事例報告

メチレンジフェニルイソシアネート曝露による急性呼吸器症状および慢性化学物質過敏症状を呈した3例に関する検討

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イソシアネートは，ポリウレタン系の塗料，接着剤，合成皮革等に使用される低分子無機化合物であり，気管支喘息や過敏性肺炎などのアレルギー性呼吸器疾患の原因物質としてよく知られる(1, 2)．今回，著者らはメチレンジフェニルジイソシアネート（MDI）曝露による急性呼吸器症状の後，長期にわたり自律神経失調症状を訴え，化学物質過敏症の発症が疑われた3例を経験したので報告する．

【症例】平成14年6月3日，同一隧道工事現場で作業に従事する18名のうち，43歳（症例1），46歳（症例2），48歳（症例3）の男性3名が，目のチカチカ感・咽頭痛等の粘膜刺激症状，咳嗽・呼吸困難・胸痛等の呼吸器症状，および頭痛・項部重圧感・四肢しびれ感・嘔気・下痢等の自律神経失調症状を訴えた．同日，3名中2名（症例1と症例2）は近隣の医療施設を受診し，何らかの揮発性化学物質による急性呼吸不全と診断され，その10日後（6月13日）には，症例3と共に3名で当院を受診した．当院初診時は呼吸器症状や粘膜刺激症状は既に消失しており，理学的にも異常なく，SpO2は3例とも97～98%であった．既往歴は，症例2が中等度の糖尿病を有していたが，無治療であった．3例共，家族歴にアレルギー疾患はなかった．隧道工事に従事した期間は各々，5年間，20年間，7年間であった．

【原因物質】使用した地盤強化剤（ウレタンフォーム）の中で粘膜刺激作用を有する物質はMDIあるいはアンモニアと考えられたが，6月14日（当院初診日の翌日）に隧道工事現場において測定されたこれらの揮発物質の濃度はいずれも許容濃度以下であった（表1）．しかしながら，測定箇所によっては注入作業中のMDI濃度が比較的許容濃度に近い区域も存在し，実際の作業中には時に許容濃度を上回るMDI曝露が存在した可能性は否定できないものと考えられた．

表1　原因と考えられる物質の濃度測定（初診日の翌日）

測定個所	坑外	原液タンク上部	リーク フォーム直上	切羽直下	切羽から約 30m付近	許容濃度 (ACGIH)
MDI濃度	ND	0.002 ppm	ND	0.002 ppm	0.001 ppm	< 0.005 ppm
MDI濃度（注入中）	0 ppm	0.002 ppm	0.004 ppm	0.002 ppm	0.002 ppm	< 0.005 ppm
アンモニア	ND	< 0.2 ppm	< 0.2 ppm	< 0.2 ppm	< 0.2 ppm	< 25 ppm

【検査成績】症例1と症例2が近隣の医療施設において発症当日に撮影された胸部CTでは過敏性肺炎等の所見は認められなかったが，軽～中等度の低酸素血症がみられた（各々，55.6mmHgと75.9mmHg）．当院初診時の呼吸機能検査は異常なく，生化学検査でも，症例1の脂肪肝，症例2の糖尿病が確認されたのみであった（表2）．MDI－RASTは，症例1と症例2で陽性であった（表3）．発症後3～7週間目に施行された気管支肺胞洗浄液の分析では3例共に，隧道工事への従事期間に比例して細胞数の増加がみられたが，気管支喘息や過敏性肺炎を思わす好酸球やリンパ球の増多は認めなかった（表4）．しかしながら，アストグラフ法による気道過敏性は症例2と症例3で亢進していた（表4）．

表2　血液生化学

CASE 1	CASE 2	CASE 3
WBC: 5,700	WBC: 7,200	WBC: 7,500
Hb: 15.5	Hb: 16.8	Hb: 15.9
Eos : 6.2 %	Eos : 2.8 %	Eos : 2.9 %
TP: 7.0	TP: 7.2	TP: 7.7
GOT: 39	GOT: 12	GOT: 23
GPT: 124	GPT: 15	GPT: 25
LDH: 193	LDH: 122	LDH: 195
CHE: 436	CHE: 484	CHE: 623
Cr: 0.75	Cr: 0.74	Cr: 0.73
CK: 109	CK: 41	CK: 199
FBS: 88	FBS: 236	FBS: 94
Electrolyte:WNL	Electrolyte:WNL	Electrolyte:WNL
Chol: 283	Chol: 209	Chol: 209
fT3 fT4: WNL	fT3 fT4: WNL	fT3 fT4: WNL
TSH: WNL	TSH: 0.31	TSH: WNL
CRP: 0.15	CRP: 0.08	CRP: 0.19

表3　アレルギー・呼吸機能検査

CASE 1		CASE 2		CASE 3	
IgE:	42	IgE:	195	IgE:	351
RAST:		RAST:		RAST:	
Mite	0	Mite	0	Mite	0
Fungi	0	Fungi	0	Fungi	0
FA	0	FA	0	FA	0
Isoc TDI	0	Isoc TDI	2	Isoc TDI	0
Isoc MDI	2	Isoc MDI	3	Isoc MDI	0
Isoc HDI	0	Isoc HDI	2	Isoc HDI	0
IgG:	689	IgG:	723	IgG:	n.d.
IgA:	91	IgA:	393	IgA:	n.d.
IgM:	15	IgM:	71.7	IgM:	n.d.
CD4/8:	0.7	CD4/8:	3.2	CD4/8:	1.9
%VC	97.3 %	%VC	102.6 %	%VC	110.7 %
$FEV_{1.0\%}$	82.2 %	$FEV_{1.0\%}$	85.1 %	$FEV_{1.0\%}$	74.3 %
V25/H	0.97	V25/H	0.94	V25/H	0.46
SpO_2	98 %	SpO_2	98 %	SpO_2	97 %

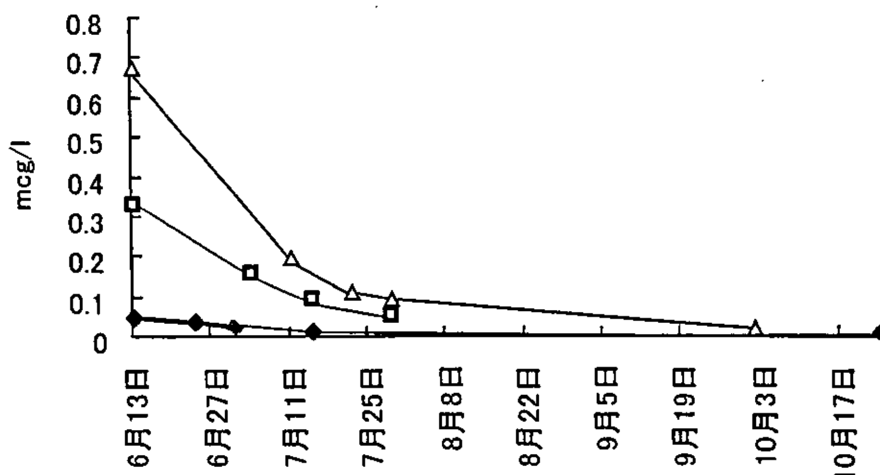
表4　気管支肺胞洗浄液細胞分類・気道過敏性検査結果

CASE 1 (6.24.02)	CASE 2 (7.8.02)	CASE 3 (7.22.02)
• Recovery 100/150	▪ Recovery 73/150	▪ Recovery 64.5/150
• Cell $1.33x10^5$/ml	▪ Cell $3.37x10^5$/ml	▪ Cell $6.53x10^5$/ml
• Mφ 76.5 %	▪ Mφ 89.0 %	▪ Mφ 83.0 %
• Lym 17.5 %	▪ Lym 11.0 %	▪ Lym 14.0 %
• Eos 5.0 %	▪ Nt 0 %	▪ Nt 2.0 %
• Nt 1.0 %	▪ Eos 0 %	▪ Eos 1.0 %
• CD4 57.6 %	▪ CD4 30.4 %	▪ CD4 37.4 %
• CD8 30.1 %	▪ CD8 51.9 %	▪ CD8 49.3 %
• CD4/CD8 1.9	▪ CD4/CD8 0.6	▪ CD4/CD8 0.8

気道過敏性試験（アストグラフ）

Dmin=7.556 U	Dmin=2.695 U	Dmin=0.234 U

表5　血中メチレンジアニリン（MDA）濃度の推移



【臨床経過】呼吸器症状や粘膜刺激症状は比較的早期に消失したが，頭痛，項部重圧感，四肢しびれ等の自律神経失調症状はその後も回復せず，様々な臭いにも過敏な状態となり，化学物質過敏症の発症が疑われた．入院の上，マルチビタミン，微量元素の補給や低出力レーザー等の対症療法を実施したが，効果はみられず，その後は自宅療養による経過観察のみとした．後日，測定されたMDIの代謝物であるメチレンジアニリン（MDA）の初診時における血清中濃度は，各々0.045，0.329，0.671 μg/lであり，曝露数日から1ヶ月または3ヶ月の間の測定で指数関数的に減衰した（表5）(3)．

【化学物質過敏症に関する検査】当施設および化学物質過敏症の専門施設で実施された電子瞳孔計，眼球電位図（滑動性追従眼球運動検査），視空間コントラスト感度等による自律神経機能検査で，3例とも何らかの異常を認めたが，トルエン負荷試験では，自覚症状，他覚所見（近赤外

表6　自律神経機能検査（負荷トルエン濃度は厚生労働省の室内VOC濃度の指針値である0.07ppm）

	症例1	症例2	症例3
電子瞳孔計	対光反応の不安定性	対光反応の不安定性	対光反応の不安定性
眼球電位図（滑動性追従眼球運動検査）	水平、垂直方向ともに階段状変化	水平、垂直方向ともに階段状変化	水平、垂直方向ともに階段状変化
視空間コントラスト感度	全領域で軽度低下	全領域で低下	異常なし
トルエン負荷試験（自覚症状）	変化なし	変化なし	変化なし
トルエン負荷試験（近赤外線脳酸素モニター）	変化なし	変化なし	変化なし

線脳酸素モニター）共に陰性であった（表6）.

【考察】3種類のイソシアネートすなわち，MDI, toluene diisocyanate（TDI），hexamethylene diisocyanate（HDI）のうち今回の曝露で問題となったのは，トンネル工事現場で使用される地盤強化剤に含まれるMDIである。MDIによる障害の報告としてはアレルギー性呼吸器疾患としての気管支喘息や過敏性肺炎が広く知られる（1, 2）. 今回の3例のうち2例でみられた発症直後の呼吸不全症状は理学的にも画像診断上もそれらのいずれにも合致していなかったが，イソシアネートの気道粘膜に対する直接刺激作用としての呼吸困難や胸痛であったとも考えられる．同一隧道工事現場で作業をしていたにもかかわらず，18名のうち3名のみにこれらの症状が出現したのは，MDIに対する反応性に個人差があったものと考えられるが，この個人差が症例1と症例2におけるMDI－RASTの陽性所見や症例2と症例3における気道過敏性の亢進所見に関連するか否かは，他の現場作業者で検査を実施していないため不明である．いずれにしろ，発症10日目の当施設受診時は3例とも目のチカチカ感，咽頭痛，咳嗽，呼吸困難，胸痛等は消失しており，MDIによる気道をはじめとする粘膜に対する障害は急性かつ一過性のものであった．

しかしながら，当初から存在した頭痛，項部重圧感，四肢しびれ等の自律神経失調症状は血中MDAの低下にもかかわらず，その後も回復せず，化学物質過敏症の発症が疑われた．化学物質過敏症は未だ国際的にも統一された診断基準が存在しない概念ではあるが，通常，揮発性化学物質への低濃度長期曝露あるいは高濃度短期曝露を契機として発症し，不定愁訴に近い慢性の多彩な症状が持続することが知られている．一度発症すると，原因と思われる化学物質を回避したとしても，健常人では問題とならないほどの極く低濃度でも過敏に反応するため，通常の環境においても症状が持続するとされる（4）．今回の3例においても，発症後13ヶ月目に実施した神経眼科的な各種検査の結果は，何らかの自律神経機能障害を示していた．これに対し，揮発性化学物質負荷試験の結果は典型的な陽性所見を示さなかったが，原因物質のMDIを負荷することが技術的に不可能であったため代替負荷物質としてトルエンを使用したことや，負荷試験前に実施すべき長期間の完全な化学物質からの回避（アンマスキング）が現実的には不可能なことを考えると，これらの結果から化学物質過敏症の存在を否定することはできないものと考えられる．

【結語】化学物質過敏症は未だ概念自体が確立しておらず，統一された診断基準が存在しないため，不定愁訴との鑑別が難しい．しかしながら，本症例のように特殊な環境で揮発性化学物質の明らかな曝露があった場合は，中毒やアレルギー以外

にも考慮に入れるべき病態である．本症例でイソシアネートは地盤強化剤という特殊な物質に含まれていたが，本来は，塗料，接着剤，合成皮革等，一般の日常生活でも遭遇する物質であり，今後はシックハウス症候群やシックビルディング症候群の原因物質としても注目すべきである．また，このような様々な化学物質の血中濃度測定システムの開発やこの領域に関する幅広い臨床医と研究者の育成が待たれるところである．

【文献】

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Original Article

Improvement in the GC–MS method for determining urinary toluene-diamine and its application to the biological monitoring of workers exposed to toluene-diisocyanate

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Abstract *Objectives:* To develop a simple and sensitive GC–MS method for determining toluene-diamine (TDA) in urine and to apply the method for biological monitoring of workers exposed to toluene-diisocyanate (TDI). *Methods:* After acid hydrolysis of 0.1 ml of urine, diluted tenfold with water, for 1.5 h, the free TDA formed was extracted with dichloromethane, and the heptafluorobutyric anhydride derivative was determined by GC–MS. Using an 80:20 mixture of 2,4-TDA and 2,6- TDI we applied the method to the biological monitoring of 18 workers. *Results:* 2,6-TDA and 2,4-TDA were simply determined in 7 min by GC–MS. TDA levels in post-shift urine were well correlated with personal exposure levels of TDI. The correlation was improved by correction with creatinine or specific gravity in the 2,6-isomer, but not in the 2,4-isomer because of low exposure levels. From the correlation equation, the 2,6-TDA level (corrected with creatinine), corresponding to the TDI level of 5 ppb, was calculated to be 31.6 μg/l. TDAs in pre-shift urine also correlated significantly with the personal exposure levels of TDIs, although the slope of the correlations for pre-shift samples was 60%–70% of those for post-shift samples. The correlation between 2,4-TDA and 2,6-TDA levels was significant, although the levels of the 2,4-isomer were less than one-tenth of the 2,6-isomers in both air (personal exposure) and urine. *Conclusion:* The present method is simple and practicable and can be useful for biological monitoring of TDI workers.

Keywords Toluene-diisocyanate - Toluene-diamine - Biological monitoring - Personal exposure - Urine

Introduction

Toluene-diisocyanate (TDI) is an important industrial chemical used in the production of polyurethane foams, elastomers, paints and coatings. TDI used in industry is an isomeric mixture of 2,6-TDI and 2,4-TDI in ratios of 20:80 or 35:65 [World Health Organisation (WHO) 1987]. Diisocyanates are very reactive compounds, which covalently bind to endogenous macromolecules such as carboxyl groups and amino groups. Thus, skin irritation (Calas et al. 1977), conjunctivitis (Luckenbach and Kieler 1980), upper respiratory impairments and allergic asthma (Banks et al. 1986; Baur et al. 1994; Hagmar et al. 1987) are well known as occupational health problems for workers using TDI. An obstructive pattern of pulmonary dysfunction was suggested to be shown in workers exposed to these chemicals (Omae et al. 1992). The threshold limit value (TLV) of 2,4-TDI is 0.005 ppm [American Conference of Governmental Industrial Hygienists (ACGIH) 2003]. The occupational exposure limit of TDI (the sum of 2,4-TDI and 2,6-TDI) is also recommended to be 0.005 ppm [Japanese Society for Occupational Health (JSOH) 1992].

During the last decade, several methods were developed for the determination of the urinary metabolite (toluene-diamine, TDA) of TDI (Lind et al. 1997; Maitre et al. 1993; Morita et al. 2003; Sakai et al. 2002; Sandstrom et al. 1989). The biological exposure index (BEI) of TDA has not been adopted by the ACGIH (ACGIH 2003). The limited application of those methods to actual workers might be because the methods reported, so far, were of time-consuming hydrolysis (overnight) and needed a deuterated substance as the internal standard, which was expensive and not easily obtained commercially. In many of those methods, more than 1 ml (largest 3 ml) of urine was also needed for the analysis, and the hydrolyzed TDA was extracted with toluene, which was relatively less effective than other solvent (such as dichloromethane, diethyl ether, ethyl acetate, and so on) for TDA extraction (Sakai et al. 2002).

In this study, we developed a sensitive and practicable GC-MS method for determining TDA after short-time hydrolysis of urine and applied the method to the biological monitoring of workers exposed to TDI.

Materials and methods

Subjects

Urine samples were collected from 18 male workers exposed to TDI in two factories and from 20 control subjects with no history of occupational exposure to TDI. TDI workers were engaged in the urethane painting of musical instruments. They were spraying urethane paints on musical instruments such as piano and guitar. TDI used in the factories was a mixture of two isomers, 2,4-TDA and 2,6-TDI, in the ratio 80:20. Urine was collected before and after the work shift (pre-shift and post-shift urine), and the samples were kept frozen at –20°C until required for analysis. All subjects gave informed written consent to participate in the study and agreed with the procedures. The medical ethics committee of Tokyo Rosai Hospital approved the study procedures, which conformed to the principals outlined in the Declaration of Helsinki.

Chemicals

2,6-TDA, 2,4-TDA, 3,4-TDA, and heptafluorobutyric anhydride (HFBA) were obtained from Tokyo Kasei (Japan). Sulfuric acid and dichloromethane (DCM) were purchased from Wako Pure Chemicals (Japan). The water used in the present study was either distilled water or water purified with a Millipore Milli-Q system (Nippon Millipore, Japan).

Sample preparation

The standard conditions used for the sample preparation in the present study were as follows: 0.1 ml of urine was diluted with 0.9 ml of water and then hydrolyzed by being boiled for 1.5 h with 0.1 ml of concentrated sulfuric acid in a total volume of 1.1 ml (11-fold dilution and a final concentration of 1.8 mol/l sulfuric acid). After hydrolysis, the urine samples were cooled and 0.7 ml of 8 mol/l sodium hydroxide solution was added. The alkalinized hydrolysate was then extracted with 2 ml of DCM for 20 min. After centrifugation at 3,000 rpm for 5 min, the organic layer was transferred to another tube. Fifty microliters of internal standard (200 ppb 3,4-TDA) and 50 μl of HFBA were added to each tube. The tubes were capped and shaken for 30 s. Derivatization with HFBA was carried out at 55°C for 1 h. After cooling, the DCM layer was dried to approximately 20–30 μl under a nitrogen steam. The residue was dissolved in 200 μl of toluene and used for GC-MS analysis.

In order to assess the standard method (the above-mentioned 11-fold dilution method), a 1.1-fold dilution was also examined in a total volume of 1.1 ml (1 ml of urine and 0.1 ml of concentrated sulfuric acid) and in a final concentration of 1.8 mol/l sulfuric acid. Hydrolysis was also carried out for 16 h for the comparison of hydrolysis yield to 1.5 h hydrolysis.

Analysis

Of the sample solution, 1 μl was injected into the GC-MS (Model GC-17A and QP5050A, Shimadzu, Japan) in splitless mode. The capillary column used was a DB-1 (30 m×0.25 mm, 0.25 μm film thickness; J&W Scientific, USA). Injection temperature was set at 280°C, and the column temperature was programmed from 100°C to 280°C at 20°C/min. Helium gas was used as the carrier, at a constant flow (1.3 ml/min). The samples were injected by splitless mode with a sampling time of 1 min. The GC-MS interface temperature was 280°C. The pressure in the ion source, in the case of NCI with iso-butane, was kept at ca. 1.2×10^{-3} Pa, and the detector gain was 1.25 kV. Under our condition as the routine, contamination of the ion source is not so fast. We can use the ion source more than 200 times without washings. The column can be used for more than 1,000 analyses. The TDA derivative was detected with the ion, m/z=494, in negative mode. Urinary creatinine concentrations were determined by Jaffe's method with a creatinine determination kit (Wako Pure Chemical, Japan: based on a colorimetric method). The specific gravity of urine was determined by protein refractometer (D type, Erma, Japan).

Determination of personal exposure levels of TDIs

Personal exposure levels were determined by the method of the Occupational Safety and Health Administration (OSHA; no. 42) (OSHA 1989). Air samples were collected by use of a

personal sampling pump (GilAir, Gilian, USA) with the sampling device in line. The sampling device was a three-piece styrene cassette (225-3-01, SKC, USA) containing a glass-fiber filter (225–9002, SKC, USA) coated with 0.4 mg of 1-(2-pyridyl) piperazine and backup pad. Sampling was carried out for 4–6 h at a flow rate of 1 l/min. Samples were extracted with 2 ml of the mixture of acetonitrile/dimethyl sulfoxide (90:10) for 1 h, and analyzed by a high performance liquid chromatograph (HPLC; Gilson, France) with an ODS column (Symmetry C18, 3.9 mm×150 mm×5 μm, Waters, USA). TDI derivatives were detected by UV/VIS·detector at 254 nm (119UV/VIS, Gilson, France). The mobile phase was the mixture of acetonitrile/10 mmol/l ammonium acetate (30:70) at flow rate of 1 ml/min.

Results

Figure 1 shows the chromatogram of three isomers of standard TDA and TDA isomers in urine from a TDI worker and a control subject. Three kinds of standard TDAs were eluted in the order of 3,4-, 2–6, and 2,4-TDA (Fig. 1c). 2–6-TDA, and 2,4-TDA were not detected in control urine (n=20), and 3, 4-TDA (internal standard) was detected neither in the hydrolyzed urine of workers nor in that of control subjects. The calibration curves of 2,6-TDA and 2,4-TDA were linear up to 400 μg/l. The detection limit of the TDAs was 0.1 μg/l. When 100 μg/l of individual TDA standard was added to ten control urine samples, the mean recovery rate ± standard deviation of 2,6-TDA and 2,4-TDA were 99.5±6.1% and 102.6±5.1%, respectively. When 25 μg/l of individual TDA standard was added to one of the control urine samples, the coefficient of variations of 2,6-TDA and 2,4-TDA determination were 5.7% and 6.3% (n=10), respectively.

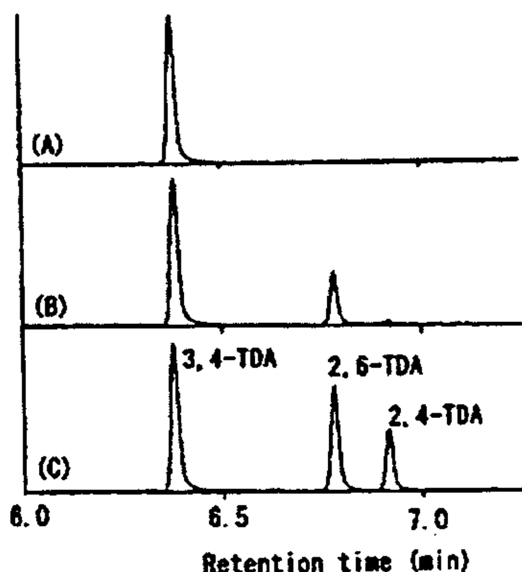

Fig. 1 Chromatographic separations of TDA isomers. **a** Control subject, **b** TDI worker, **c** standard TDIs. Concentrations of 2,6-TDA and 2,4-TDA in the worker were 29.1 μg/l and 3.2 μg/l, respectively, and those of standard TDAs were equivalent to 50 μg/l

Table 1 indicates correlation equations among TDA values obtained by various hydrolysis conditions (urine dilution and hydrolysis time). Some of the correlations are shown in Fig. 2. When 11-fold diluted urine was used, the 2, 6-TDA levels obtained by 1.5 h hydrolysis were approximately 46.1%, as compared to the correlation slope of those by 16 h hydrolysis (Fig. 2a). This indicates that prolonged hydrolysis increases the yield of hydrolysis product 2,6-TDA. However, shortening the hydrolysis time is favorable for the time-consuming procedure of TDA analysis, although the values are nearly or less than half the complete hydrolysis (16 h). 2,6-TDA levels obtained in 11-fold diluted urine were slightly higher than those in 1.1-fold diluted urine when the hydrolysis time was 1.5 h (Fig 2b). This finding indicates that urine dilution is slightly effective for the hydrolysis of TDA-forming compound. Similar results were obtained for 2, 4-TDA, although the levels were as low as one-tenth of 2,6-TDA (Table 1). Based on these results, hydrolysis time can be shortened to 1.5 h with 11-fold diluted urine.

Table 1 Correlation equation ($y=ax+b$) among TDA values (x and y) by various hydrolysis conditions [*A* 1.5 h hydrolysis (y) versus 16 h hydrolysis (x) with 11-fold diluted urine, *B* 11-fold diluted (y) versus 1.1-fold diluted (x) with 1.5 h hydrolysis urine, and *C* 1.5 h hydrolysis of 1.1-fold diluted urine (y) versus 16 h hydrolysis of 11-fold diluted urine (x)]. a slope, b intersects the y-axis, r correlation coefficient, x and y TDA levels (μg/l)

Isomer	Urine used	Hydrolysis	a	b	r
2,6-TDA	Post-shift urine (n=18)	A	0.477	−0.313	0.984
		B	1.120	0.389	0.973
		C	0.395	0.138	0.942
	Pre-shift urine (n=18)	A	0.424	0.071	0.929
		B	1.120	−0.232	0.985
		C	0.353	0.735	0.879
	Both urine (n=36)	A	0.461	−0.247	0.966
		B	1.130	0.025	0.977
		C	0.381	0.360	0.923
2,4-TDA	Post-shift urine (n=18)	A	0.396	−0.072	0.839
		B	1.320	0.201	0.902
		C	0.307	−0.225	0.952
	Pre-shift urine (n=18)	A	0.326	−0.227	0.642
		B	0.430	0.285	0.683
		C	0.511	−0.547	0.633
	Both urine (n=36)	A	0.378	−0.193	0.778
		B	0.695	0.322	0.713
		C	0.360	−0.260	0.723

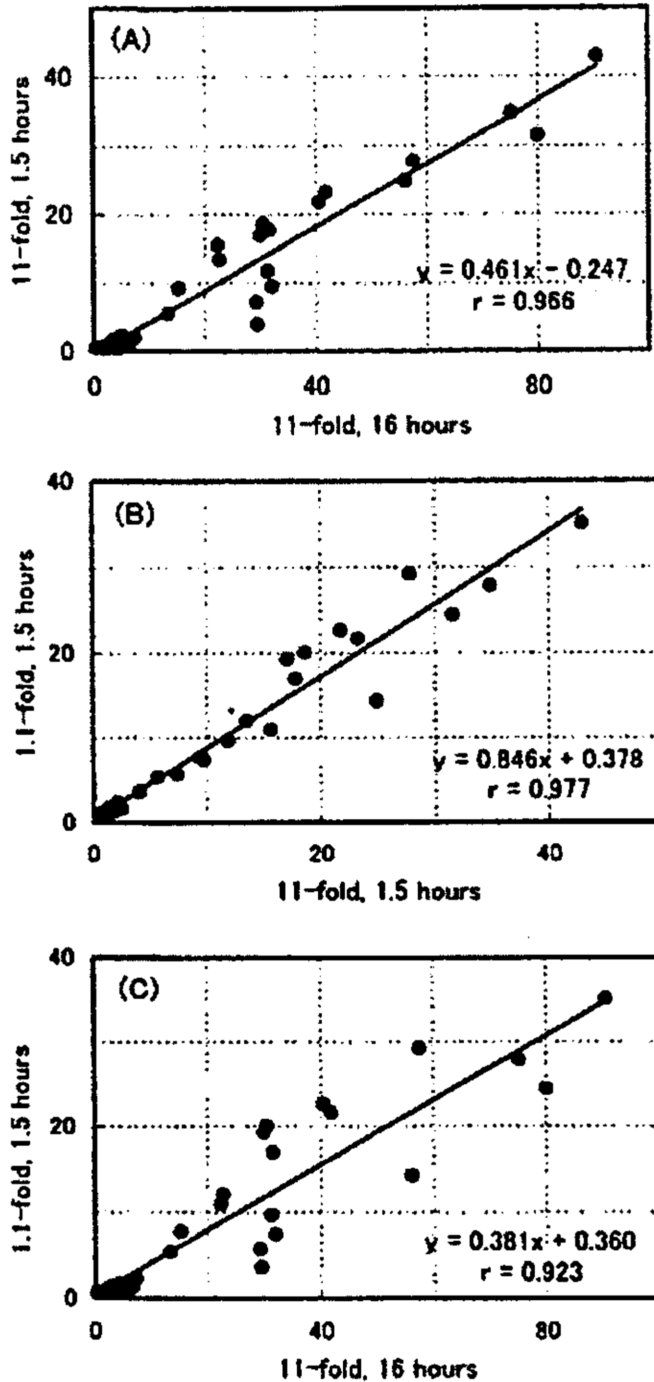

Fig. 2 Relationship between TDA values (*x* and *y*) by various hydrolysis conditions. Concentration of 2,6-TDA (μg/l) were compared among the hydrolysis condition of 11-fold or 1.1-fold diluted urine for 1.5 or 16 h. Both pre-shift and post-shift urine (n=36) was investigated together. **a** 1.5 h hydrolysis (*y*) versus 16 h hydrolysis (*x*) with 11-fold diluted urine. **b** 1.1-fold diluted (*y*) versus 11-fold diluted (*x*) with 1.5 h hydrolysis urine. **c** 1.5 h hydrolysis of 1.1-fold diluted urine (*y*) versus 16 h hydrolysis of 11-fold diluted urine (*x*)

When 11-fold diluted urine was hydrolyzed for 1.5 h, the TDA levels in post-shift urine were well correlated with personal exposure levels of TDI (Table 2). To investigate which is the better index for airborne TDI exposure, we investigated the relationships between airborne

TDI levels (x) and TDA levels in urine (y), both uncorrected and corrected concentrations. If the 2,6-TDI levels were corrected with creatinine or specific gravity (1.020), the correlation was improved, although the correlation in the 2,4-isomer was not improved because of low exposure levels. Creatinine correction showed the highest correlation coefficient (r=0.905) in the 2,6-isomer (Table 2 and Fig. 3b). TDAs in pre-shift urine were also correlated significantly with the personal exposure levels of TDIs, although the slope of the correlations in pre-shift urine was 60%–70% of that in the post-shift samples (Table 2). The correlation between 2,4-TDA and 2,6-TDA levels was significant (Fig. 4), although 2,4-TDA levels were less than one-tenth of 2,6-TDA levels. Thus, the levels of the 2,4-isomer were lower than those of the 2,6-isomer, not only in the urine but also in the work environment.

Table 2 Correlation equation ($y=ax+b$) between urinary TDA levels (y) and personal exposure levels of TDI (x). a slope, b intersects the y-axis, and r correlation coefficient. x personal exposure levels of TDI (ppb), y TDA levels in various correction of urine [A no correction (μg/l), B creatinine correction (μg/g creatinine), and C specific gravity (1.020) correction (μg/l)]

Parameter	Urine used	Correction	a	b	r
2,6-TDA vs 2,6-TDI	Post-shift urine (n=18)	A	10.160	–1.160	0.701
		B	6.600	–1.430	0.905
		C	8.130	–1.190	0.807
	Pre-shift urine (n=18)	A	6.090	–0.306	0.614
		B	4.930	–1.690	0.856
		C	5.920	–1.450	0.811
2,4-TDA vs 2,4-TDI	Post-shift urine (n=18)	A	8.330	0.231	0.835
		B	3.190	0.385	0.639
		C	5.340	0.334	0.782
	Pre-shift urine (n=18)	A	5.010	0.174	0.785
		B	2.900	0.124	0.847
		C	4.020	0.152	0.844

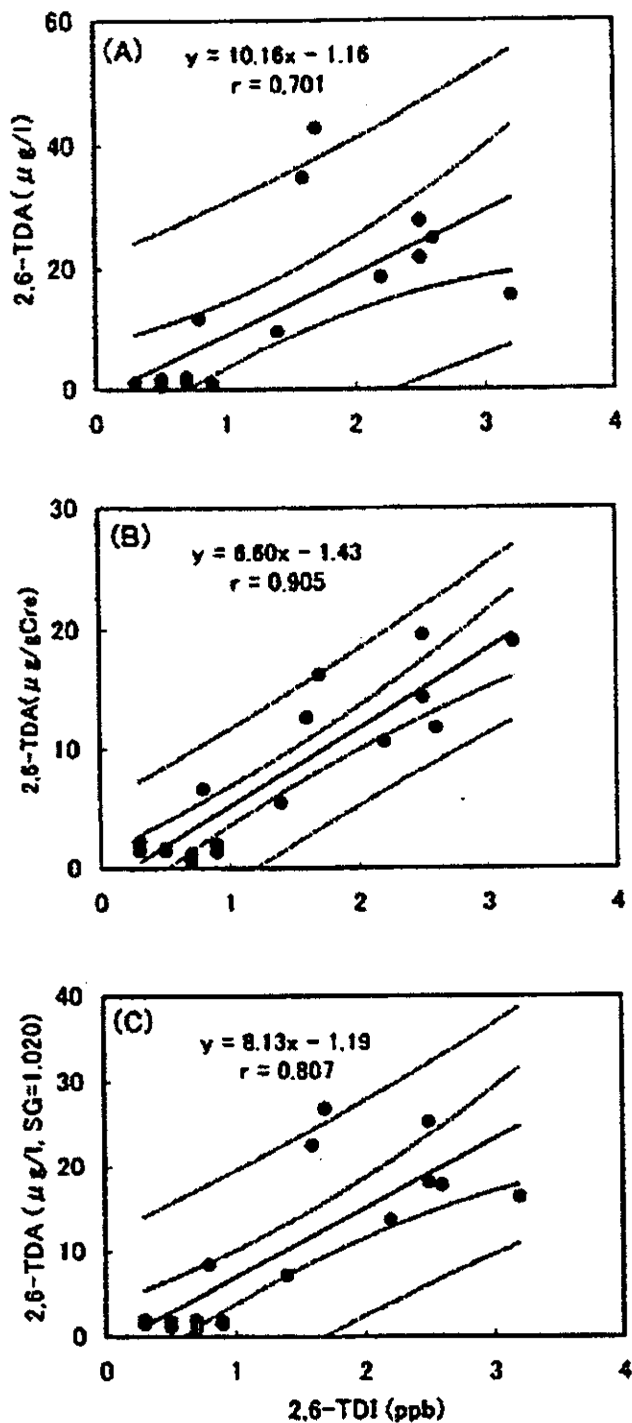

Fig. 3 Relationship between 2,6-TDA levels in post-shift urine (*y*) acquired by various corrections and personal exposure levels of 2,6-TDI (*x*) (*n*=18). **a** No correction (μ/l), **b** creatinine correction (μ/g creatinine), **c** Specific gravity (1.020) correction (μ/l). *Solid lines* show regression lines. *Curves* (*dotted lines*) close to the regression line shows 95% confidence ranges of the regression line, and outermost curves (*dotted lines*) indicate 95% predictive intervals of individual values. *Cre* creatinine, *SG* specific gravity

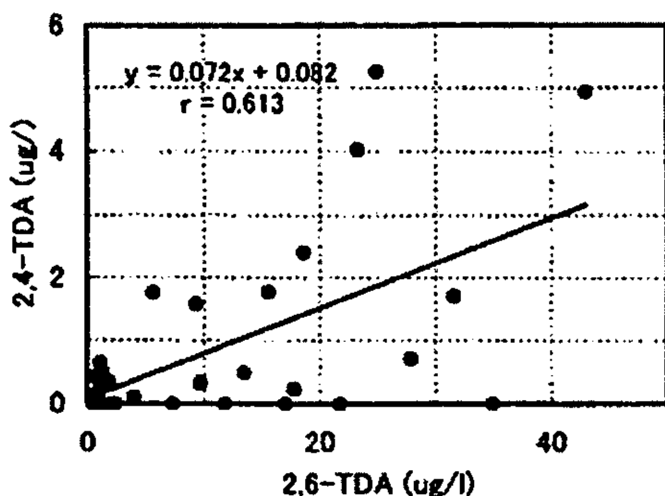

Fig. 4 Relationship between 2,4-TDA (*y*) and 2,6-TDA (*x*) in urine from workers. The data include both pre-shift and post-shift measurements (n=36)

Discussion

The most important improvement in the present method was the decreased volume of urine sample used (0.1 ml). More than 1 ml of urine (largest 3 ml) is used in the conventional methods (Brorson et al. *1991*; Lind et al. *1996*, *1997*; Maitre et al. *1993*; Persson et al. *1993*; Sandstrom et al. *1989*; Skarping et al. *1991*, *1994*). The decreased volume of urine used for the present analysis was attained by the use of DCM as the extraction solvent, which was highly effective for TDA extraction (Sakai et al. *2002*). Various conditions of hydrolysis, extraction and detection have been reported for the determination of TDA, so far (Table 3). Most analyses preferentially used toluene as the extraction solvent (Brorson et al. *1991*; Lind et al. *1996*, *1997*; Maitre et al. *1993*; Persson et al. *1993*; Sandstrom et al. *1989*; Skarping et al. *1991*, *1994*). However, the extraction efficiency of TDA with toluene was very much lower than that with DCM. We confirmed the high efficiency of extraction of TDA with DCM by a LC-MS method, which does not need the derivatization step (Sakai et al. *2002*). The extraction efficiencies of 2,6-TDA and 2,4-TDA by DCM were 76.2% and 74.6%, respectively. By contrast, those by toluene extraction were 27.1% and 26.9%, respectively (Sakai et al. *2002*).

Table 3 Comparison of various methods of TDA determination previously reported

Author	Volume of urine (ml)	Volume of acid (ml)	Acid	Dilution of urine (fold)	Final concentration of acid (mol/l)	Hydrolysis time (hours at 100°C)	Extraction solvent	[illegible]
Sandstrom et al. (*1989*) and Brorson et	2	3	6 mol/l HCl	2.5	3.6	Overnight	Toluene	[illegible]

al. (1991)								
Maitre et al. (1993)	3	0.75	12 mol/l HCl	1.28	2.3	2	Toluene (twice)	C
Lind et al. (1997)	1	1.5	3 mol/l H_2SO_4	2.5	1.8	16	Toluene	C
Sakai et al. (2002)	1	0.1	18 mol/l H_2SO_4	1.1	1.8	1.5	Dichloromethane	L
Morita et al. (2003)	1	0.1	18 mol/l H_2SO_4	1.1	1.8	1.5	Diethyl ether	C
This issue	0.1	0.1	18 mol/l H_2SO_4	1.1	1.8	1.5/16	Dichloromethane	C

The conventional GC-MS methods required the use of deuterated isomers of TDA as internal standards (Brorson et al. 1991; Lind et al. 1996, 1997; Maitre et al. 1993; Persson et al. 1993; Sandstrom et al. 1989; Skarping et al. 1991, 1994). It is generally difficult to obtain deuterated internal standards, and it may not be possible to obtain the standard material in many laboratories, because they are very expensive. This may be one of the most important limitations of the application to biological monitoring. We have previously developed alternative substances as internal standards for TDA determination instead of deuterated ones (OSHA 1989; Sakai et al. 2002). In the present GC-MS method, we also eliminated the use of deuterated standards by substituting with 3,4-TDA as the internal standard.

We have previously demonstrated the effects of urine dilution and shortened hydrolysis time on the yield of hydrolysis. The data indicated that a long hydrolysis time and hydrolysis without urine dilution were not favorable for high yields of TDA (Sakai et al. 2002). In many of the GC-MS methods, 2.5-fold diluted urine was hydrolyzed for quite long times (Table 3), such as 16 h or overnight (Brorson et al. 1991; Lind et al. 1996, 1997; Maitre et al. 1993; Persson et al. 1993; Sandstrom et al. 1989; Skarping et al. 1991, 1994). In the previous study (Sakai et al. 2002). When we used 1.1-fold diluted urine, the yield of TDA hydrolysis reached the plateaux at approximately 4 h and decreased with prolonged hydrolysis time, especially at more than 10 h. Thus, the hydrolysis time should be less than 4 h if the 1.1-fold urine is used. When we used an 11-fold dilution of urine, 16-h dilution, showed the highest yield of TDA. In the present GC-MS method, we hydrolyzed 11-fold diluted urine for 1.5 h. The TDA levels after 1.5 h hydrolysis correlated well with those after complete hydrolysis (16 h), although the former levels were less than half of the latter. Both levels are comparable, from the correlation equation (Fig. 2a).

For practical purpose, it is difficult to hydrolyze all samples for 16 h because it is very time consuming. This might be one of the reasons why biological monitoring by TDA is not routinely used. TDA levels obtained by 1.5 h of hydrolysis can be converted by use of the correlation equation (Fig. 2a). When we need the exact TDA levels we can hydrolysis urine for 16 h. However, to monitor many workers, the TDA levels acquired by 1.5 h hydrolysis are sufficient to prevent TDI-induced respiratory disease.

Ours is the first report on the relationship between urinary TDA levels and personal exposure levels of TDI in the respective isomer in actual workers, although Maitre et al. (1993) presented a linear relationship between TDA and TDI levels in the form of combined isomers (r=0.910). They examined samples from only nine workers, and the range of total TDI (2,4-

TDA and 2,6-TDA) was 9.5–94 $\mu g/m^3$ (1.33–13.2 ppb). TDA levels ranged between 6.5 $\mu g/g$ creatinine (Cre) and 31.7 $\mu g/g$ Cre, and a TDI concentration of 38 $\mu g/m^3$ (5.34 ppb) corresponded to a TDA excretion of 18 $\mu g/g$ Cre. In the present study a 2,6-TDA level that corresponded to the 5.34 ppb of 2,6-TDI was calculated as 33.8 $\mu g/g$ Cre, if the 2,6-TDI level was extrapolated in the correlation equation in Fig. 3b. From this comparison, TDA levels in the present study were 1.9-times higher than those found by Maitre et al. (1993). The higher levels in the present study might be partly attributed to the fact that a more complete hydrolysis was archived by 11-fold dilution of urine and high concentration of acid (1.8 mol/l H_2SO_4 in the final solution) used for hydrolysis. Maitre et al. (1993) hydrolyzed 1.28-fold diluted urine with HCl (2.3 mol/l final concentration) for 2 h. The difference might be because there were fewer subjects (n=9) in their study (Maitre et al. 1993). However, there should be further investigation at higher exposure levels than in our study and that of Maitre et al. (1993).

Brorson et al. (1991) carried out test-chamber exposure of TDI to two volunteers. From their data, the correlation equation can be calculated between TDI exposure levels (x ppb) and urinary TDA levels (y $\mu g/g$ Cre): y=8.29x–10.2 for the 2,6-isomer (n=6, r=0.942) and y=3.97x–1.33 (n=6, r=0.990) for the 2,4-isomer. These relationships between exposure and urinary metabolites are similar to our present data (Table 2): y=6.6x–1.43 (n=18, r=0.905) and y=3.19x+0.385 (n=18, r=0.639), respectively. In the correlation equation, the TDA levels at 5 ppb exposure of TDI in their study are near to those in our study, even though the exposure time in their study was only 4 h (half of that in our study). It might be because of the longer hydrolysis time (overnight) in their study (Brorson et al. 1991) (see Table 3).

The threshold limit value [or occupational exposure limit OEL)] of TDI was set at 0.005 ppm for the prevention of respiratory disease such as asthma (ACGIH 2003; JSOH 1992). If we can determine the TDA level that corresponds to the TLV level of TDI (biological threshold value or BEI), it would be possible to prevent TDI-induced asthma by simply determining urinary TDA levels instead of determining airborne TDI, which require complex procedures. Furthermore, biological monitoring by TDA levels is useful for estimating the intake value of TDI by all pathways, including airways, skin, and digestive organs. In addition, we can prove the TDI exposure by determining TDA, even if the airborne TDI level had not been determined when the symptoms developed.

From Fig. 3a (uncorrected urine), it can be estimated that the TDI levels calculated from TDA might have had wide range, especially over the TLV of 5 ppb. It is better to use creatinine-corrected urine for estimating TDI levels, from the view of confidence limit (Fig. 3b). From the correlation equation, the 2,6-TDA level (corrected with creatinine) that corresponded to the TLV (or OEL) level of TDI (5 ppb) was calculated to be 31.6 $\mu g/l$. The relationship between TDA levels and asthma is now under investigation by questionnaire, health examinations, IgE test, and determination of TDA and TDI.

In the present study, TDA levels, even in pre-shift urine, also correlated well with the personal exposure levels of TDI, although the slope of the correlation in pre-shift urine was 60%–70% of that in the post-shift samples (Table 2). The data might suggest that each worker was constantly exposed to TDI in every day work and that the excretion of TDA due to the exposure during the previous day's work continued in the pre-shift urine. Urinary excretion of TDA was lasting for more than 20 h after the beginning of 4-h exposure to 2,4-

TDI and 2,6-TDI (Brorson et al. 1991). In a study by Tinnerberg et al. (1997) within a flexible-foam plant, considerable concentrations of TDA in hydrolyzed urine were reported, even on Monday mornings before the start of the shift, and TDA levels in pre-shift urine gradually increased during the continuing working days. This finding might indicate that the TDA-forming compound is accumulated in the body, maybe in the form of protein adducts such as albumin (Brorson et al. 1991; Tinnerberg et al. 1997). Some part of the TDA found in pre-shift urine might be due to those protein adducts. In the data from Tinnerberg et al. (1997), 2,6-TDA levels in pre-shift urine were nearly half the levels of 2,6-TDA in the previous post-shift urine. These data are also compatible with the present findings. Since the exposure conditions in the work rooms were constant and continued for consecutive work days, TDA levels in pre-shift urine are relatively high and correlated with the exposure levels of the day, which might be not so different from those of the preceding day. However, in the work place where exposure levels are not constant from day to day, the relationship between TDA in pre-shift and post-shift urine will not be observed.

In our study we found that the levels of 2,4-isomer were less than one-tenth of the 2,6-isomer in both air (personal exposure) and urine (Fig. 3), although the plant used an 80:20 mixture of 2,4-TDI and 2,6-TDI. This remarkable difference between materials used in the factory and both levels in environment and urine might be attributable to the grater reactivity of the 2,4-isomer in the polymerization reaction. The 2,4-isomer was predominant in the air at the start of the polymerization process, and 2,6-isomer was the major isomer at the end of the process (Rando et al. 1984). In the flexible-foam plant using an 80:20 mixture of 2,4-TDI and 2,6-TDI, the 2,6-TDA levels were more than ten-times higher than the 2,4-TDA levels in the urine of a worker processing the product (polyurethane resin blocks), while both isomer levels were nearly same in the urine of workers close to the foaming nozzle or at the beginning of the curing tunnel (Tinnerberg et al. 1997). In the factory examined here, intermittent polymerization was carried out at many point in the large work room, resulting in 2,6-isomer predominance in the work environment and urine.

In summary, we have developed a simple and sensitive GC-MS method for the determination of TDAs in urine and have applied it to workers exposed to TDI. The TDA levels in post-shift urine were well correlated with personal exposure levels of TDI. The correlation was improved by correction with creatinine or specific gravity in the 2,6-isomer, but not in the 2,4-isomer because of low exposure level. From the correlation equation, the 2,6-TDA level (corrected with creatinine) that corresponded to the TDI level of 5 ppb was calculated to be 31.6 μg/l. TDAs in pre-shift urine also correlated significantly with the personal exposure levels of TDIs. The correlation between 2,4-TDI and 2,6-TDA levels was significant, although the levels of the 2,4-isomer were less than one-tenth of the 2,6- isomers in both air (personal exposure) and urine.

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Microbial metabolite of dimethylarsinic acid is highly toxic and genotoxic

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Abstract

Dimethylarsinic acid [DMA, $(CH_3)_2AsO(OH)$] causes cancer in the urinary bladder of rats. However, its mechanism of cancer or the ultimate carcinogenic form is not yet known. Rats administered dimethylarsinic acid excrete three unknown arsenic compounds (termed M-1, M-2, and M-3) in urine or feces, and these compounds are presumed to be produced by intestinal bacteria. *Escherichia coli* A3-6 isolated from a rat yielded two unknown arsenic compounds (M-2 and M-3) from dimethylarsinic acid and M-1 from trimethylarsine oxide (TMAO) in the presence of cysteine (Cys). Contents of M-2 and M-3 varied with cysteine concentration. The cytotoxicity and genotoxicity of the bacteria-free solution of dimethylarsinic acid or trimethylarsine oxide metabolized by *E. coli* A3-6 were studied using V79 cells. Dimethylarsinic acid (1 mM) metabolized by *E. coli* A3-6 in the presence of cysteine (1 mM) was highly cytotoxic (50% survival reduction concentration; 2.1 μM As) in V79 cells, and the toxic substance appeared to be M-2. The metabolite solution (at 2.5–10 μM total As) induced c-mitosis and tetraploids, and caused mitotic arrest, since it increased mitotic cells at the cytotoxic dose. The metabolite solution also significantly increased sister chromatid exchange (SCE) and chromosomal aberrations, most of which were chromatid gaps and chromatid breaks. A3-6 converted 96.1% of trimethylarsine oxide to M-1 in the presence of cysteine. This metabolite solution did not exhibit cytotoxicity or genotoxicity. The reported M-2 concentration in urine of rats administered levels of DMA via drinking water known to cause bladder tumors was sufficient to exhibit cytotoxic and genotoxic effects in urinary bladder. Thus, we hypothesize that intestinal bacteria play an important role in carcinogenicity of dimethylarsinic acid.
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Keywords: Dimethylarsinic acid; *Escherichia coli*; SCE; Chromosomal aberration; Tetraploid; Mitotic arrest; C-mitosis

Introduction

Dimethylarsinic acid [DMA, $(CH_3)_2AsO(OH)$] is a tumor promoter and a complete carcinogen in the urinary bladder in rats (Li et al., 1998; Wanibuchi et al., 1996; Wei et al., 1999, 2002; Yamamomto et al., 1995). However, its mechanisms for the induction of the bladder cancer are not yet known (Kitchin, 2001). When arsenate is introduced into the mammalian body, it is reduced to arsenite, and then is methylated to monomethylarsonic acid (MMA) and DMA (Thompson, 1993). DMA is the major metabolite of inorganic arsenic in humans (Buchet and Lauwerys, 1987; Vahter, 1999).

DMA is a mitotic poison, causing mitotic arrest, inducing tetraploids (Eguchi et al., 1997; Endo et al., 1992) and c-mitosis (Iwami et al., 1997), and inducing abnormal spindles in mitotic cells (Kawata et al., 2001). DMA also induces aneuploids in mouse bone marrow cells (Kashiwada et al., 1998). Our previous studies using V79 cells and HL-60 cells showed that cysteine (Cys) enhanced cytogenetic toxicity of DMA including mitotic arrest, tetraploid formation, and induction of chromosomal aberrations (Kitamura et al., 2002) and also enhanced cytotoxicity and induction of apoptosis (Zhou et al., 2003). We found dimethylarsinous acid (DMAIII), a reduced form of DMA, in the mixture of Cys and DMA, and concluded that the enhancement by Cys of DMA-induced cytogenetic toxicity was due to the formation of DMAIII. Methylated trivalent arsenicals such as DMAIII and monomethylarsonous acid (MMAIII), intermediates reduced in the metabolic processing of DMA and TMAO, are more cytotoxic than arsenite (Cohen et al., 2002; Petrick et al., 2000; Styblo et al., 2000, 2002). They are very

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