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Direct methyl esterification with 2,2-dimethoxypropane for the simultaneous determination of urinary metabolites of toluene, xylene, styrene, and ethylbenzene by gas chromatography-mass spectrometry

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Abstract

Objectives: The purpose of this study was to develop a simple and accurate gas chromatography-mass spectrometry (GC-MS) method for simultaneous determination of four urinary metabolites from four organic solvents, that is, hippuric acid (HA) from toluene, methylhippuric acid (MHA) from xylene, and mandelic acid (MA) and phenylglyoxylic acid (PGA) from styrene or ethylbenzene for biological monitoring.

Methods: The four metabolites were directly methyl-esterified with 2,2-dimethoxypropane and analyzed using GC-MS. The proposed method was validated according to the US Food and Drug Administration guidance. The accuracy of the proposed method was confirmed by analyzing a ClinChek®—Control for occupational medicine (RECIPE Chemicals +Instruments GmbH).

Results: Calibration curves showed linearity in the concentration range of 10-1000 mg/L for each metabolite, with correlation coefficients >0.999. For each metabolite, the limits of detection and quantification were 3 mg/L and 10 mg/L, respectively. The recovery was 93%-117%, intraday accuracy, expressed as the deviation from the nominal value, was 92.7%-103.0%, and intraday precision, expressed as the relative standard deviation (RSD), was 1.3%-4.7%. Interday accuracy and precision were 93.4%-104.0% and 1.2%-9.5%, respectively. The analytical values of ClinChek obtained using the proposed method were sufficiently accurate.

Conclusions: The proposed method is a simple and accurate which is suitable for routine analyses that could be used for biological monitoring of occupational exposure to four organic solvents.

KEYWORDS

2,2-dimethoxypropane, gas chromatography-mass spectrometry, hippuric acid, mandelic acid, methylhippuric acid, phenylglyoxylic acid

1 | INTRODUCTION

Various organic solvents are used in industrial workplaces, among which toluene, xylene, styrene, and ethylbenzene are the most widely used.[1] Toluene and xylene are used separately or in mixtures as organic solvents in paints, coatings, thinners, inks, and adhesives.[1-3] Ethylbenzene is not only used primarily as an intermediate in the production of styrene, but also contained approximately 20% in industrial grade xylene.[4] Styrene is used to produce polystyrene plastics and resins, especially in fiber-reinforced plastics production.[1,5] Workers in these industries are frequently co-exposed to these solvents because these solvents are often used in combination with each other.[1]

Toluene is excreted in urine almost exclusively as hippuric acid (HA).[2] Xylene, which exists as three isomers (*o*-, *m*-, and *p*-xylene), is excreted in urine as the corresponding *o*-, *m*-, and *p*-methyl hippuric acids (*o*-, *m*-, and *p*-MHA).[3] The major urinary metabolites of ethylbenzene are mandelic acid (MA) and phenylglyoxylic acid (PGA),[4] which are the same for styrene.[5] These metabolites are indicators of internal exposure because they reflect the total absorbed dose from all routes of exposure, including inhalation, and dermal and oral absorptions. Therefore, these metabolites have been widely used in the biological monitoring of exposure to these solvents to prevent occupational diseases of workers. The occupational exposure limit based on biological monitoring (OEL-B) of urinary MHA (total of all isomers) for xylene proposed by the Japan Society for Occupational Health (JSOH) is 800 mg/l.[6] The biological exposure index (BEI) of xylene proposed by the American Conference of Governmental Industrial Hygienists (ACGIH) is 1.5 g/g creatinine.[3] The OEL-B and BEI for styrene are 430 mg/L[6] and 400 mg/g creatinine,[5] respectively, as the sum of urinary MA and PGA. The BEI for ethylbenzene is 0.15 g/g creatinine as the sum of urinary MA and PGA,[4] yet the OEL-B has not been proposed. The JSOH and the ACGIH no longer adopt urinary HA as a biomarker for toluene and propose other biomarkers such as urinary unmetabolized toluene and urinary *o*-cresol.[2,6] This reason is that the occupational contribution to low-level toluene exposure is masked by the background concentration of urinary HA that is mainly attributable to diet.[2]

For accurate and comprehensive evaluation of combined exposure, simultaneous determination of these urinary metabolites is desired for routine biological monitoring. Several simultaneous determination methods for these urinary metabolites have been reported, including gas chromatography-mass spectrometry (GC-MS),[7-11] high-performance capillary electrophoresis (HPCE),[12] high-performance liquid chromatography-ultraviolet (HPLC-UV) detection,[13,14] high-performance liquid chromatography-hybrid quadrupole time-of-flight mass spectrometry (HPLC-QqTOF-MS),[15] and ultra-high-performance liquid chromatography-electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS).[16] However, each of these methods has disadvantages. The GC-MS methods require complex and time-consuming sample preparation procedures including extraction, evaporation, or derivatization. Although sample preparation for the HPCE and the HPLC-UV methods is easier than that for the GC-MS methods, the selectivity is not sufficient for urine sample analysis. The HPLC-QqTOF-MS and the UPLC-ESI-MS/MS methods are not commonly used in many laboratories due to remarkably expensive equipment. Therefore, the aim of the present study was to develop and validate a simple and accurate method for simultaneous determination of these four urinary metabolites for routine analysis. To achieve this aim, we used GC-MS and a sample preparation method with 2,2-dimethoxypropane (DMP). DMP served a dual role as a reagent for dehydration and derivatization and enabled direct methyl esterification of these metabolites. The sample preparation by using DMP was simple, and an aliquot after preparation was directly injected into the GC without an extraction or evaporation procedure, which were bottlenecks of the conventional sample preparation. A complex and time-consuming procedure was avoided while maintaining reproducibility.

2 | MATERIALS AND METHODS

2.1 | Materials

HA, MA, and PGA were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). *o*-MHA, *m*-MHA, *p*-MHA, and DMP were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). N-Benzoyl-DL-alanine was used as an internal standard (IS) and purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Hydrochloric acid (HCl) and pyridine were analytical grade. Water was purified with an Elix 5 system (Millipore, Bedford, MA, USA). A ClinChek®—Control (Urine Control lyophilized) for occupational medicine was purchased from RECIPE Chemicals +Instruments GmbH (Munich, Germany). A mixed standard stock solution of MA, PGA, HA, and MHAs (each 10 g/L) and an IS solution (5 g/L) were prepared in methanol and stored at 4°C. Blank urine samples were collected from healthy adult volunteers. These volunteers were not occupationally exposed to toluene, xylene, styrene, or ethylbenzene. Informed consent was obtained from the volunteers before collection of urine. This study was approved by the Ethics Committee of the Japan Industrial Safety and Health Association.

2.2 | Instruments

The GC-MS system was a 7890A gas chromatograph equipped with a 5975C inert XL mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). The column was a

20 m × 0.18 mm ID InertCap 17 narrow bore column with 0.18-μm film thickness (GL Sciences Inc, Tokyo, Japan). Helium was used as the carrier gas at a flow rate of 1.0 mL/minute. The temperatures of the injection port and the transfer line were set at 250 and 300°C, respectively. The oven temperature was set at 100°C for 0.5 minute and then increased to 300°C at a rate of 20°C/minute. Samples (1 μL) were injected in the pulsed split mode (split ratio, 5:1; pulse pressure, 50 psi; pulse time, 0.5 minute). 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 as follows: *m/z* 107 and 79 for the methyl (Me) derivative of MA (MA-Me); *m/z* 105 and 77 for PGA-Me, IS-Me, and HA-Me; and *m/z* 119 and 91 for MHAs-Me where the former was selected as the quantifier ion and the latter as the qualifier ion. The mean of ion ratios (%) [(area of the qualifier ion/area of the quantifier ion) × 100] in all the calibration standard were 68.93 for MA-Me, 55.15 for PGA-Me, 35.43 for IS-Me, 43.27 for HA-Me, 56.62 for *o*-MHA-Me, 35.33 for *m*-MHA-Me, and 32.33 for *p*-MHA-Me. For quantification, the acceptance criteria of ion ratios for MA-Me, PGA-Me and *o*-MHA-Me were within ±10% of the ion ratios in the calibration standard, and those for IS-Me, HA-Me, *m*-MHA-Me, and *p*-MHA-Me were within ±15% accordingly.[17] Data were obtained in scan mode with a scan range from *m/z* 40-250 to confirm the mass fragmentation of the derivatives.

2.3 | Sample preparation

Urine (100 μL) was placed in a glass test tube. IS (5 g/L, 10 μL), HCl (35.0%-37.0%, 50 μL), and DMP (1 mL) were added to the tube, which was vortexed and then heated in an aluminum block bath (Dry Thermo Unit DTU-2C, TAITEC Co., Saitama, Japan) for 30 minute at 100°C. After cooling to room temperature, the excess HCl in the sample was neutralized by adding pyridine (100 μL). After 5 minutes, the sample was centrifuged at 1870 *g* for 10 minutes to remove urinary sediment. A 1-μL aliquot of the sample was injected into the GC-MS system.

2.4 | Method validation

Method validation was conducted according to the US Food and Drug Administration (FDA) guidance.[18] For calibration, urine samples spiked with each metabolite at six concentrations (matrix-matched standard) at 10, 50, 100, 250, 500, and 1000 mg/L were prepared in triplicate, and the samples were prepared and analyzed by the procedure described above. Calibration curves were obtained by plotting the peak area ratio of Me-derivatives of each metabolite to Me-derivative of IS against their respective concentrations. The reproducibility of the developed method, defined as precision, was evaluated by analyzing urine samples containing three concentrations (10, 250, and 1000 mg/L) of each metabolite 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 Me-derivatives of each metabolite 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 each metabolite in urine that corresponded to three and ten times the baseline noise, respectively. Finally, the accuracy of the proposed method was confirmed using ClinChek. The obtained results were compared with the certified values.

3 | RESULTS

3.1 | Optimization of methyl esterification reaction conditions

We examined the influences of the volume of HCl, the reaction temperature, and the reaction time using urine samples spiked with 1000 mg/L of each metabolite to determine the optimal methyl esterification reaction conditions. The peak area of each Me-derivative reached a plateau at 30 μL, except for PGA-Me, when the volume of HCl ranged from 10-50 μL at 100°C for 30 minutes. The peak area of PGA-Me increased as HCl volume increased (Figure 1A). When the effects of reaction temperature were examined at four different temperatures (40, 60, 80, and 100°C) for 30 minutes with an HCl volume of 50 μL, the peak area of each Me-derivative reached a plateau at 80°C (except for MA-Me). The peak area of MA-Me increased as reaction temperature increased (Figure 1B). When the reaction time ranged from 5 to 60 minutes at 100°C with an HCl volume of 50 μL, the peak area of each Me-derivative reached a maximum after 10 minutes and remained constant for 60 minutes (Figure 1C).

3.2 | Validation

Calibration curves showed linearity in the concentration range of 10-1000 mg/L for each metabolite, with correlation coefficients >0.999. For each metabolite, the LOD and LOQ were 3 mg/L and 10 mg/L, respectively. The recovery was 93%-117%. Intraday accuracy, expressed as the deviation from the nominal value, was 92.7%-103.0%. Intraday precision, expressed as the relative standard deviation (RSD), was 1.3%-4.7%. Interday accuracy and precision were 93.4%-104.0% and 1.2%-9.5%, respectively (Table 1). The analytical values of ClinChek obtained by the proposed method are shown in Table 2. Typical mass chromatograms of blank urine, standard spiked urine, and ClinChek Level I

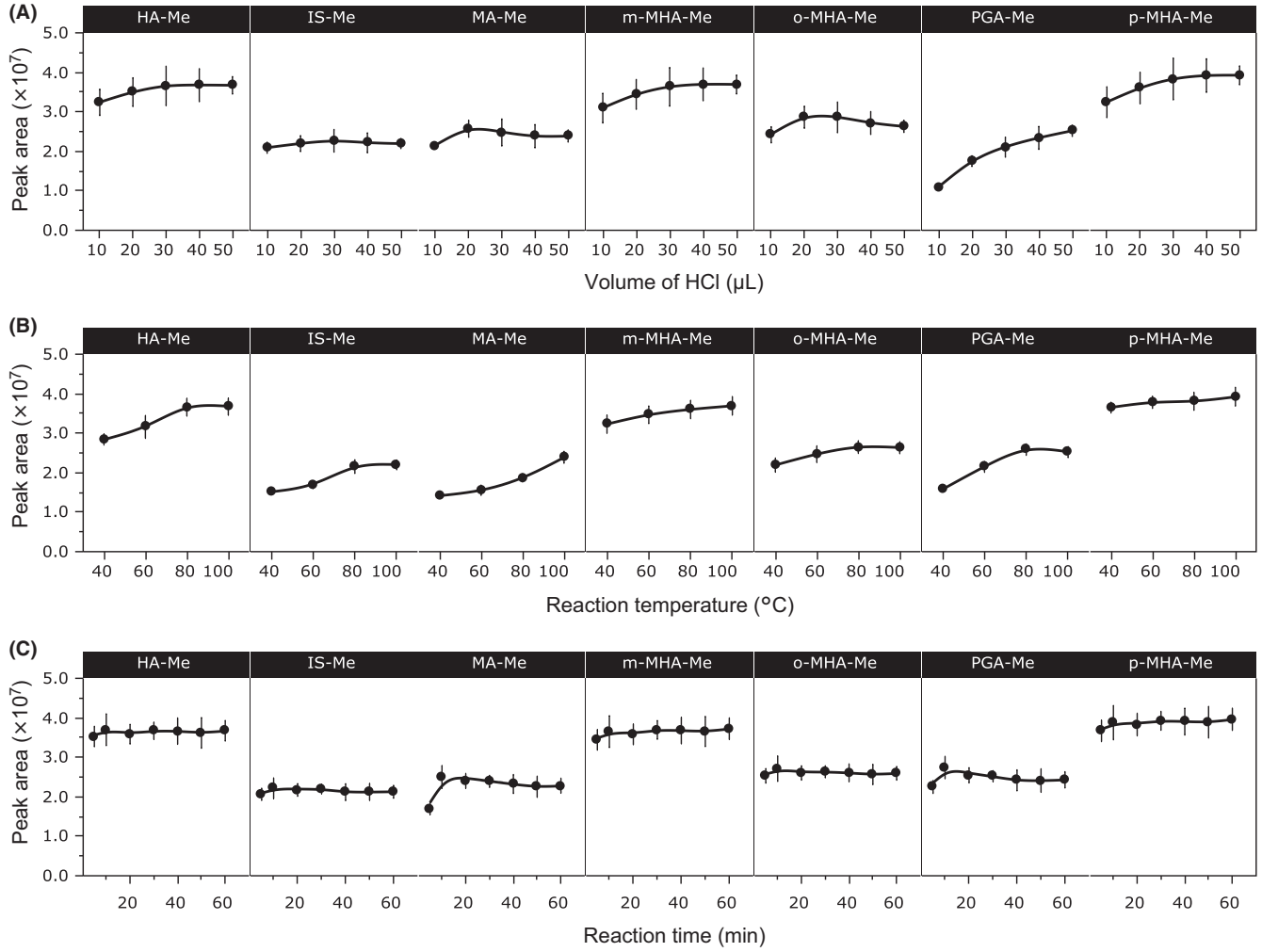


FIGURE 1 Effects of the (A) volume of HCl, (B) reaction temperature, and (C) reaction time on the production of the methyl derivatives of hippuric acid (HA-Me), internal standard (IS-Me), mandelic acid (MA-Me), *m*-methyl hippuric acid (*m*-MHA-Me), *o*-methyl hippuric acid (*o*-MHA-Me), phenylglyoxylic acid (PGA-Me), and *p*-methyl hippuric acid (*p*-MHA-Me). Error bars show the standard deviation (n = 3)

are shown in Figure 2. Sharp and symmetrical peaks of Me-derivatives of metabolites were obtained without disturbance by endogenous interference. N-Benzoyl-DL-alanine showed similar behavior to target metabolites during the derivatization and was well separated from the Me-derivatives of target metabolites in the chromatogram. Therefore, it was suitable as an IS.

4 | DISCUSSION

We developed and validated a simple and accurate GC-MS method for the simultaneous determination of toluene, xylene, styrene, and ethylbenzene urinary metabolites. Several simultaneous GC-MS methods have been reported.[7-11] In these methods, several derivatization procedures such as alkylation,[7,9] methyl esterification,[8] silylation,[10] and alkoxycarbonylation[11] were used to enable GC analyses because these metabolites have low volatility. However, most of these derivatization procedures were accompanied by an extraction procedure and an evaporation procedure. Therefore, the reported methods were not suitable for routine analyses because the extraction and evaporation procedures were time-consuming.

We adopted a sample preparation using DMP to overcome these disadvantages based on our previous study for the determination of urinary dimethyl sulfoxide and dimethyl sulfone.[19] The reaction scheme for urinary metabolites with DMP is presented in Figure 3. Although DMP was not miscible with water (ie, urine), the addition of HCl increased miscibility and the sample solution changed to a uniform phase. DMP served a dual purpose as a dehydration and derivatization reagent. Extraction procedures were not needed with DMP because it reacted with equimolar amounts of water to form methanol and acetone in a molar ratio of 2:1 in the presence of hydrogen ions that catalyzed the reaction (Figure 3 Reaction step 1). Moreover, HCl used as a catalyst in reaction step 1 and the methanol produced by reaction step 1 were

TABLE 1 Intra- and interday coefficients of variation of the proposed method

Spiked urine concentration (mg/l)	Recovery (n = 5)		Intraday (n = 5)[a]			Interday (n = 15)[b]		
	Mean ± SD (%)	RSD (%)	Mean ± SD (mg/l)	RSD (%)	Accuracy (%)	Mean ± SD (mg/l)	RSD (%)	Accuracy (%)
MA								
10	100 ± 1.9	1.9	9.83 ± 0.18	1.9	98.3	9.84 ± 0.25	2.5	98.4
250	95 ± 1.2	1.3	257.39 ± 3.25	1.3	103.0	256.79 ± 4.43	1.7	102.7
1000	97 ± 1.6	1.7	1002.58 ± 16.60	1.7	100.3	1001.43 ± 12.94	1.3	100.1
PGA								
10	97 ± 3.8	3.9	9.62 ± 0.38	3.9	96.2	9.59 ± 0.37	3.9	95.9
250	93 ± 2.2	2.4	253.57 ± 5.97	2.4	101.4	252.93 ± 5.04	2.0	101.2
1000	95 ± 2.3	2.4	997.74 ± 23.81	2.4	99.8	996.65 ± 14.58	1.5	99.7
HA								
10	95 ± 4.5	4.7	9.27 ± 0.43	4.7	92.7	9.34 ± 0.58	6.2	93.4
250	100 ± 1.6	1.6	253.81 ± 4.15	1.6	101.5	252.88 ± 4.72	1.9	101.2
1000	104 ± 1.8	1.8	999.01 ± 17.66	1.8	99.9	998.72 ± 11.78	1.2	99.9
o-MHA								
10	109 ± 1.0	1.0	10.22 ± 0.20	2.0	102.2	10.00 ± 0.95	9.5	100.0
250	102 ± 1.9	1.8	257.34 ± 4.89	1.9	102.9	255.24 ± 5.73	2.2	102.1
1000	103 ± 1.5	1.5	999.82 ± 14.77	1.5	100.0	998.15 ± 13.14	1.3	99.8
m-MHA								
10	113 ± 3.0	2.6	10.26 ± 0.27	2.6	102.6	10.40 ± 0.28	2.7	104.0
250	103 ± 1.9	1.9	251.68 ± 4.70	1.9	100.7	252.52 ± 4.97	2.0	101.0
1000	106 ± 2.0	1.9	999.55 ± 18.78	1.9	100.0	998.98 ± 12.41	1.2	99.9
p-MHA								
10	117 ± 3.3	2.8	9.31 ± 0.26	2.8	93.1	9.72 ± 0.69	7.1	97.2
250	102 ± 1.9	1.8	251.53 ± 4.62	1.8	100.6	252.90 ± 4.36	1.7	101.2
1000	106 ± 1.8	1.7	996.29 ± 16.88	1.7	99.6	996.27 ± 12.28	1.2	99.6

RSD relative standard deviation.

[a]Intraday reproducibility analysis was performed on a single day.

[b]Interday reproducibility analysis was performed over three consecutive days in five replicates.

TABLE 2 Results for urinary metabolites in ClinChek (Levels I and II)[a]

	Level I			Level II		
	This method (n = 5)	Certified		This method (n = 5)	Certified	
Metabolites	Mean ± SD	Mean	Confidence intervals	Mean ± SD	Mean	Confidence intervals
MA	99.2 ± 1.9	104	84.9-124	566.3 ± 14.9	603	527-679
PGA	34.7 ± 0.7	38.6	27.5-49.7	194.2 ± 7.2	207	165-249
HA	408.4 ± 3.7	412	349-475	2040.7 ± 19.6	2140	1910-2380
MHA	496.8 ± 8.6	484	400-568	1297.9 ± 21.9	1370	1169-1572

[a]Concentrations in mg/l.

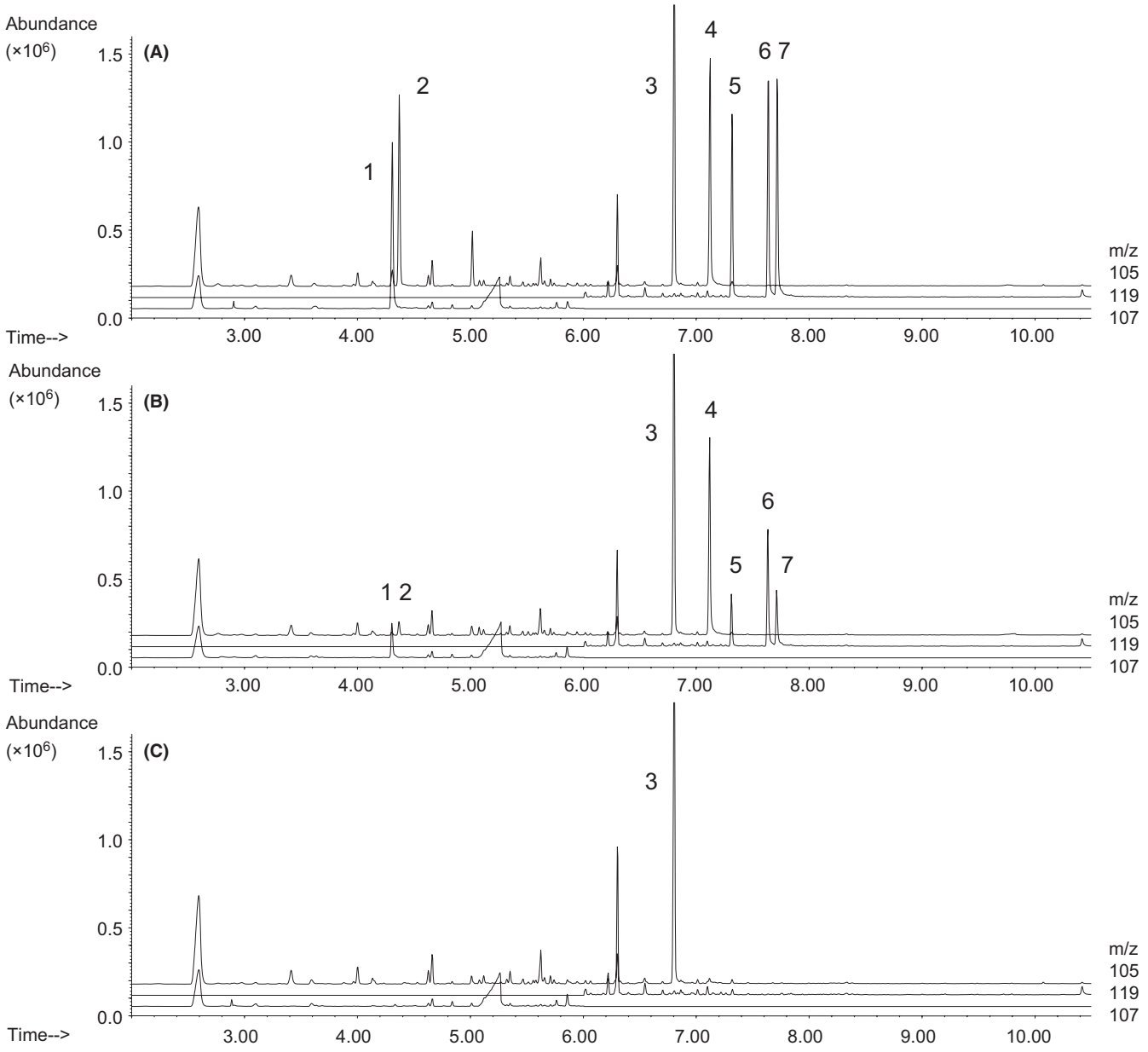
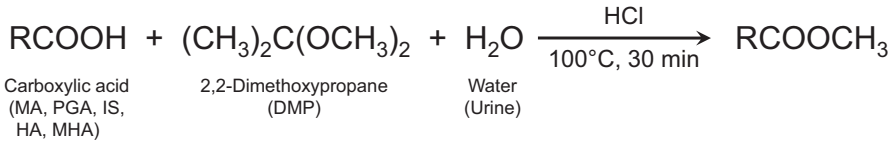
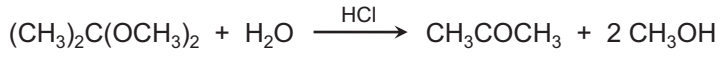


FIGURE 2 Typical reconstructed mass chromatograms of a (A) standard spiked urine (500 mg/L for each), (B) ClinChek Level I (RECIPE Chemicals +Instruments GmbH) (MA, 97.8 mg/L; PGA, 34.5 mg/L; HA, 407.5 mg/L; MHA, 489.7 mg/L), and (C) blank urine. The peaks of methyl derivative of (1) mandelic acid (MA-Me), (2) phenylglyoxylic acid (PGA-Me), (3) internal standard (IS-Me), (4) hippuric acid (HA-Me), (5) *o*-methyl hippuric acid (*o*-MHA-Me), (6) *m*-methyl hippuric acid (*m*-MHA-Me), and (7) *p*-methyl hippuric acid (*p*-MHA-Me) are indicated



(Reaction step 1)



(Reaction step 2)

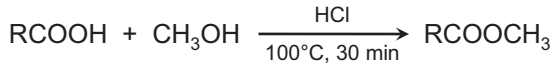


FIGURE 3 Reaction scheme for urinary metabolites with 2,2-dimethoxypropane. Mandelic acid (MA), phenylglyoxylic acid (PGA), internal standard (IS), hippuric acid (HA), methyl hippuric acid (MHA)

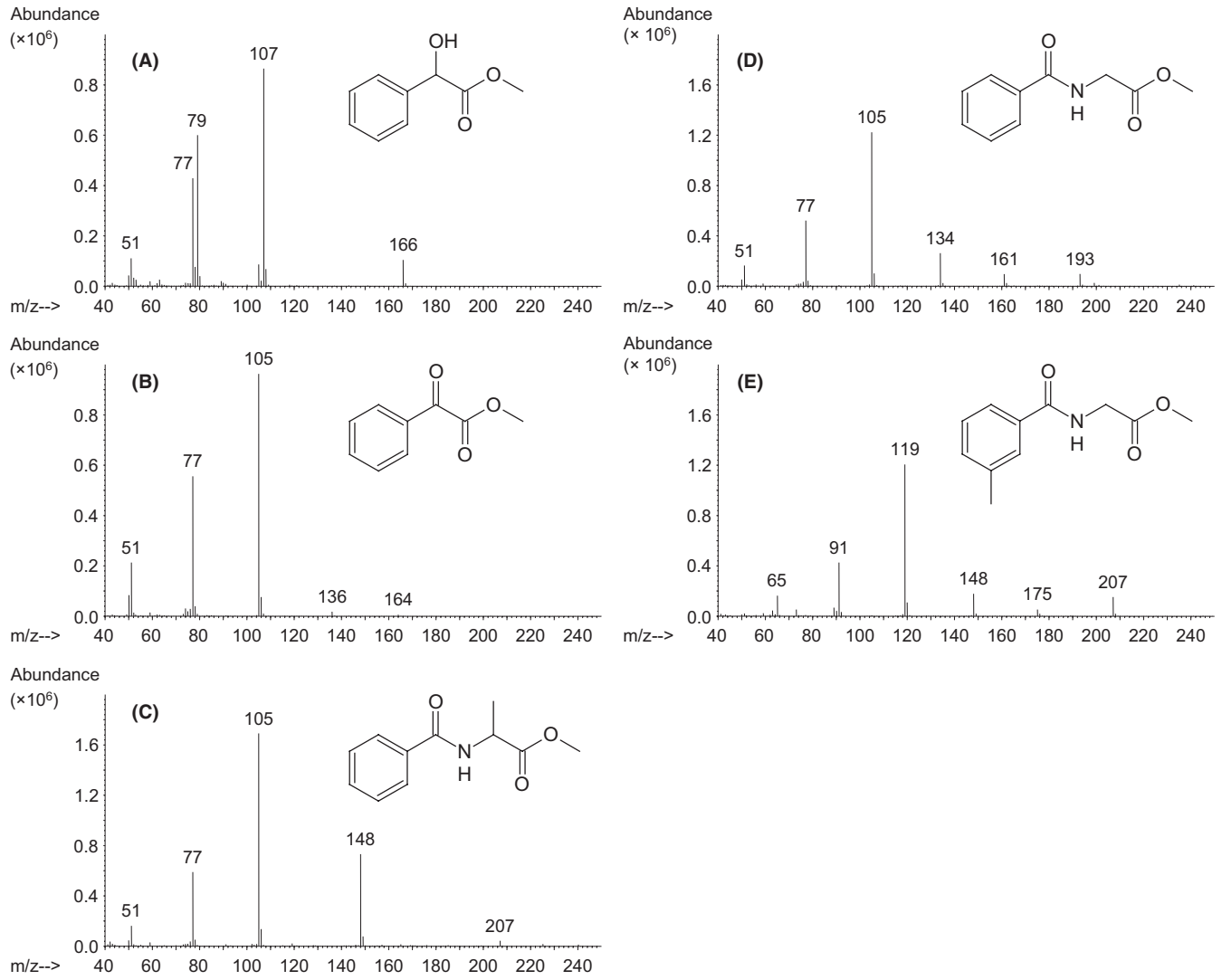


FIGURE 4 Typical mass spectra and expected structures of the methyl derivatives of (A) mandelic acid (MA-Me), (B) phenylglyoxylic acid (PGA-Me), (C) internal standard (IS-Me), (D) hippuric acid (HA-Me), and (E) *m*-methyl hippuric acid (*m*-MHA-Me). The mass spectra of the methyl derivatives of *o*- and *p*-methyl hippuric acid were similar to that of *m*-MHA-Me

used to methyl esterify the metabolites (Figure 3 Reaction step 2). Derivatization by methyl esterification was used in a previous study.[8] However, because the reaction condition was different from the previous study, it was necessary to optimize the methyl esterification reaction conditions using DMP. Therefore, we examined the influence of HCl volume,

the reaction temperature, and the reaction time. The plateaus of the PGA-Me and MA-Me peak areas were not observed in the experimental ranges of optimal HCl volume and reaction temperature determination, respectively (Figure 1A,B). We chose an HCl volume of 50 μL and a reaction temperature of 100°C to gain the maximal peak area for all metabolites. Although the methyl esterification reaction was complete within 10 minutes at 100°C with an HCl volume of 50 μL, we chose a reaction time of 30 minutes to ensure completion of the reaction (Figure 1C).

Routine analysis methods for periodic health checkups require high sample throughput because clinical laboratories need to analyze a large number of samples quickly. Therefore, we used a fast GC column. The analysis time was within 15 minutes, including the oven cool-down time, and reduced compared to that of previous methods. The EI mass spectra and expected structures of each of the Me-derivatives of metabolites are shown in Figure 4. The molecular ions $M^{+\cdot}$ of the expected structures of each of the Me-derivatives were observed at *m/z* 166, 164, 193, and 207 for MA-Me, PGA-Me, HA-Me, and IS-Me and MHAs-Me, respectively. The base peaks were also observed at *m/z* 107 for MA-Me, *m/z* 105 for PGA-Me, IS-Me, and HA-Me, and *m/z* 119 for MHAs-Me, respectively. These corresponded to $[C_6H_5CHOH]^+$ for *m/z* 107, $[C_6H_5CO]^+$ for *m/z* 105, and $[CH_3C_6H_4CO]^+$ for *m/z* 119. Other major fragment ions were observed at *m/z* 77 of $[C_6H_5]^+$ for MA-Me, PGA-Me, IS-Me, and HA-Me or *m/z* 91 of $[CH_3C_6H_4]^+$ for MHAs-Me. Our mass spectrum agreed with the data of Ohashi et al.[8] From these mass spectra, each Me-derivative peak was assigned on the chromatograms, and each of base peaks was selected as the quantifier ion.

The proposed method was validated according to the US FDA guidance. The accuracy and precision values of the proposed method met the FDA criteria (Table 1). The proposed method was further validated by analyzing ClinChek. The obtained results showed that the proposed method had sufficient accuracy as the obtained mean values were within the certified confidence intervals (Table 2).

The proposed GC-MS method did not require complex and time-consuming sample preparation procedures (such as extraction and evaporation), a long analysis time, or large amounts of organic solvent compared to those of previous methods. The LOQs of the proposed method were higher than those of the previous methods, except for the methods reported by Szucs et al (GC-MS)[10] and Sperlingova et al (HPLC-UV),[14] but they were lower than 1/10 of the OEL-B or BEI values of each metabolite (calculated as that creatinine concentration is 1 g/L). If necessary, a lower LOQ could be achieved by the addition of the evaporation procedure after the dehydration reaction. If the analyte concentration exceeded the calibration range, the urine sample would then be diluted appropriately and re-analyzed.

When styrene and ethylbenzene are used at the same time in a workplace, it is difficult to determine which is a dominant solvent contributing to urinary concentrations of MA and PGA. A personal exposure monitoring, work environmental measurement, or measurement of unmetabolized solvent in urine are useful tools to determine the dominant solvent. Therefore, in addition to the proposed method, it is better to use such tools to evaluate exposure to each solvent.

In conclusion, we developed and validated a GC-MS method to determine urinary metabolites of toluene, xylene, styrene, and ethylbenzene simultaneously. The metabolites were methyl esterified in a single step with our procedure, and the sample was directly injected into GC-MS without further preparation. The proposed method is suitable for routine analysis, and potentially useful for biological monitoring of a large number of workers exposed to these solvents.

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DISCLOSURE

Approval of the research protocol: The protocol of this study was approved by the Ethics Committee of the Japan Industrial Safety and Health Association. *Informed consent*: Informed consent was obtained from the volunteers. *Registry and the registration no. of the study/trial:* N/A. *Animal studies:* N/A.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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