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Journal of Chromatography B, 857 (2007) 123–129
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Direct determination of glucuronide and sulfate of 4-hydroxy-3-methoxymethamphetamine, the main metabolite of MDMA, in human urine

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Received 30 January 2007; accepted 3 July 2007
Available online 10 July 2007

Abstract

A sensitive and reliable LC-ESI–MS procedure for the simultaneous determination of MDMA and its five metabolites including 4-hydroxy-3-methoxymethamphetamine (HMMA) conjugates has been established following the synthesis of two HMMA conjugates, 4-hydroxy-3-methoxymethamphetamine-glucuronide (HMMA-Glu) and 4-hydroxy-3-methoxymethamphetamine-sulfate (HMMA-Sul). Pretreatment of urine samples with methanol and LC–MS employing a C_{18} semi-micro column with a gradient elution program provided the successful separations and MS determinations of these analytes within 20 min. Upon applying the method to MDMA users' urine specimens, HMMA-Glu and HMMA-Sul have been directly determined, suggesting the superiority of sulfation to glucuronidation in the HMMA phase II metabolism.

Keywords: MDMA; 4-Hydroxy-3-methoxymethamphetamine; Sulfate; Glucuronide; LC–MS

1. Introduction

3,4-Methylenedioxymethamphetamine (MDMA), which was described as a new drug class "entactogens" by Nichols, has been abused to enhance understanding, communicativeness and empathy almost without showing hallucinogenic effects [1]. It is well-known by the street name of "Ecstasy" and is recently the most popular recreational drug worldwide.

MDMA is also well-known as a potent releaser and/or reuptake inhibitor of presynaptic serotonin, dopamine and norephedrine, and these actions contribute stimulation of the central nervous system [2–4]. The most frequent pharmacological effects after its administration are euphoria, well-being, stimulation, increased sociability, changed perception of colors and sounds, heart rate increase, and lack of appetite. Its overdose often causes severe intoxication effects such as coma, hallucinogenic symptoms and seizure [4–7].

In 1985, the U.S. Drug Enforcement Administration (DEA) classified MDMA as a Schedule 1 drug due to its high abuse potential, lack of clinical application, lack of accepted safety for use under medical supervision. In 1989, the Japanese authorities also banned this substance under the narcotics control law to prevent its recreational use. Nevertheless, the abuse among young people has been increasing throughout the world, and deaths by acute intoxications are frequently reported. Thus, the proof of MDMA use is indispensable for enforcing strict regulations of the abuse [8–16].

For unequivocal proof of MDMA use, detection of its metabolites along with MDMA is generally performed in urine samples. Several metabolites including 3,4-methylenedioxyamphetamine (MDA), 4-hydroxy-3-methoxymethamphetamine (HMMA), 3,4-dihydroxymethamphetamine (DHMA), 3-hydroxy-4-methoxymethamphetamine (3-OH-4-MeO-MA) have been identified in human urine by GC–MS (Fig. 1) [17]. In particular, HMMA, a compound resulting from the O-methylation of DHMA, appears as a major metabolite in urine samples, and its urinary recovery in 24 h accounted for a 23% of the dose [11]. Several studies [12,18–20] have reported

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doi:10.1016/j.jchromb.2007.07.003

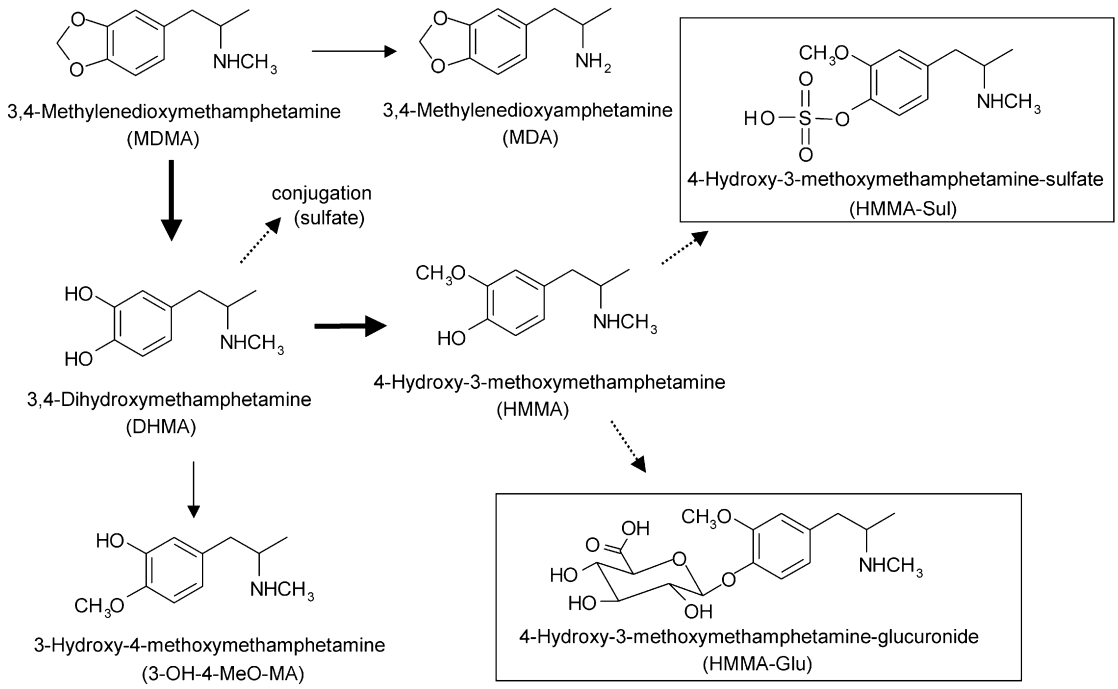

Fig. 1. Principal metabolic pathways of MDMA in humans.

that HMMA notably increased after acid/enzymatic hydrolysis, suggesting that the majority of HMMA was eliminated as the glucuronide/sulfate. These reports compared the urinary HMMA level with and without hydrolysis, but did not directly prove the existence of conjugates. No discussion has appeared on the precise concentrations and ratios of HMMA-glucuronide (HMMA-Glu) and HMMA-sulfate (HMMA-Sul).

In the present study, the authentic standards for HMMA-Glu and HMMA-Sul were, for the first time, synthesized and used in establishing the LC-electrospray ionization (ESI)–MS procedure and simultaneously and directly determining MDMA and its five metabolites including the conjugates. Utilizing the optimized method, urinary levels of HMMA-Glu and HMMA-Sul have been directly measured in MDMA users. Further studies utilizing many more urine samples will provide better knowledge of the MDMA metabolism in human.

2. Experimental

2.1. Chemicals and reagents

HMMA-Glu and HMMA-Sul were both synthesized in our laboratory by modifying our previously described methods [21] as detailed in the subsequent section. HMMA and 3-OH-4-MeO-MA were synthesized according to the methods of Lim and Foltz [22]. MDA and MDMA were synthesized according to the methods of Shulgin et al. [23,24]. All synthesized standards were ensured to be >98% pure based on percentage peak area of the target product from total peak area obtained by LC–MS analysis (a flow-injection method). *N*-methylbenzylamine (MBA), used as an internal standard (IS), was obtained from Wako Pure Chemical Industries. Methyl (2,3,4-tri-O-acetyl-1-bromo-1-deoxy-alpha-D-glucopyranosid)uronate was synthesized according to the methods of Bollenback et al. [25]. Sulfur trioxide pyridine complex was purchased from Aldrich. Chromatorex® NH-DM 1020 (aminosilicagel) and Wakogel® C-200 (silicagel) were obtained from Fuji Silysia Chemical (Kasugai, Japan) and Wako Pure Chemical Industries, respectively.

All organic and inorganic reagents used were of analytical grade or better. Distilled water and HPLC-grade methanol were used throughout the experiments.

2.2. Chemical syntheses of glucuronide and sulfate of HMMA

2.2.1. Synthesis and characterization of 4-hydroxy-3-methoxymethamphetamine-O-β-glucuronide (HMMA-Glu)

To 2.5 ml of ice-cold dry methanol solution containing 500 mg of HMMA was added 0.5 ml of a solution of 28% sodium methoxide in methanol. The mixture was stirred at 0 °C for 30 min under an atmosphere of nitrogen and then added all at once to a solution of 1 g of methyl (2,3,4-tri-O-acetyl-1-bromo-1-deoxy-alpha-D-glucopyranosid)uronate in 5 ml of methanol at 0 °C. The mixture was stirred under nitrogen for 2 h, during which time the mixture was allowed to reach room temperature. The reaction mixture was adjusted to pH 12 with 1N NaOH and stirred for 1 h. The mixture was passed through an aminosilicagel column after it was neutralized with acetic acid. The eluate by methanol was evaporated, and the residue was successively purified by silicagel column chromatography utilizing methanol as the effluent solvent. The fractions were combined, and the sol-

vent was evaporated to dryness *in vacuo* to give 56 mg (total yield is 6%) of colorless needles.

^{13}C-NMR (75 MHz, D2O) δ 175.68 (C), 149.25 (C), 144.88 (C), 131.81 (C), 122.53 (CH), 116.93 (CH/2), 116.82 (CH/2), 114.22 (CH/2), 114.12 (CH/2), 100.66 (CH), 76.77 (CH), 75.75 (CH), 73.09 (CH), 72.08 (CH), 56.69 (CH_3), 56.28 (CH), 38.952 (CH_2), 30.43 (CH_3), 15.43 (CH_3).

^{1}H-NMR (300 MHz, D2O) δ 7.14(1H, d, J = 8.3), 6.96(1H, d, J = 1.9), 6.85(1H, dd, J = 1.9, 8.3), 5.12, (1H, d, J = 6.6), 3.86(3H, s), 3.87–3.81 (1H, m), 3.68–3.53 (3H, m), 3.49–3.4 (1H, m), 2.97(1H, dd, J = 6.5, 14.0), 2.83 (1H, dd, J = 7.5, 14.0), 2.65(3H, s), 1.24 (3H, d, J = 6.9).

2.2.2. Synthesis and characterization of 4-hydroxy-3-methoxymethamphetamine-O-sulfate (HMMA-Sul)

To the solution of 1 g HMMA in 20 ml of pyridine was added 1.8 g of a pyridine-sulfur trioxide complex, and the mixture was stirred at 0–5 °C for 48 h. After stirring, the precipitating solid in the reaction mixture was filtered off and washed with diethyl ether, then dried *in vacuo* to give 920 mg (total yield is 65%) of colorless leaflets.

^{13}C-NMR (75 MHz, D2O) δ 151.90 (C), 139.52 (C), 135.34 (C), 123.51 (CH), 122.31 (CH), 114.82 (CH), 56.75(CH_3), 56.49 (CH), 39.044(CH_2), 30.37(CH_3), 15.35(CH_3).

^{1}H-NMR (300 MHz, D2O) δ 7.43 (1H, d, J = 8.1), 7.13 (1H, d, J = 1.8), 6.99 (1H, dd, J = 1.8, 8.1), 3.96(3H, s), 3.68–3.6 (1H, m), 3.13 (1H, dd, J = 13.8, 6.5), 3.01 (1H, dd, J = 7.5, 13.8), 2.782 (3H, s), 1.379 (3H, d, J = 6.6).

2.3. Preparation of standard solutions

Individual standard stock solutions of 50 mmol/l for HMMA-Glu, HMMA-Sul, HMMA, 3-OH-4-MeO-MA, MDA and MDMA were prepared in methanol. Subsequently, standard mixtures (0.3, 1, 2, 5, 10, 20, 50, 100, 200, 300, 400 and 500 μmol/l each) were obtained by mixing six standard solutions and distilled water. In addition, MDMA working solutions were separately prepared at 1000, 3000, 8000, and 10,000 μmol/l from the stock solution by serial dilutions with distilled water. Each spiked urine specimen used for the method validation was prepared by addition of 100 μl of a standard mixture or working solution to 900 μl of a drug-free volunteer's urine.

2.4. Sample preparation

To 100 μl of a urine sample, 100 μl MBA aqueous solution (10 μmol/l) was added as the IS. After briefly mixing, 500 μl of methanol was added, and the mixture was vortex mixed for 1 min. The mixture was centrifuged for 10 min at 1500 × g, and the supernatant was transferred to a stoppered glass test tube. This was then evaporated to dryness under a nitrogen stream at 60 °C. The residue was dissolved in 100 μl of distilled water. After filtration through a 0.45 μm membrane filter, a 5 μl aliquot was injected into the LC–MS and LC–MS–MS systems.

2.5. Instrumentation

LC–MS was performed using a Shimadzu LCMS 2010A high-performance liquid chromatograph mass spectrometer equipped with an SIL-HTc auto sampler, three LC-10AD pumps, a CTO-10A column oven and an ESI interface (Shimadzu, Kyoto, Japan). For optimization of MS conditions, the ion intensity in the positive and negative ionization modes was measured by varying the Q-array voltage, which significantly affects the sensitivity and the fragmentation. Standard aqueous solutions of HMMA-Glu and HMMA-Sul (10 μmol/l each) were used as samples, and 5-μl aliquots were injected in the flow-injection mode. ESI-MS was performed in the positive mode under the following operating parameters: probe voltage, 4.5 kV; nebulizer nitrogen gas, 1.5 l/min; curved desolvation line (CDL) temperature, 200 °C; Q-array voltage, 5 V; Q-array RF, 150 V. Quantitative analysis was performed by monitoring six target ions for analytes (m/z 372 for HMMA-Glu, m/z 276 for HMMA-Sul, m/z 196 for HMMA and 3-OH-4-MeO-MA, m/z 194 for MDMA, m/z 180 for MDA and m/z 122 for IS) in the selected-ion monitoring (SIM) mode. The following qualifier ions were also monitored in the SIM mode: m/z 196 and 165 for HMMA-Sul, m/z 165 and 137 for HMMA and 3-OH-4-MeO-MA, m/z 163 and 135 for MDMA and MDA, m/z 91 for IS.

LC–MS–MS was carried out on an Agilent 1100 HPLC system (Agilent, Palo Alto, CA, USA) linked to a Quattro LC (Micromass, Manchester, UK) triple quadrupole mass spectrometer equipped with an ESI interface. ESI-MS was performed in the positive mode under the following operating parameters: capillary voltage, 3.3 kV; cone voltage, 30 V; ion source temperature, 280 °C. Collision-induced dissociation (CID) was performed using argon as the collision gas at a collision energy of 15 eV and a pressure of 2.0×10^{-3} mbar. MS–MS was run in the product ion scan mode, and ions of m/z 372 and 276, corresponding to the protonated molecules of HMMA-Glu and HMMA-Sul, respectively, were selected as precursor ions.

The chromatographic separation was performed on an L-column ODS semi-micro column (1.5 mm i.d. × 150 mm, 5 μm, Chemicals Evaluation and Research Institute, Tokyo, Japan). Each 25-min chromatographic run was carried out with a binary mobile phase of methanol and 10 mM ammonium formate buffer (pH 3.5) using a linear gradient (15–35% methanol). The column was equilibrated with 15% methanol for 15 min before the subsequent injection.

All nuclear magnetic resonance (NMR) spectra of the synthetic compounds were acquired using a JEOL (Kyoto, Japan) JNM-ECA700 in D_2O.

2.6. Method validation

Extraction efficiency was evaluated using a drug-free urine sample spiked with the analytes at 10 μmol/l each (n = 5). The recoveries were calculated by comparing the peak areas of the analytes extracted from a spiked urine sample and those from a diluted standard solution (10 μmol/l each). Limit of detection (LOD) were defined as the detection limits of the target and qualifier ion peaks on each mass chromatogram in the SIM mode

(S/N ≧ 3). Calibration curves ranged from 0.1 to 50 μmol/l (0.1, 0.2, 0.5, 2.0, 5.0, 20 and 50 μmol/l) for HMMA-Glu, HMMA-Sul and MDA, from 0.03 to 20 μmol/l (0.03, 0.1, 0.2, 0.5, 2.0, 5.0 and 20 μmol/l) for HMMA and 3-OH-4-MeO-MA, and from 10 to 1000 μmol/l (10, 30, 100, 300 and 1000 μmol/l) for MDMA. Accuracy and precision were estimated at two or three different concentrations (1, 10, 40 and 800 μmol/l) with five replicates for each level, analyzed in the same day (the within-day study) and on five different days (the between-day study). Precision was calculated in terms of relative standard deviation (RSD, %), and accuracy in terms of relative error (%) between the measured and the spiked concentrations. To assess any potential suppression of ionization from components present in the extracted urinary matrix, a decrease in response was calculated by comparing the ion intensity of each analyte (10 μmol/l each, $n = 5$) spiked in distilled water and urinary extract from five different urine samples.

2.7. Urine specimens

Two MDMA users' urine specimens included in this study had been submitted to our laboratory for forensic analysis. These samples were stored at −20 °C until analysis.

3. Results and discussion

3.1. Procedure optimization

3.1.1. MS conditions

The optimization of the ESI-MS conditions for direct determination of HMMA-Glu and HMMA-Sul was performed using a Shimadzu LC–MS QP2010A.

HMMA-Glu contains both an amino and a carboxyl group, which would be easily protonated and deprotonated, respectively. HMMA-Sul similarly has an amino and a sulfate group. The selection of a suitable ionization mode will be therefore required for the sensitive detection of the conjugates. The predominant $[M+H]^+$ and $[M-H]^-$ ions were observed in the positive and negative modes, respectively. As shown in Fig. 2, over 30 and 10 times higher sensitivities for the sulfate and glucuronide have been respectively obtained in the positive mode than in the negative mode.

The highest intensity of $[M+H]^+$ for either conjugate was attained at a 5 V Q-array voltage in the positive mode (Fig. 2), and the mass spectra were obtained as shown in Fig. 3. The sodium-adducted molecule $[M+Na]^+$ was observed for HMMA-Glu at *m/z* 394. The ion intensity decreased significantly at voltages higher than 30 V. The higher voltage resulted in a higher cleavage to produce a few structural fragment ions at *m/z* 196 and 165 for HMMA-Sul, but lowered the sensitivity. On the other hand, no fragment ion for HMMA-Glu was observed at any voltage. Based on these results, we have finally chosen the following parameters as the optimum conditions for determination in the SIM mode: ionization mode, positive; Q-array voltage, 5 V; target ion, *m/z* 372 for HMMA-Glu and *m/z* 276 for HMMA-Sul.

3.1.2. LC conditions

In order to simultaneously determine MDMA and its five metabolites, MDA, HMMA, 3-OH-4-MeO-MA, HMMA-Glu and HMMA-Sul, the optimization of the mobile phase was explored. In our previous study on the LC-MS determination

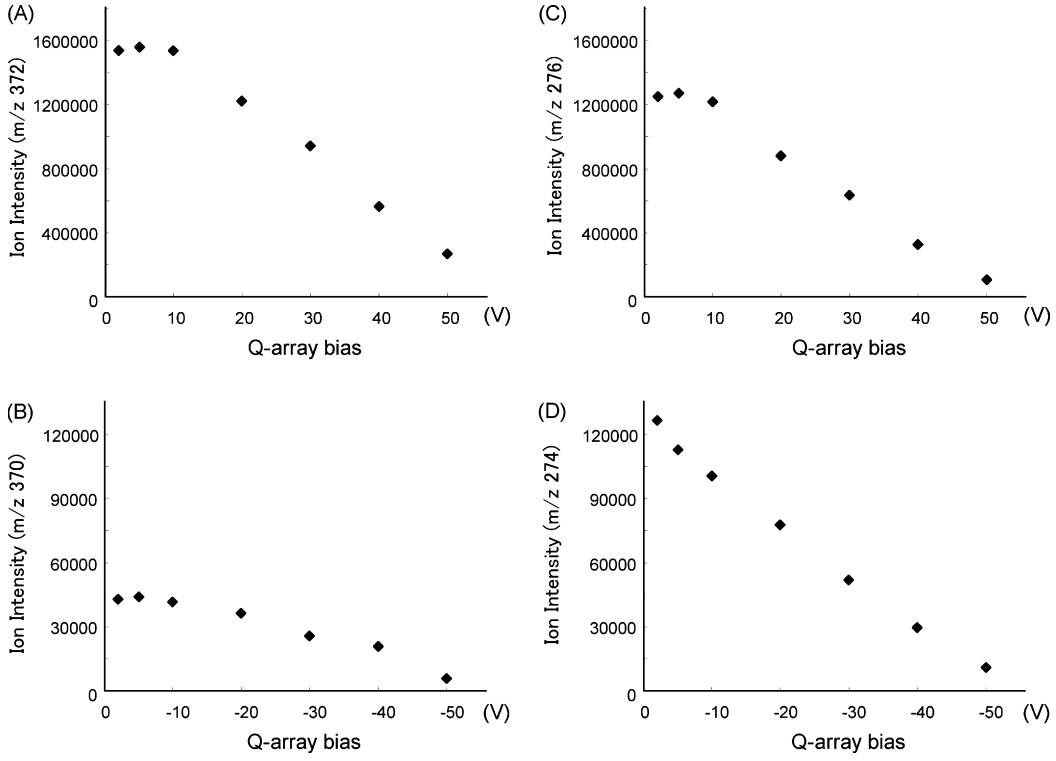

Fig. 2. Effect of Q-array voltage on ion intensity obtained from HMMA-Glu in the positive (A) and the negative (B) modes, and HMMA-Sul in the positive (C) and the negative (D) modes.

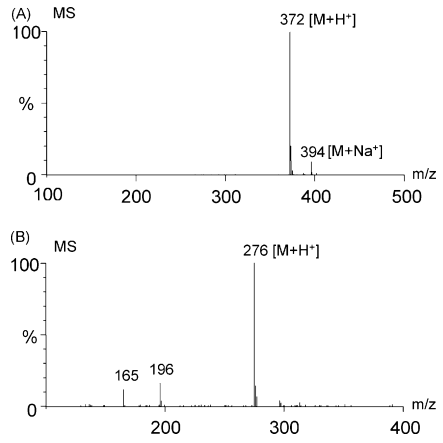

Fig. 3. Mass spectra obtained from HMMA-Glu (A) and HMMA-Sul (B) in the full-scan mode. ESI-MS was performed at the Q-array voltage of 5 V in the positive mode.

of MA and its metabolites including *p*-OHMA-Glu and *p*-OHMA-Sul [21], the linear gradient eluent program of 10 mM ammonium formate buffer (adjusted to pH 3.5 with formic acid)-methanol (5% to 40% methanol in 20 min) was proposed as the optimal eluent. In the pre-experiment, we tested this eluent to examine if it is applicable to the present study. However, more than 20 min was required for the elution of MDA and MDMA.

For improvement of the retention characteristics, the gradient eluent systems were investigated. Consequently, the linear gradient eluent program (15–35% methanol in 25 min) provided successful elutions of MDMA and its five metabolites along with the IS within 20 min (Fig. 4).

3.1.3. Extraction process

All of the six analytes showed high recoveries by the pretreatment with methanol (Table 1). In addition, the technique is extremely simple and rapid, however, it allowed us to analyze without any significant disturbance by the urinary components obtained from a drug-free urine sample ($n = 10$). We then chose the pretreatment with methanol as the sample preparation method in the present study.

3.2. Method validation

Table 1 lists the validation data evaluated by analyzing the spiked urine samples and diluted standard solutions at known concentrations. The obtained values for accuracy and precision were less than 15% for all concentration levels, falling within the criteria accepted internationally [26]. Ion suppression can lead to variable sensitivities and decreased precision and accuracy. Therefore, a decrease in response was calculated as described in the experimental section. Consequently, no significant changes in responses were observed (less than 10%, data not shown). The results confirm the usefulness of pretreatment procedure to obtain reproducible and reliable quantitative results for all analytes without major interference of matrix compounds.

When the values for urinary concentrations of analytes in MDMA user's urine are above the calibration range shown in Table 1 the concentrations should be determined after diluting with a drug-free urine to appropriate concentration.

3.3. Analysis of HMMA-Glu and HMMASul in urine from MDMA users

The optimized LC-ESI–MS analysis was applied to two MDMA users' urine samples, which were already identified as MDMA positive by GC–MS in our laboratory. Consequently, HMMA-Glu and HMMA-Sul were detected at the retention time of 3.6 and 6.1 min, respectively (Fig. 5). The urinary concentrations of these conjugates were much higher than that of HMMA (free form) in both samples as shown in Table 2. These data indicate that the glucuronidation and sulfation are the major routes of phase II conjugation of HMMA and are quite consistent with previous reports [18–20] that showed the majority of HMMA being excreted as the conjugates in human urine. The levels of the two conjugates suggest that the sulfation is quantitatively more important than glucuronidation for the conjugation of HMMA in humans.

On the other hand, 3-OH-4-MeO-MA, the HMMA isomer, was also eliminated in both samples; however, the levels were less than one-tenth of HMMA (free form) (Table 2). These data lend support to a previous report [17] that showed DHMA, the demethylanated metabolite of MDMA, is O-methylated by the enzyme catechol-O-methyltransferase (COMT) mainly to HMMA. In careful examination, two specific peaks (Peaks a′ and b′) were observed in the extracted mass chromatograms (*m/z* 372 and 276) as shown in Fig. 5. Unknown components corresponding to Peak a' and b' were therefore subjected to an LC-ESI–MS–MS along with HMMA-Glu (Peak a) and HMMA-

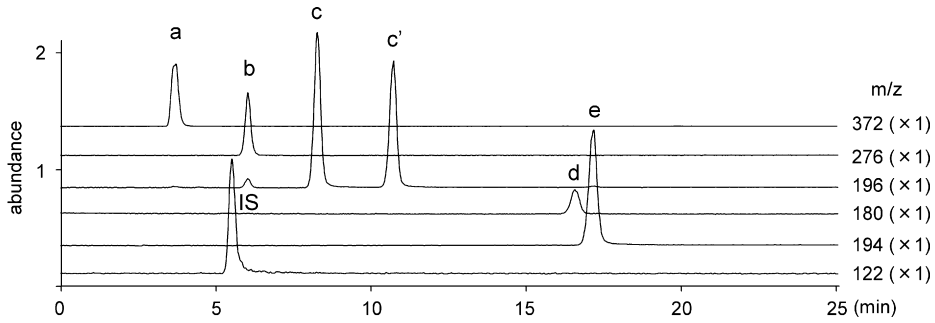

Fig. 4. Extracted mass chromatograms obtained from the standard mixed solution of MDMA and its five metabolites (10 μmol/l each) with the linear gradient eluent program (15%–35% methanol in 25 min). The numbers in parentheses represent magnifications. Peaks: (a) HMMA-Glu; (b) HMMA-Sul; (c) HMMA; (c′) 3-OH-4-MeO-MA; (d) MDA; and (e) MDMA.

Table 1
Validation data for the established procedure

Analyte	HMMA-Glu	HMMA-Sul	HMMA	3-OH-4-MeO-MA	MDA	MDMA
Recovery (%) (10 μmol/l)[#]	89	85	95	93	87	95
Estimated limits of detection (nmol/l)						
SIM mode	10	10	5	5	50	5
Linearity range (μmol/l)	0.1–50	0.1–50	0.03–20	0.03–20	0.1–50	10–1000
RSD (%)	4.1	5.8	6.3	4.3	5.1	3.8
Accuracy relative error (%)						
(1.0 μmol/l)[#]	8.1	2.7	−1.2	1.4	4.3	–
(10 μmol/l)[#]	3.4	4.2	−2.2	3.1	−1.3	2.8
(40 μmol/l)[#]	2.3	−5.2	–	–	−2.5	4.3
(800 μmol/l)[#]	–	–	–	–	–	−4.2
Pecision RSD (%)						
Within-day						
(1.0 μmol/l)[#]	7.2	5.4	7.9	6.1	10.1	–
(10 μmol/l)[#]	4.8	6.6	7.2	6.9	5.8	5.1
(40 μmol/l)[#]	5.5	8.3	–	–	9.8	8.8
(800 μmol/l)[#]	–	–	–	–	–	8.7
Between-day						
(1.0 μmol/l)[#]	9.3	4.2	8.8	5.3	9.8	–
(10 μmol/l)[#]	6.9	8.3	8.2	8.9	7.7	6.1
(40 μmol/l)[#]	5.2	8.2	–	–	9.2	8.5
(800 μmol/l)[#]	–	–	–	–	–	9.2

[#] Evaluated using a drug-free urine sample spiked with the analytes ($n = 5$). (–: not calibrated).

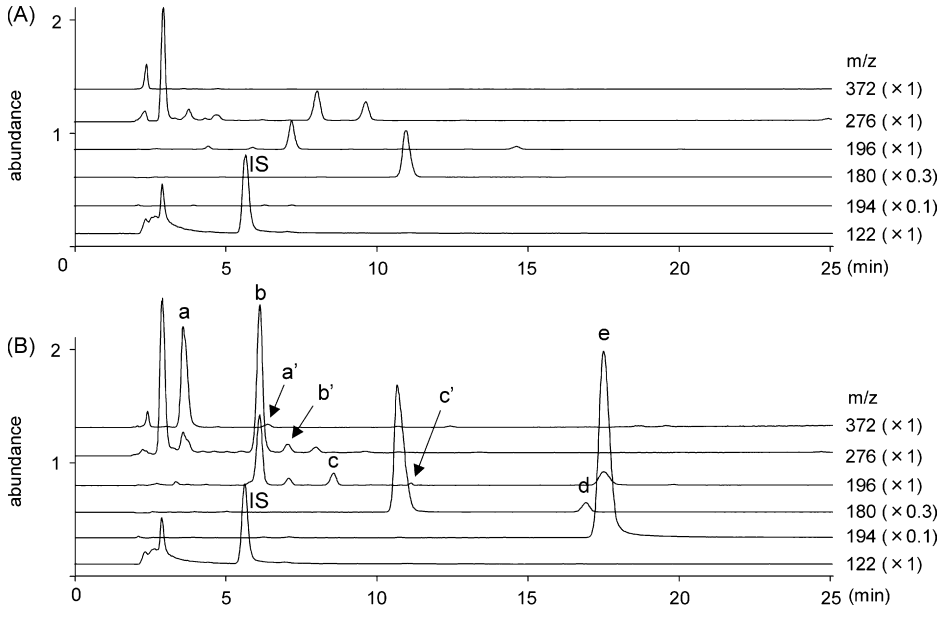

Fig. 5. Extracted mass chromatograms obtained from (A) a drug-free and (B) an MDMA user's (Subject 1) urine sample. The samples were prepared and analyzed as described in the Experimental section. The numbers in parentheses represent magnifications. Peaks: (a) HMMA-Glu; (b) HMMA-Sul; (c) HMMA; (c′) 3-OH-4-MeO-MA; (d) MDA; and (e) MDMA.

Table 2
Urinary concentrations of MDMA and its metabolites in urine samples from two MDMA users

Subject	MDMA (μmol/l)	MDA (μmol/l)	HMMA (free) (μmol/l)	HMMA-Glu (μmol/l)	HMMA-Sul (μmol/l)	3-OH-4-MeO-MA (free) (μmol/l)
1	562	45.3	2.2	37.1	71.6	0.13
2	254	7.6	7.2	37.0	91.4	0.30

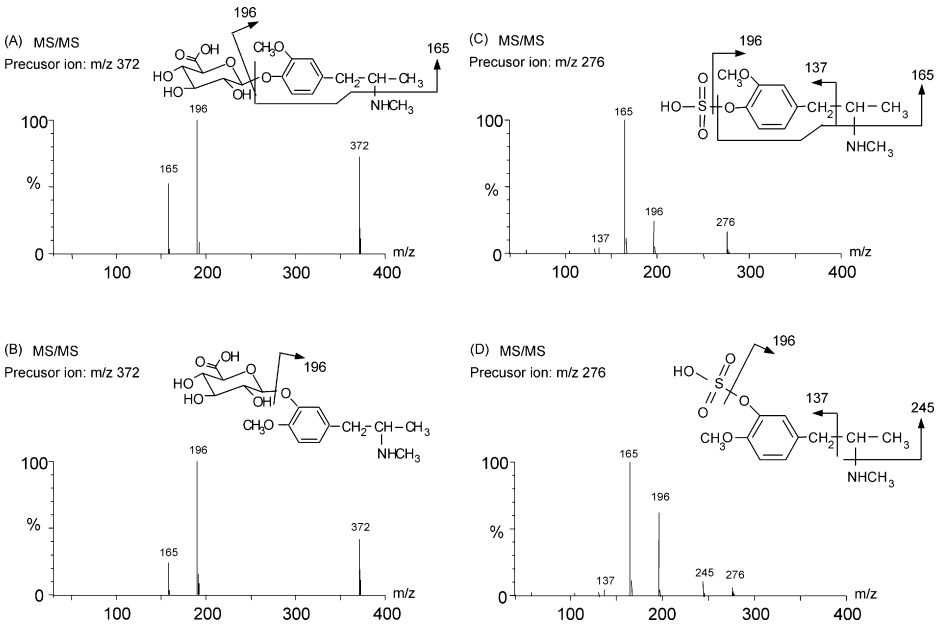

Fig. 6. Product ion spectra obtained from HMMA-Glu (A), Peak a′ in Fig. 5 (B), HMMA-Sul (C) and Peak b′ in Fig. 5 (D). Peaks a′and b′ were assigned to glucuronide and sulfate of 3-OH-4-MeO-MA, respectively.

Sul (Peak b) by selecting the protonated molecules (*m/z* 372 for Peak a and a′, *m/z* 276 for Peak b and b′) as the precursor ions. As shown in Fig. 6, the product ion spectra from Peaks a′ (B) and b′ (D) were closely similar to those from HMMA-Glu (A) and HMMA-Sul (D), respectively. Based on these data, we have assigned glucuronide and sulfate of 3-OH-4-MeO-MA to Peaks a′ and b′, respectively.

4. Conclusion

This is a report of the direct detection of HMMA-Glu and HMMA-Sul in human urine as well as their synthesis. In the present study, a sensitive LC-ESI–MS procedure with a simple pretreatment was established for the simultaneous determination of MDMA and its five metabolites, MDA, HMMA, 3-OH-4-MeO-MA, HMMA-Glu and HMMA-Sul, in urine. In addition, the conjugates of HMMA, HMMA-Glu and HMMA-Sul, have been directly identified in MDMA users' urine.

Based on the urinary levels of the conjugates in two MDMA users, the sulfation may be superior to the glucuronidation in humans. No obvious differences in the ratios of the urinary conjugate levels were observed between two samples employed in this study. Interindividual variation may, however, exist in the phase II metabolism of HMMA in humans. Further studies utilizing many more urine samples from various ages and races of MDMA users are now in progress.

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