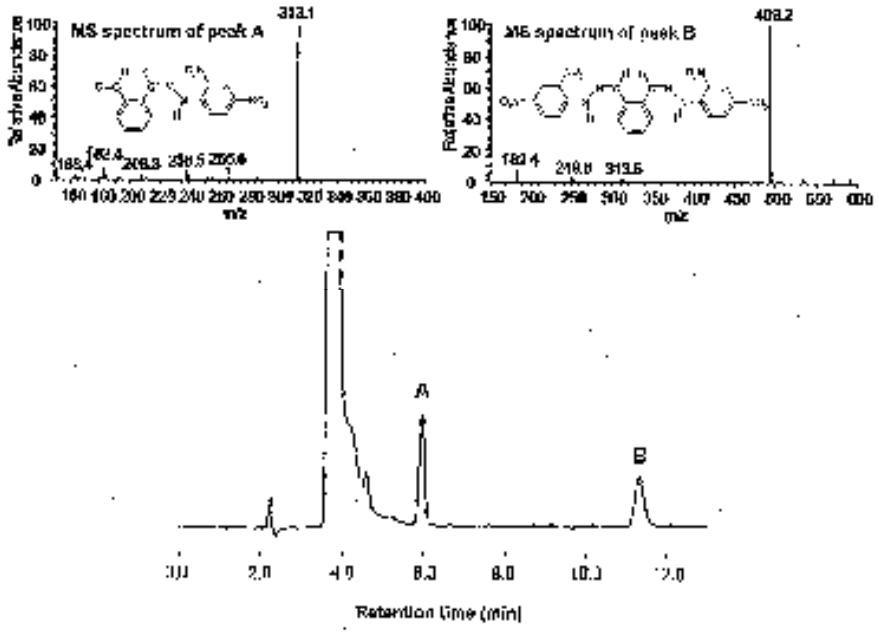

Fig. 2. HPLC-UV chromatogram and ion trap APCI mass spectra of the test solution, in which OPA standard solution was reacted with DNPH. Peak (A), OPA mono-DNPhydrazone; Peak (B), OPA bis-DNPhydrazone.

acetonitrile solution (2%, v/v) was added to 475 μl of the eluent solution. The sample was allowed to stand at room temperature for 0 to 4 h and aliquots were subsequently analyzed by HPLC-UV.

In this HPLC analysis, the peak corresponding to OPA mono-DNPhydrazone overlapped with the tail of the large peak due to the derivatizing reagents. Therefore, the time course of the derivatization reaction was traced by measuring OPA bis-DNPhydrazone.

Results and Discussion

Characterization of hydrazone derivatives of OPA with DNPH by LC-MS/MS

The HPLC-UV chromatogram and the ion trap APCI mass spectra of the test solution, in which OPA standard solution was reacted with DNPH, are shown in Fig. 2. Analysis of the ion trap APCI mass spectra showed that m/z=313.1 and m/z=493.2 were the precursor ions of peaks (A) and (B), respectively. Both peaks have mass fragments of m/z=182.4, which originated from DNPH. Peaks (A) and (B) correspond to OPA mono-DNPhydrazone and OPA bis-DNPhydrazone, respectively. These findings are in agreement with those of a previous HPLC analysis of the eluate from a DNPH-silica cartridge after passage of OPA standard vapor[9], which found one peak each for OPA mono- and OPA bis-DNPhydrazone.

Derivatization of OPA with 2,4-dinitrophenylhydrazine in phosphoric acid solutions

In the presence of catalytic amounts of acid, carbonyl compounds, including carboxylic acids, aldehydes and ketones, react with DNPH to form hydrazones[9]. Although non-volatile phosphoric acid is the usual catalytic agent for these reactions, the optimal acid concentration for the reaction of a carbonyl compound with DNPH differs for each compound, and the optimal concentration for derivatization of OPA has not yet been reported. We therefore assessed the optimum concentration of phosphoric acid and reaction time to reach a plateau value for the ratio of OPA mono-DNPhydrazone to OPA bis-DNPhydrazone. The effect of phosphoric acid concentration on the derivatization reaction of OPA with DNPH is shown in Fig. 3. The peak area of mono derivative increased with increasing phosphoric acid concentration, reaching a maximum value at 0.0075%, but decreasing thereafter. In contrast, the peak area of the bis-derivative began to increase dramatically at about 0.0075% phosphoric acid and plateaued at a concentration higher than 0.075%. These results suggest that the amounts of mono- and bis-derivatives produced by the reaction of OPA with DNPH

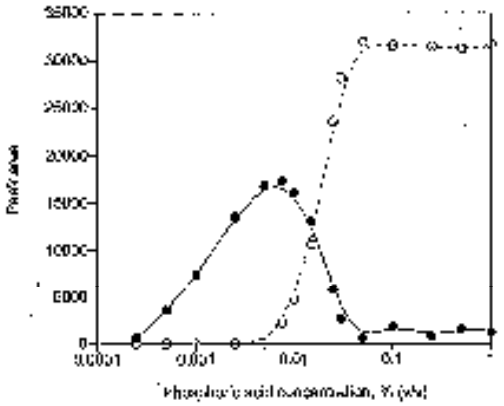

Fig. 3. Effect of phosphoric acid concentration on the formation of hydrazone derivatives. A 50-μl aliquot of phosphoric acid/acetonitrile solution, the phosphoric acid concentration of which ranged from 0.005 to 20% (v/v), was added to test tube containing 50 μl of OPA standard solution and 0.9 ml of DNPH solution. The final concentration of phosphoric acid in test tubes ranged from 0.00025 to 1.0% (v/v). OPA mono-DNPhydrazone (●), OPA bis-DNPhydrazone (○).

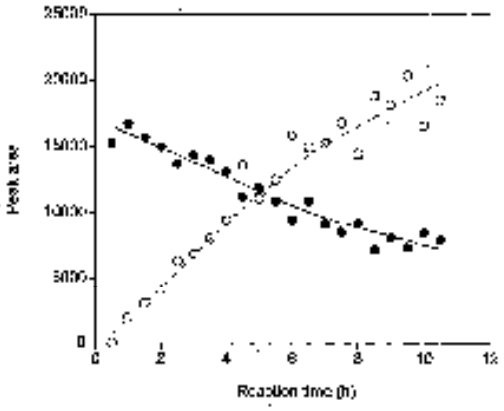

Fig. 4. Transformation of OPA mono-DNPhydrazone derivatives to OPA bis-DNPhydrazone derivatives in acetonitrile solution. The reaction time was ranged from 0 to 10.5 h. OPA mono-DNPhydrazone (●), OPA bis-DNPhydrazone (○).

depend on the concentration of phosphoric acid.

Figure 4 shows the time dependence of the transformation of OPA mono-DNPhydrazone to OPA bis-DNPhydrazone. Although no appreciable amount of the bis-derivative was detected immediately after the addition of 0.1% phosphoric acid, the amount of the bis-derivative increased over time, while the amount of the mono-derivative decreased.

These results suggest that the reaction of 1 mol of OPA with 1 mol of DNPH first yields the mono-derivative, later forming the bis-derivative by reacting with another 1 mol of DNPH and agree with findings using OPA standard vapor[8].

To minimize analytic errors, it is important to reach equilibrium in OPA bis-DNPhydrazone formation, and the optimum concentration of phosphoric acid we determined, is expected to reduce the time needed to reach equilibrium.

Generation of OPA test gas and collection with DNPH-silica cartridges

Based on the measurements of the phosphoric acid contents of various commercially available DNPH-silica cartridges, the optimal acid concentration and reaction time have been determined for the analysis of ketone-2,4-dinitrophenylhydrazones by HPLC[9]. The optimal concentration of phosphoric acid and the reaction time necessary for the determination of OPA are expected to be different from those for ketones. It is also unclear

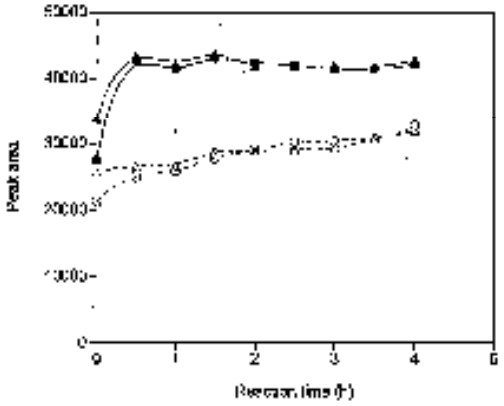

Fig. 5. Variations of peak areas of OPA bis-DNPhydrazone in eluates from DNPH-silica cartridges in the presence (●: LpDNPH S10, ■: GL Pak mini AERO DNPH) or absence (○: LpDNPH S10, □: GL-Pak mini AERO DNPH) of additional phosphoric acid. The DNPH-silica cartridges used in this study were LpDNPH S10 and GL-Pak mini AERO DNPH, respectively.

whether the formation of OPA bis-DNPhydrazone is complete in the presence of phosphoric acid in DNPH-silica cartridges.

We therefore assessed the formation of OPA bis-DNPhydrazone using two kinds of commercially available DNPH-silica cartridges, after sampling of OPA

test gas. Figure 5 shows the HPLC analyses of OPA bis-DNPhydrazone in the eluates of DNPH silica cartridge. The formation of OPA bis-DNPhydrazone varied according to the presence or absence of phosphoric acid in the eluates and with the time of reaction. When phosphoric acid was added to the eluate, the peak areas of OPA bis-DNPhydrazone reached a plateau after 30 min for both DNPH silica cartridges. In the absence of added phosphoric acid, however, the peak areas gradually increased over time, up to at least 4 h after elution. Relative to the peak area of OPA bis-DNPhydrazone in the presence of phosphoric acid at 4 h, the peak area in the absence of phosphoric acid at 4 h was approximately 80%. These results indicate that the derivatization reaction is not completed in the cartridge and continues in the effluent. The presence of phosphoric acid markedly accelerates the reaction rate of OPA mono-DNPhydrazone to OPA bis-DNPhydrazone. While a previous study[11] reported that the transformation reaction of the mono- to the bis-derivative occurred rapidly in acetonitrile and was completed in 4 h, we found that this transformation reaction was not completed within 4 h. This discrepancy may be due to differences in the phosphoric acid concentration in the cartridges. We used commercially available DNPH silica cartridges, whereas the previous study used hand-made cartridges, with different concentrations of phosphoric acid.

Conclusion

We have determined the optimal analytical conditions for the measurement of OPA in air of work environments using commercially available DNPH silica cartridges. OPA mono- and OPA bis-DNPhydrazone of OPA-DNPH were found in the effluent from these DNPH-silica cartridges, to which the test atmosphere had been introduced. The HPLC analysis of OPA shows that measurements of OPA bis-DNPhydrazone are suitable for obtaining reliable data for OPA in air. We also demonstrated that the degree of transformation of OPA mono-DNPhydrazone to OPA bis-DNPhydrazone depends on the reaction time and the concentration of phosphoric acid in the sample solution. To obtain complete formation of OPA bis-DNPhydrazone, (1) a 25-μl aliquot of phosphoric acid (2%, v/v) should be added to 475 μl of effluent from DNPH-silica cartridges and (2) the solution should be allowed to stand for more than 30 min at room temperature in order to reach equilibrium in the reaction between OPA and DNPH.

The present method for the determination of OPA is based on practically complete formation of OPA bis-DNPhydrazone and is expected to minimize analytical errors.

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3-8)　迅速で正確な低濃度尿中カドミウム(Cd)測定法の開発

第 1 期においては、固相抽出法と ICP-MS の組み合わせによる低濃度尿中 Cd 測定法を開発した。第 2 期においては、迅速で正確な測定法として、コリジョンセルを用いた ICP-MS 法を開発し報告した(Biomed Res Trace Elements, 24:1-6, 2013)。

Original Article

Development of a Simple and Precise Method to Determine Low Urinary Cadmium Levels by Using Inductively Coupled Plasma-Mass Spectrometry

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Abstract

Cadmium (Cd), a ubiquitous environmental pollutant, is a carcinogenic substance. Cd in the urine reflects an individual's cumulative exposure and the Cd concentration in the kidney. Inductively coupled plasma-mass spectrometry (ICP-MS) can detect Cd levels at the nanogram per liter level; however, mass spectral interference by molybdenum oxide (MoO) has been observed in biological samples. We developed an analytical method that did not include solid phase extraction ICP-MS (SPE-ICP-MS); rather, it used ICP-MS and octapole collision cell (OCC-ICP-MS) technology using helium (He) gas. The measurement mass numbers were ^{111}Cd and ^{98}Mo, and we used ^{103}Rh as an internal standard. Method accuracy was assessed using reference urine (SRM 2670a; National Institute of Standards and Technology, Gaithersburg, MD, USA). The method detection and quantification limits of Cd in the urine were 0.0097 and 0.038 μg/L, respectively. Inter-day accuracy and precision were 100.7–102.1% and 0.9–4.9%, respectively. The analytical values of Cd and Mo in SRM 2670a reference urines obtained by the proposed method were within the allowable errors for the certified values. Significantly correlated Cd concentrations of urine samples from healthy 20 women was found between the present OCC-ICP-MS method and the SPE-ICP-MS method ($r = 0.866$). ICP-MS analysis using He as a collision gas is useful for determining low urinary Cd levels without requiring pretreatment.

Keywords: cadmium, urine, ICP-MS, molybdenum oxide, spectral interference

Introduction

Cadmium (Cd) and its compounds have been widely dispersed throughout the environment via a range of human activities including mining, industrial emissions, and the use of Cd-containing fertilizers [1]. Chronic exposure to Cd can result in kidney disease, lung disease, and bone damage [1]. Based on animal experiments and epidemiological studies of highly exposed populations, the International Agency for Research on Cancer has classified Cd as a Group 1 human carcinogen [2]. The major route of Cd exposure in the general population is via food, although smoking is also an important source. Accurate Cd measurements in both dietary sources and biological fluids are needed to assess exposure and toxicological effects [3].

Urinary Cd is used as an indicator of cumulative exposure and the Cd concentration within the kidney [2]. Urinary Cd is conventionally measured by graphite furnace atomic absorption spectroscopy (GF-AAS), the

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detection limit of which is reported to be 0.5 μg/L [4]. Inductively coupled plasma-mass spectrometry (ICP-MS) can detect Cd at the level of nanogram per liter [5]; however, mass spectral interferences (SI) by molybdenum oxide (MoO) generated in hot plasma has been observed in both sediment analysis [5] and biological samples [6, 7].

Molybdenum (Mo), an essential elemental nutrient, is incorporated into the human body primarily through the diet with an average dietary daily intake of approximately 100 μg/day [8]. Urinary Mo levels have been reported to be 10–120 μg/L in Europeans [9] and 10–200 μg/L in the Japanese [10]; a geometric mean (GM) of 39.6 μg/L (95% confidence interval (CI): 37.5–41.8 μg/L) was reported in US adults (n = 3019) [11]. In contrast, the GM of urinary Cd in the general population was reported to be 0.99 ± 2.34 μg/L in Japanese women (n = 9720) [12] and 0.229 μg/L (95% CI: 0.213–0.245 μg/L) in US adults (n = 2019) [11]. Thus, the urinary Mo concentration in the general population is 10–100 times higher than the urinary Cd concentration.

ICP-MS suffers from several types of spectral and non-spectral interferences originating from plasma, water, and reagents used as well as the matrix of biological samples. For this reason, numerous elements cannot be determined in human fluids with instruments equipped with a quadrupole mass filter (ICP-QMS) without extensive sample pretreatment. Sector-field ICP-MS (SF-ICP-MS) has high-resolution capabilities that overcome most SIs [13]. However, the interference species ^{96}Sn, $^{96}Mo^{16}O$ and $^{96}Mo^{16}O$ could not be separated from ^{96}Cd and ^{97}Cd even in the high-resolution mode [14–16]. Isotope dilution ICP-MS (ID-ICP-MS) is considered a primary method of quantification since it is not affected by matrix effects (at least to a first approximation) and allows for relatively straightforward budgeting of measuring uncertainties; thus, it possesses better metrological properties than external calibration or standard addition [13]. However, at low Cd concentrations, SI and sample loading of the plasma can play a role in limiting ICP-MS Cd measurement accuracy, which makes matrix separation using chromatographic separation necessary [5]. To determine low levels of Cd in urine samples, we removed the interference from the coexisting Mo using solid-phase extraction (SPE) using a functional chelating resin [17].

Collision or reaction cell technology for ICP-QMS is an alternative solution for achieving high resolution to overcome SIs. It provides a way to improve isotope abundance ratio measurements via collisional damping of ion-beam fluctuations (flicker noise) as well as signal-to-noise ratio enhancement using collisional focusing. dynamic reaction cell (DRC) technology or octopole collision cell (OCC) technology, an innovation in ICP-MS that has been successfully used to eliminate isobaric interferences [13]. Although Chang et al successfully measured Cd in rice flour reference material (NIST SRM 1568a) with the presence of excess Mo using the DRC-ICP-MS method [18], we failed to determine low Cd levels in urine reference material (NIST SRM 2670a) due to low sensitivity and standard variance [17]. In this paper, we examined the efficacy of the OCC technology (OCC-ICP-MS) in urinary Cd measurements and compared the urinary Cd concentrations of healthy women obtained using OCC-ICP-MS with those obtained using SPE before conducting ICP-MS measurements.

Materials and methods

Reagents and chemicals

A single-element standard Cd solution for AAS (1011.0 mg/L; Wako Pure Chemicals, Osaka, Japan) was diluted with 0.15 M nitric acid (HNO_3) prepared from TAMAPURE AA-100 nitric acid (68%; Tama Chemicals, Kawasaki, Japan) and used for calibration and as a standard addition solution for Cd measurement. The rhodium (Rh) standard solution for AAS (1000 mg/L) used as an internal standard and Mo standard solution for AAS (1000 mg/L) were purchased from Wako Pure Chemicals (Osaka, Japan) and diluted with 0.15 M HNO_3. For the sample dilution, TAMAPURE AA-100 ammonium solution (20%; Tama Chemicals, Kawasaki, Japan), Triton X-100 (scintillation grade; ACROS ORGANICS, Antwerp, Belgium), and EDTA (ethylene diaminetetraacetic acid; Titriplex2, Merck, Darmstadt, Germany) were used. The ultra-pure water used for the trace element analysis was prepared using an Ultrapure Water Purification System (Milli-Q-ICP-MS; Millipore, Tokyo, Japan).

Urine samples

The urine samples used in the experiment were obtained from 20 healthy women of Tokyo who were 26–43 years old (average, 31.7 ± 4.6 years). Urine voided in the morning was placed in a sealed plastic tube and stored at −80°C in a freezer until the analysis. This study was approved by the institutional review board of the National Cancer Center.

SPE of urine samples

The SPE was performed using a functional chelating resin column (NOBIAS Chelate PA-1; Hitachi High Technology, Tokyo, Japan). The details were described in our previous report [17].

ICP-MS measurements

After being diluted 1/50 with a mixture of 0.5% NH_4, 0.5g/L of Triton X-100, and 0.5g/L of EDTA, the urine sample was introduced into the ICP-MS system. The National Institute of Standard & Technology (NIST) SRM2670a low urine sample was prepared as a 1/5 dilution of the mixture.

ICP-MS measurements were performed using an Agilent 7500ce ICP-MS with OCC technology (Agilent, Santa Clara, CA, USA). The instrumental conditions of the Agilent 7500ce ICP-MS were as follows: forward power, 1550 W; sample depth, 8.0 mm; carrier gas flow rate, 0.9 L/min; make-up gas flow rate, 0.23 L/min; sample flow rate, 0.4 mL/min; helium (He > 99.999%) as collision gas; He flow rate, 4.5 mL/min; kinetic energy discrimination (KED), 3 V; skimmer and sample cones were nickel; elemental Cd and Mo were measured at *m/z* values of 111 and 98, respectively; and these signals were corrected against an internal, standardized Rh signal at *m/z* = 103.

Validation

The limit of detection (LOD) of the Cd standard solution and the method limit of quantification (MLOQ) of Cd in the urine were calculated in accordance with the Japanese Industrial Standards (JIS) definition [19]. LOD and MLOQ were calculated based on 3 and 4.1 times the standard deviation (SD) of the results of 10 repeated runs using a blank solution, respectively. Method accuracy was assessed using the SRM 2670a (Toxic Elements in Urine) reference material from NIST (Gaithersburg, MD, USA). The reproducibility of the developed method defined as precision was evaluated by analyzing the reference urine samples containing 2 concentrations of Cd (0.0591 and 4.862 µg/L) over 3 consecutive days (every 5 replicates; inter-day reproducibility). (Table 1)

Results and Discussion

The most commonly used isotopes in ICP-MS analysis are ^{114}Cd and ^{111}Cd. ^{114}Cd has the highest abundance but has an isobaric overlap with ^{114}Sn. Both ^{111}Cd and ^{114}Cd have a spectral interference from $^{95}Mo^{16}O$ and $^{98}Mo^{16}O$, respectively. For the separation of Cd from

Table 1. Inter-day reproducibility of the proposed method obtained from 3-day repeated

Day	Cadmium concentration (µg/L)	
	Low (0.0591)	High (4.862)
1	0.0584 ± 0.0037*	4.9411 ± 0.0711*
2	0.0628 ± 0.0020*	5.039 ± 0.0554*
3	0.0559 ± 0.0053*	4.671 ± 0.0754*
Mean	0.0595	4.880
SD	0.0029	0.045
RSD** (%)	4.9	0.9
Accuracy (%)	100.7	102.4

*: The values are expressed as mean ± SD (n = 5)
**: Relative standard deviation

MoO interference, a resolution of at least more than 8000 is required, thus making it impossible to separate the two with SF-ICP-MS even in a resolution mode (*m/Δm* resp. 8000) [15,16]. The analysis of urinary Cd due to a high-resolution SF-ICP-MS (*m/Δm* > 8000) is expensive and thus is not practical technique. Accordingly, mathematical correction to the metal in urine of the low-resolution (*m/Δm* = 300) measurement in the determination of ^{114}Cd was generally used [14].

Our previous study [17] has clarified that Mo was a major source of interference in the determination of low urinary Cd levels and that the DRC method could not reduce the interference. The OCC technology can reduce polyatomic species interference but not isobaric interference [20]. As shown in Table 2, the most abundant Cd isotope was ^{114}Cd and the SIs from ^{114}Sn and $^{98}Mo^{16}O$ were as expected. Since the only isotope of Cd without isobaric interference is ^{111}Cd, we monitored ^{111}Cd isotope in this study.

Table 2. Spectral interferences for cadmium (Cd) analysis using inductively coupled plasma mass spectrometry

Mass number of Cd	Isotopic abundance (%)	Interfering ions
108	0.89	$^{108}Pd,^{92}MoO$
110	12.49	$^{110}Pd,^{94}MoO$
111	12.8	^{95}MoO
112	24.13	$^{112}Sn,^{96}MoO$
113	12.22	$^{113}In,^{97}MoO$
114	28.73	$^{114}Sn,^{98}MoO$
116	7.49	$^{116}Sn,^{100}MoO$

The LOD of Cd was 0.0002 μg/L. The LOD and MLOQ of Cd in the urine were 0.0097 and 0.038 μg/L, respectively. The LOD for the SF-ICP-MS determination of Cd in the urine was reported to be 0.007 μg/l [14]. The sensitivity of our method in the present study is almost equal to that of SF-ICP-MS described by Bocca et al. [14], and its sensitivity is sufficient to measure urinary Cd levels in the general population. Furthermore, these values obtained by this method are 10 times more sensitive than those by GF-AAS.

With regard to the treatment of human fluids, simple specimen dilution may be preferred to reduce manipulation. Chemical, physical, and SIs are reduced by sample dilution, but this approach has the trade-off of sensitivity loss. Therefore, the standard addition method is often employed to determine the amount of a target element in a matched matrix. When the NIST SRM 2670a standard urine samples were prepared as a 1/5 dilution using pure water and then analyzed by the standard addition method using ICP-MS, the Cd concentrations were higher and beyond the range of the certified values [17].

It has been reported that numerous elements cannot be determined in human fluids by using instruments equipped with ICP-QMS without extensive sample pretreatment, because ICP-MS suffers from several types of spectral and non-spectral interferences originating from the biological sample matrix [14]. In fact, we had to use the SPE technique for urinary Cd analysis pretreatment in an previous report because we failed to obtain an accurate analysis using a DRC system with O_2 as a reaction gas [17]. As such, we tried to use the OCC system because the collision mode using He gas removes polyatomic interferences by using a process known as KED [21]. An energy differential was applied to prevent the lower energy polyatomic ions from entering the mass filter [21]. The Cd and Mo concentrations determined by the OCC operating in He collision mode were within the allowable error for certified values (Table 3).

Table 3. Analysis of cadmium and molybdenum in NIST reference urine SRM 2670a (Toxic Elements in Urine) by octopole collision cell-inductively coupled plasma-mass spectrometry (OCC-ICP-MS), and solid phase extraction-inductively coupled plasma-mass spectrometry (SPE-ICP-MS) methods (μg/L)

Methods	Cadmium		Molybdenum	
	Low level urine	High level urine	Low level urine	High level urine
OCC-ICP-MS (He)	0.0584 ± 0.0057	4.940 ± 0.07	46.9 ± 0.1	133.6 ± 0.5
SPE-ICP-MS*	0.056 ± 0.003	4.897 ± 0.368	-	-
Certified value	0.0591 ± 0.0034	4.862 ± 0.264	47**	114.1 ± 4.3

The values are expressed as mean ± SD (n = 5)
*: The data are described in our previous report (ref. 17)
**: informative value

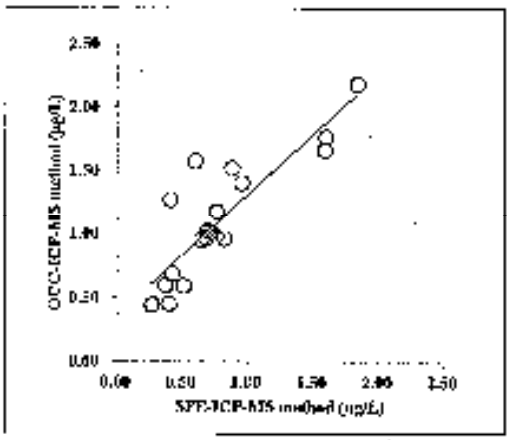

Fig. 1 The relationship between Cd concentrations in 20 urine samples analyzed using the octopole collision cell-inductively coupled plasma-mass spectrometry (OCC-ICP-MS) and solid phase extraction-inductively coupled plasma-mass spectrometry (SPE-ICP-MS) methods (Y = 0.95x + 0.36) (n = 20, r = 0.866).

The relationship between the Cd concentrations of the 30 urine samples analyzed using OCC He collision method and those analyzed using the SPE method is described in Figure 1. The measurements of the 20 general urine samples by both methods were related using the equation $Y = 0.95x + 0.16$ ($r = 0.866$) and showed good agreement. The OCC He collision method can analyze low levels of urinary Cd without requiring pretreatment by using a specific chelating resin. Therefore, ICP-MS analysis using the OCC He collision method is simple and adequate for an assay of trace urinary Cd levels.

Acknowledgements

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3-9) 急性アルシン中毒症例

原因不明の溶血症患者が急性アルシン中毒である事を血液中形態別ヒ素分析により証明したので報告した(J Occup Health 53: 45-49, 2011)。

J Occup Health 2011; 53: 45–49

Case Study

Acute Arsine Poisoning Confirmed by Speciation Analysis of Arsenic Compounds in the Plasma and Urine by HPLC-ICP-MS

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Abstract: Acute Arsine Poisoning Confirmed by Speciation Analysis of Arsenic Compounds in the Plasma and Urine by HPLC-ICP-MS: Yukihiro Yoshikawa, *et al.* Department of Infectious Disease, Yokohama Municipal Citizen's Hospital—Objectives: Arsine, a potent hemolytic agent, is widely used in the semiconductor industry. We report a case of arsine poisoning confirmed by arsenic speciation analysis in serum and urine that occurred in a recycling factory. **Case:** A male worker in his twenties noticed hematuria 3 h after finishing work and was admitted into our hospital 24 h later. Speciation analysis of arsenics in serum and urine samples was performed using HPLC-ICP-MS. On admission, anemia, hematuria, and renal and liver dysfunction were observed. His clinical condition had improved remarkably after 5 days of transfusion and 4 units of RBC transfusion. The total arsenic content in the serum was 244.8 μg/l at admission and 97.1 μg/l at discharge. In the speciation analysis, four kinds of As compounds derived from arsine metabolism were detected in serum and urine. The concentrations of arsenite (AsIII), arsenate (AsV), monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) in serum at admission were 45.8, 5.2, 17.9 and 9.3 μg/l, respectively. The concentrations of AsIII, AsV and MMA decreased with biological half-lives (BHL) of 30.1, 43.0, and 96.3 h, respectively, while that of DMA was increased towards discharge. The urinary AsIII, AsV, MMA, and DMA concentrations at discharge were 384.5, 20.8, 547.4 and 1816.3 μg/g creatinine, respectively. These concentrations in urine subsequently decreased and their BHL was 15 days. **Conclusion:** The results of the present study suggest that arsine is quickly metabolized to AsIII and then metabolized via MMA to DMA in humans.
(J Occup Health 2011; 53: 45–49)

Key words: Arsenic compounds, Arsine, Gallium, Plasma, Speciation analysis, Urine

Arsine is a colorless gas and one of the most potent hemolytic agents used in industry. Arsine is extensively used for epitaxial growth of gallium arsenide (GaAs) and as a dopant for silicon based electronic devices in the semiconductor industry[1].

Scrap recycling accounts for 40% of the total production of high-purity gallium in Japan[2]. Arsine generation may occur during the recycling process of various grades of GaAs scraps. Here, we report a case of arsine poisoning that occurred in a recycling factory.

Case

A man in his twenties visited our hospital emergency room because of hematuria and vomiting; he was immediately admitted.

On admission, his temperature was 37.5°C, bulbar conjunctiva was icteric, palpebral conjunctiva was not anemic, and pharynx was reddish in color. His breath sounds were normal, and there were no crackles or wheezes. Other prominent physical findings were normal. Anemia, renal and liver dysfunction, and hematuria were confirmed on the basis of laboratory analysis of blood and urine samples (Table 1). Blood urea nitrogen (BUN) level was 39.8 mg/dl. The levels of inflammatory markers, such as white blood cells (14,300/μl), creatine kinase (406 U/l), and C-reactive protein (7.0 mg/dl) were high. The patient provided informed consent for publishing a case report.

Since the patient could not provide any information about arsine exposure, initially, we suspected that his hemolytic anemia had been caused by a mycoplasma or viral infection and therefore started supportive treatment such as intravenous (IV) therapy. His clinical course and laboratory findings are described in Table 1. His hemoglobin value decreased to 5.9 g/dl on day 3; therefore, blood transfusion was performed (2 units of packed red blood cells daily for 2 days). His general condition, anemia, and other laboratory data improved, and he was discharged on day 7. When he

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visited our clinic after discharge (day 18), his physical condition was good, and his laboratory data were within the normal ranges, except for slightly elevated serum creatinine (CRE) level; therefore, we allowed him to resume work. When he visited our clinic for examinations on days 68 and 110, his anemia, hematuria, renal dysfunction, and liver injury had completely disappeared, and no exacerbation was observed.

Work environment and working condition

The patient started working in a factory, which extracts of Ga from GaAs scraps, 3 mo before the accident. At the factory, waxed residue of GaAs-epitaxial substrate containing Ga-As solution is mixed with water and crushed in a ball-mill for 12 h. Degassing is necessary to release the excess pressure that develops during crushing.

The patient worked on a crusher for 1–2 h a day, 2–3 days per week. He wore protective gloves and a gas respirator to prevent inhalation of acid gases. He worked with another worker but the worker did not complain of any symptoms.

He first noticed hematuria 3 h after completing a 2-hour work shift and was admitted to our hospital 34 h later. After discharge, he was transferred to a workshop with no arsine exposure.

In order to gather more information in regard to his working situation, industrial hygienists at the factory reproduced his accidental situation and exposure to arsine gas. It was considered that arsine gas was produced by As reduction and Ga oxidization from the mixture of Ga-As solution and water, because the ionization tendency of As is lower than that of Ga. We confirmed that he was accidentally exposed to arsine gas during the degassing process. Therefore, the gas-respirators of the workers involved in the degassing process were replaced with air-line respirators.

Determination of arsenic compounds in serum and urine

Total arsenic (T-As) and Ga concentrations in serum were determined using an Agilent 7500ce ICP-MS fitted with an octopole-based collision/reaction cell (Agilent Technologies, Santa Clara, USA). The limits of detection (LOD) for As and Ga were 0.005 and 0.007 $\mu g/l$, respectively.

We observed a significant decrease in the T-As concentration with time and estimated its biological half-life (BHL) to be 59.2 h. The concentrations of Ga in serum measured at 34 and 153 h were <0.14 and 0.59 $\mu g/l$, respectively.

Arsenic speciation analysis was performed according to our previously reported method[3] after deproteinization with Microcon YM-10 (Millipore, MA, USA). The recovery rates for As species from the filtration ranged from 93 to 106%.

Arsenic speciation analysis of the serum and urine samples revealed the presence of 4 kinds of As compounds derived from arsenic metabolism and arsenobetaine (AsBe). The variations in serum concentrations of As species during hospitalization are shown in Table 1. We found that arsenite (AsIII) had the highest concentration at 34 h. It had a short BHL of 30.1 h. All 5 arsenate (AsV) concentrations were much lower than those of AsIII, and their BHL was slightly longer at 43.0 h. The concentration of methylarsonic acid (MMA) showed a slight increase, but it decreased later; its BHL was estimated to be 96.3 h. The concentration of dimethylarsinic acid (DMA) increased with time during the 6-day hospitalization; hence, its BHL could not be calculated. The patient's AsBe concentration was almost constant throughout his hospitalization.

The variations in urinary arsenic concentrations are described in Table 1. The sum of AsIII, AsV, MMA, and DMA concentrations sampled 6 days after the accident was 29-fold higher than that of the 95 percentile reference value of 96.7 $\mu g/g$ CRE obtained in our previous study[2]. The urinary concentrations of all the As compounds, except AsBe, subsequently decreased; and their BHL was 15 days. The urinary Ga concentrations at 6, 68, and 110 days after the accident were <0.34, 3.79, and 0.57 $\mu g/g$ CRE, respectively.

Discussion

Speciation analysis revealed significant amounts of arsenic compounds in the patient's serum and urine samples; therefore, we diagnosed the patient with acute arsine poisoning. An experiment conducted at the factory also confirmed the generation of arsine.

Symptoms of arsine poisoning appear 2–24 h after exposure, depending on the concentration and time of exposure[4]. Our patient showed symptoms of poisoning 3 h after exposed to arsine for a duration of 2 h. His serum T-As concentration was 244.8 $\mu g/l$. He also developed anemia, renal and liver dysfunction, and hematuria. He did not develop acute oliguric renal failure, and therefore, exchange transfusion was not required. He was adequately treated by early IV fluid therapy and RBC transfusion for severe anemia.

This is the first report of speciation analysis of arsenic in serum from a patient with acute arsine poisoning. In the speciation analysis of the patient's serum, we detected 4 kinds of As compounds derived from arsine metabolism and AsBe. Although we used 2 different columns, we did not detect other forms of As compounds in the serum. Yoshino *et al.*[5] detected arsenite (AsIII), arsenate, MMA and DMA, in the plasma of a patient who received arsenic trioxide treatment, and reported that T-As in blood cells was 4–10 times higher than those in plasma. The proportion of the sum of the concentrations of the four As species,

Table 1. Time course changes of laboratory values and As concentrations in serum and urine and clinical course of the patient with acute arsine poisoning

Day (h*)		2 (34)**	3 (64)	4	5 (112)	6	7***	18	68	110
Laboratory data	Reference value									
Hb (g/dl)	13.5–17.6	9.1	5.9	6.6	7.7	ND	7.4	13	16.6	16.6
T-Bil (mg/dl)	0.2–0.9	8.1	3.5	ND	2.0	ND	1.0	0.4	0.4	0.7
γ-GTP (IU/l)	0–60	81	52	ND	35	ND	35	25	17	17
AST (IU/l)	8–38	580	139	ND	29	ND	28	21	18	20
ALT (IU/l)	4–44	135	60	ND	38	ND	41	35	21	23
LDH (IU/l)	106–211	7,405	2,413	ND	1,156	ND	856	200	154	186
Creatinine (mg/dl)	0.52–1.15	1.33	1.23	ND	1.31	ND	1.30	1.21	1.07	1.06
RBC in urine (/HPF)	≤5	>100	ND	ND	10–19	ND	5–9	1–4	1–4	1–4
Occult blood in urine	–	4+	ND	ND	3+	ND	1+	–	–	
Urine output (l)		ND	1.8	1.7	5.2	3.9	ND	ND	ND	ND
As concentration										
		serum As (μg/l)				urinary As (μg/g CRE)				
AsV		5.2	3.3		1.5	20.8			0.5	0.7
AsIII		15.8	29.5		7.9	382.5			2.5	3.2
MMA		17.0	18.9		10.6	547.4			8.2	4.2
DMA		9.2	21.3		29.1	1,816.3			38.5	26.2
AsBe		3.4	2.9		2.8	1.8			6.1	3.0
Total As excluding AsBe		241.4	154.1		94.3	2,769.3			53.7	28.6
Clinical course										
Intravenous feeding (l)		2	3.3	2.5	2.5	1.5				
RBC transfusion (unit)			5	3						

*: Hours after cessation of arsine exposure, **: Admission, ***: Discharge, ND: Not Determined.

expressed as extracted As (E-As), to T-As were constant in their patient, while, it increased with time from 37.3% at 34 h to 53.5% at 112 h in our patient. When a human blood sample was exposed to arsine vapor, partial hemolysis was observed, and As adducts were detected in the plasma[6]. Since our patient had hemolytic anemia, T-As in his serum sampled at 34 h after the accidental exposure may have contained As-adducts bound to hemoglobin. Thus, the proportion of E-As to T-As in our arsine poisoning patient was remarkably different from that in the patient exposed to AsIII. This may be a characteristic of the metabolic profile of arsine poisoning.

Our results indicated that inhaled arsine is rapidly converted to AsIII and then metabolized via MMA to DMA. Apostoli *et al.*[7] reported that the BHL of T-As in the blood of a patient with acute arsine poisoning followed a triphasic model, with periods of 28 h, 59 h, and 9 days. Our estimated BHL of T-As in serum (59.2 h) is in good agreement with their second-phase BHL of T-As in blood. Among the As compounds, DMA alone had serum concentrations that showed a progressive increase up to 112 h. The possible reason for this finding is as follows: arsine itself[7], and/or excessive AsIII[8], and its metabolite may suppress the second methylation step from MMA to DMA that is mainly mediated by hepatic enzymes[8]. The presence of liver dysfunction in the patient also supports this possibility. In contrast, the BHLs of urinary As species were calculated to be 15–20 days. These BHLs are similar to the third-phase BHL of serum T-As reported by Apostoli *et al.*[7].

Ga concentrations in serum and urine were within the reference range[11, 12]. Since prominent acute respiratory symptoms were absent, we think that the patient did not inhaled a significant amount of GaAs.

Ingestion of seafood including seaweeds increases urinary T-As and DMA concentrations. The patient's serum and urine AsBe concentrations were low, and hence, seafood ingestion was not considered to have had any effect on his T-As and DMA concentrations. On the basis of the hypothesis the patient's urinary As level decreased with the serum T-As BHL of 59.2 h before and during hospitalization and then with the urinary T-As BHL of 15 days after hospitalization, we estimated that the patient had excreted approximately 78 mg of As. A previous study had estimated the pharmacokinetic parameters by determining the blood level of inorganic As (iAs) in patients administered arsenic trioxide[13]; on the basis of this study, we found that our patient (body weight, 58.4 kg) had absorbed approximately 63 mg of As. Both the estimates yielded almost identical values. This finding indicates that the patient could have been exposed to about 23–39 mg/m^3 (8–12 ppm) of arsine. The level of exposure is about 100 times higher than the Occupational Exposure Limit-Ceiling of 0.1 ppm recommended by The Japan Society for Occupational Health[9] and is high enough to cause arsine poisoning, because a previous study have reported the appearance of symptoms of poisoning after a few hours of exposure at 1–5 ppm[1].

In conclusion, accidental arsine poisoning occurred in a GaAs recycling factory. Although arsine toxicity is very different from iAs toxicity, the characteristics of the metabolites resulting from arsine exposure were considered to be similar to those of the metabolites resulting from iAs exposure. The effects of iAs toxicity should be considered in chronic arsine exposure. Therefore, in order to assess the adverse effects of arsine exposure, toxicity due to iAs exposure appearing in other than the hematopoietic system should be fully considered and their preventive measures should be adopted not only for acute toxicity but also for chronic toxicity.

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3-10）アルシンの経皮吸収性

前述の事例ではアルシンの経皮吸収が疑われた事から、ヘアレスマウスを用いて経皮吸収性を検討した結果、経皮吸収されなかった事を国際学会にて報告した(ISEE2013, 2013)。現在、論文を投稿中である(J Toxicol Sci)。

4）情報発信

第 1 期においては産業中毒センターのホームページに約 800 の化学物質について法的規制や毒性情報などの検索画面を作成し、管理濃度や許容濃度等の情報を掲載していたが、現在では約 3400 物質に関する情報を 2013 年度版まで更新して発信している。有害性情報については、国内では中央労働災害防止協会、(財）化学物質評価機構、独）製品評価技術基盤機構、および（財）日本中毒情報センター、海外とは米国 ACGIH およびドイツ DFG 等各団体とリンクを貼っており、様々な情報が得られるようになっており、最近 4 年間のアクセス数は 6.5 万件であった。

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※「化学物質の曝露による産業中毒」分野

テーマ：産業中毒の迅速かつ効率的な診断法に係る

研究・開発、普及