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Direct determination of glucuronide and sulfate of *p*-hydroxymethamphetamine in methamphetamine users' urine

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Abstract

Two conjugates of *p*-hydroxymethamphetamine (*p*-OHMA), *p*-OHMA-glucuronide (*p*-OHMA-Glu) and *p*-OHMA-sulfate (*p*-OHMA-Sul) have been identified in methamphetamine (MA) users' urine by using liquid chromatography-high resolution tandem mass spectrometry (LC–HRMS-MS). The synthesis of *p*-OHMA-Glu and *p*-OHMA-Sul, and an LC–MS procedure for the simultaneous determination of MA and its four metabolites, amphetamine (AP), *p*-OHMA, *p*-OHMA-Glu and *p*-OHMA-Sul, in urine have also been established. After deproteinizing urine samples with methanol, 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. Based on the established method, *p*-OHMA-Sul was detected at higher concentrations than *p*-OHMA-Glu in all of the three urine samples tested. These data suggest that sulfation is a major pathway in the MA phase II metabolism.

Keywords: Methamphetamine; *p*-Hydroxymethamphetamine; Sulfate; Glucuronide; Urine

1. Introduction

Methamphetamine (MA) is the most prevalent illicit drug in Asian countries including Japan [1], and is causing a serious social problem. At present, the identification of MA and its metabolites, amphetamine (AP) and/or *p*-hydroxymethamphetamine (*p*-OHMA) in urine by gas chromatography–mass spectrometry (GC–MS) and/or liquid chromatography–mass spectrometry (LC–MS) is employed for determining MA use.

Several studies on the metabolism of MA have been conducted since the 1940s [2–8]. Concerning the excretion of MA and its metabolites in urine, unchanged MA, which is eliminated with the highest concentrations [2,3], accounts for about 18–27% of a dose, while about 14–16% is excreted as *p*-OHMA and its conjugates, 2–3% for AP, and lesser amounts of other metabolites. Thus, *p*-OHMA is the most abundant metabolite of MA in human urine [3]. They also found that most of *p*-OHMA was eliminated as conjugates, because of its increased levels after hydrolysis with HCl [3,4]. Also, based on the comparison with the hydrolysis using β-glucuronidase, most of the conjugates were thought to be attributed to the glucuronidation [4,5]. However, it is well known that sulfation is predominant in the conjugation of phenol [9]. This suggested that sulfation may be a major phase II metabolic pathway of MA in humans. However, there has been no report on the direct identification of the conjugates of *p*-OHMA in human urine.

In this study, we identified two conjugates of *p*-OHMA, glucuronide and sulfate, in MA users' urine by utilizing liquid chromatography–high resolution tandem mass spectrometry (LC–HRMS-MS). The authentic standards of these metabolites were synthesized for the first time and used in establishing the LC–ESI-MS procedure and simultaneously determining MA and its four metabolites including the conjugates. Utilizing the optimized method, urinary excretion patterns of *p*-OHMA-Glu and *p*-OHMA-Sul have also been investigated for three MA users' urine samples.

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2. Experimental

2.1. Reagents

p-OHMA-Glu and *p*-OHMA-Sul were both synthesized in our laboratory by modifying the methods of Yoshimura et al. and Ohkawa et al. as described below [10–13]. MA hydrochloride and AP sulfate were purchased from Dainippon Pharmaceutical (Osaka, Japan) and Takeda Pharmaceutical (Osaka, Japan), respectively. The hydrochloride salt of *p*-OHMA was synthesized according to the method of Buzas et al. [14]. β-Phenethylamine (PEA), used as an internal standard (IS), was obtained from Tokyo Kasei (Tokyo, Japan). Sulfur trioxide pyridine complex was purchased from Aldrich (Milwaukee, WI, USA). Chromatorex® NH-DM 1020 (aminosilicagel) and Wakogel® C-200 (silicagel) were obtained from Fuji Silysia Chemical (Kasugai, Japan) and Wako Pure Chemical Industries (Osaka, Japan), respectively. Stock standard solutions of all these substances were prepared in methanol, and were diluted with distilled water to appropriate concentrations as needed. All organic and inorganic reagents used were of analytical grade or better quality. Distilled water and HPLC methanol were used throughout the experiments.

2.2. Instrumentation

LC–MS–MS for identification was carried out on a Shimadzu LCMS-IT-TOF equipped with an SIL-10AD auto injector, two LC-10AD pumps, a CTO-10A column oven and an electrospray ionization (ESI) interface (Shimadzu, Kyoto, Japan). 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; ion trapping time, 30 ms; isolation time, 20 ms; collision-induced dissociation (CID) energy, 50%; collision gas, 50%; CID time, 30 ms. The column used was an L-column ODS semi-micro column (1.5 mm i.d. × 150 mm, Chemicals Evaluation and Research Institute, Tokyo, Japan). The mobile phase consisted of methanol–10 mM ammonium formate buffer (pH 3.5) (5/95, v/v).

LC–MS for the quantitative analysis was performed on a Shimadzu LCMS QP2010A high-performance liquid chromatograph mass spectrometer equipped with an SIL-HTc auto sampler, three LC-10AD pumps, a CTO-10A column oven and ESI interface (Shimadzu). 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; CDL temperature, 200 °C; Q-array DC, 10 V; Q-array RF, 150 V. The column used was an L-column ODS semi-micro column. Each 20-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 (5–40% methanol). The quantitative analysis was performed by monitoring six ions of interest (*m*/*z* 342 for *p*-OHMA-Glu, *m*/*z* 246 for *p*-OHMA-Sul, *m*/*z* 166 for *p*-OHMA, *m*/*z* 150 for MA, *m*/*z* 136 for AP and *m*/*z* 122 for PEA) in the selected-ion monitoring (SIM) mode.

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

2.3. Syntheses of glucuronide and sulfate of p-OHMA

2.3.1. Synthesis and characterization of p-hydroxymethamphetamine-O-β-glucuronide (p-OHMA-Glu)

To a solution of 500 mg of *p*-OHMA in 2.5 ml of 2 N NaOH, a solution of 1.6 g of methyl (2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-alpha-D-glucopyranosid) uronate (A) in 5 ml of acetone was added and the mixture was allowed to stand overnight. It formed two layers, i.e. aqueous (lower) and acetone (upper) layer. With the addition of 1 ml of 30% NaOH, the unchanged *p*-OHMA was again dissolved by stirring. To this solution, a solution of A in 2 ml of acetone was added and the mixture was allowed to stand overnight. These procedures were repeated seven times (a total of 6.6 g of A was used against 500 mg of *p*-OHMA). The reaction mixture was adjusted to pH 9 with AcOH and extracted three times with 20 ml of $CHCl_3$–iso-PrOH (3:1) to remove any unchanged *p*-OHMA. The aqueous layer was passed through an aminosilicagel column. Elution with methanol and removal of the solvent produced a viscous residue. The residue was successively purified by aminosilicagel column chromatography and by silicagel column chromatography utilizing methanol as the effluent solvent. The fractions were combined, and the solvent was evaporated to dryness in vacuo to give 124 mg (total yield is 12%) of colorless needles.

^{13}C NMR (D_2O) δ177.95 (C), 158.41 (C), 133.40 (CH × 2), 132.92 (C), 119.63 (CH × 2), 102.65 (CH), 78.86 (CH), 77.97 (CH), 75.36 (CH), 74.32 (CH), 58.99 (CH), 40.58 (CH2/2), 40.51 (CH2/2), 32.45 (CH3), 17.38 (CH3/2), 17.36 (CH3/2).

^{1}H NMR (D_2O) δ1.206 (3H/2, d, J = 6.9 Hz), 1.211 (3H/2, d, J = 6.8 Hz), 2.644 (3H/2, s), 2.647 (3H/2, s), 2.82 (1H, dd, J = 8.1 and 14.0), 2.948 (H/2, dd, J = 6.0 and 14.0), 2.954 (H/2, dd, J = 6.4 and 14.0), 3.40–3.46 (1H, m), 3.55–3.61 (3H, m), 3.84 (1H, d, J = 9.2), 5.08 (1H, d, J = 7.2 Hz), 7.08 (2H, d, J = 8.6), 7.12 (H, d, J = 8.6).

2.3.2. Synthesis and characterization of p-hydroxymethamphetamine-O-sulfate (p-OHMA-Sul)

To the solution of 1 g *p*-OHMA in 20 ml of pyridine, 1.8 g of a pyridine–sulfur trioxide complex was added 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 960 mg (total yield is 65%) of colorless leaflets.

^{13}C NMR (D_2O) δ153.00 (C), 136.19 (C), 133.35 (CH × 2), 124.58 (CH × 2), 58.96 (CH), 40.75 (CH2), 32.48 (CH3), 17.41(CH3).

^{1}H NMR (D_2O) δ1.26 (3H, d, J = 6.4 Hz), 2.67 (3H, s), 2.92 (1H, dd, J = 7.6 and 14.0 Hz), 3.04 (1H, dd, J = 6.4 and 14.0 Hz), 3.52 (1H, ddq, J = 6.4, 6.4 and 7.6 Hz), 7.28 (2H, d, J = 8.4 Hz), 7.32 (2H, d, J = 8.4 Hz).

2.4. Sample preparation

To 100 μl of a urine sample, 100 μl of a PEA aqueous solution (1 μg/ml) was added as the IS, and the mixture was deproteinized by adding methanol (500 μl) with vortex mixing 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 automatically injected into the LC–MS and LC–MS–MS systems.

2.5. Urine specimens

Three MA 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. Spiked urine specimens used for the method validation were prepared by adding known amounts of authentic *p*-OHMA-Glu, *p*-OHMA-Sul, *p*-OHMA, MA and AP to drug-free volunteer's urine.

3. Results and discussion

3.1. Identification of p-OHMA-Glu and p-OHMA-Sul by LC–MS–MS

In order to identify the conjugates of *p*-OHMA, the MA users' urine samples were subjected to a high resolution mass spectrometric analysis using a Shimadzu LCMS-IT-TOF. As a result, two specific peaks (labeled Peaks 1 and 2) characterized by predominant ions at *m*/*z* 342.156 and 246.078 were detected at the retention time of 5 and 10.5 min, respectively. The measured accurate mass of each ion has been found to well correlate to the calculated exact mass of $[C_{16}H_{24}NO_7]^+$ (342.155) and $[C_{10}H_{16}NO_4S]^+$ (246.080), which correspond with the protonated molecules of *p*-OHMA-Glu and *p*-OHMA-Sul, respectively (Fig. 1).

The two peaks were further subjected to MS–MS analysis by selecting each base ion (*m*/*z* 342.156 for Peak 1 and *m*/*z* 246.078 for Peak 2) as a precursor ion. The results are summarized in Fig. 1 and show that the metabolite corresponding to Peak 1 was the glucuronide conjugate, as indicated by the loss of 176 of the glucuronyl group to produce the $[M+H]^+$ at *m*/*z* 166.122 of *p*-OHMA (calculated exact mass being 166.123) and its characteristic substructural ion at *m*/*z* 135.084 (calculated being 135.081). They also show that the metabolite corresponding to Peak 2 was the sulfate conjugate as indicated by the loss of 80 of the sulfonate group to produce the $[M+H]^+$ at *m*/*z* 166.120 of *p*-OHMA and its characteristic substructural ions at *m*/*z* 135.079, as well as by the loss of an amino-group and the α-cleavage to produce ions at *m*/*z* 215.037 (calculated being 215.038) and *m*/*z* 187.006 (calculated being 187.007), respectively.

In addition, the MS–MS–MS analysis was accomplished by selecting the ions at *m*/*z* 166.122 for Peak 1 and *m*/*z* 166.120 for Peak 2 as the precursor ions under the same CID conditions as those of MS–MS, and the resultant fragmentation profiles were both extremely similar to that of *p*-OHMA (data not shown).

To confirm the above identification, the authentic standards of glucuronide and sulfate of *p*-OHMA were synthesized and then subjected to the LC–MS and LC–MS–MS under the same conditions as the urine sample. The retention characteristics and mass

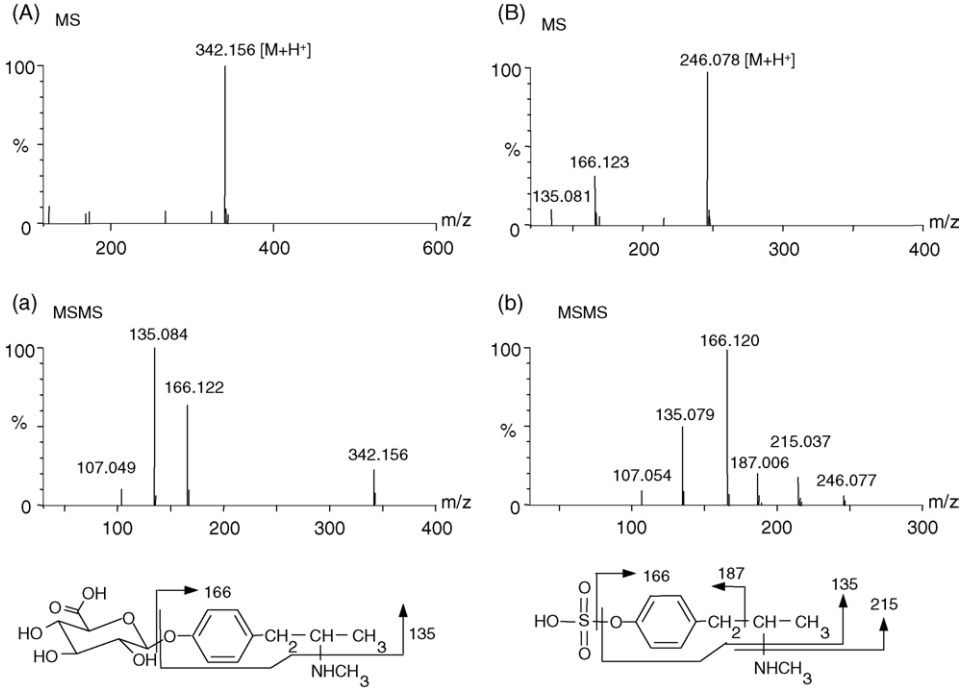

Fig. 1. Mass spectra obtained from Peak 1 (A) and Peak 2 (B), and their product ion spectra produced from their precursor ions at *m*/*z* 342.156 (a) and 246.078 (b), respectively. Peaks 1 and 2 responded to *p*-OHMA-Glu and *p*-OHMA-Sul.

spectra from Peaks 1 and 2 were identical to those of the authentic *p*-OHMA-Glu and *p*-OHMA-Sul, respectively. Finally, we have unequivocally identified the metabolites corresponding to Peaks 1 and 2 as *p*-OHMA-Glu and *p*-OHMA-Sul, respectively.

In further studies, other conjugates of *p*-OHMA including glycoside and methylate were monitored by the precursor ion scan. However, no other significant conjugates were detected in the urine samples.

Based on the above examinations, the excretion of not only *p*-OHMA-Glu, but also *p*-OHMA-Sul has been demonstrated in the MA users' urine.

3.2. Procedure optimisation

3.2.1. MS conditions

The optimization of the ESI-MS conditions for the determination of *p*-OHMA-Glu and *p*-OHMA-Sul was performed using a Shimadzu LC-MS QP2010A.

p-OHMA-Glu contains both an amino and a carboxyl group, which would be easily protonated and deprotonated, respectively. Also, *p*-OHMA-Sul has an amino and a sulfate group, and it would be easily charged in the same manner. For the sensitive detection of the conjugates, the selection of a suitable ionization mode will be required. Thus, the ion intensity obtained from the conjugates in the positive and negative ionization modes was measured by varying the Q-array voltage, which significantly affects the sensitivity and the fragmentation, and then the difference in sensitivity between both modes was compared. As samples, standard aqueous solutions of *p*-OHMA-Glu and *p*-OHMA-Sul (1 μg/ml each) were used, and 5 μl aliquots were injected in the flow-injection mode (Fig. 2). From the mass spectra of both conjugates, the predominant $[M + H]^+$ and $[M - H]^-$ ions were observed in the positive and negative modes, respectively. However, over 5 and 20 times higher sensitivities for the sulfate and the glucuronide have been obtained in the positive mode than in the negative mode, respectively.

In addition, the highest intensity of $[M + H]^+$ for either conjugate was attained at a 10 V Q-array voltage in the positive mode, while at voltages higher than 30 V, the ion intensity significantly decreased. The higher voltage resulted in a higher cleavage to produce a few structural fragment ions at *m*/*z* 166 and 135 for *p*-OHMA-Sul, but lowered the sensitivity. On the other hand, no fragment ion for *p*-OHMA-Glu was observed at any voltage. Based on these results, we have finally chosen the following parameters as the optimum conditions for the quantitative analysis in the SIM mode: ionization mode, positive; Q-array voltage, 10 V; target ion, *m*/*z* 342 for *p*-OHMA-Glu and *m*/*z* 246 for *p*-OHMA-Sul.

3.2.2. LC conditions

In order to simultaneously determine MA and its four metabolites, AP, *p*-OHMA, *p*-OHMA-Glu and *p*-OHMA-Sul, optimization of the mobile phase was explored. In our previous study [15], 10 mM ammonium formate buffer (adjusted to pH 3.5 with formic acid)–acetonitrile (87.5:12.5, v/v) was proposed as the optimal eluent for the LC–MS determination of psilocin glucuronide. In the pre-experiment, we tested this eluent to examine if it is applicable to the present study. Neither *p*-OHMA-Glu nor *p*-OHMA-Sul was retained on the analytical column, therefore, improvement of the eluent was performed.

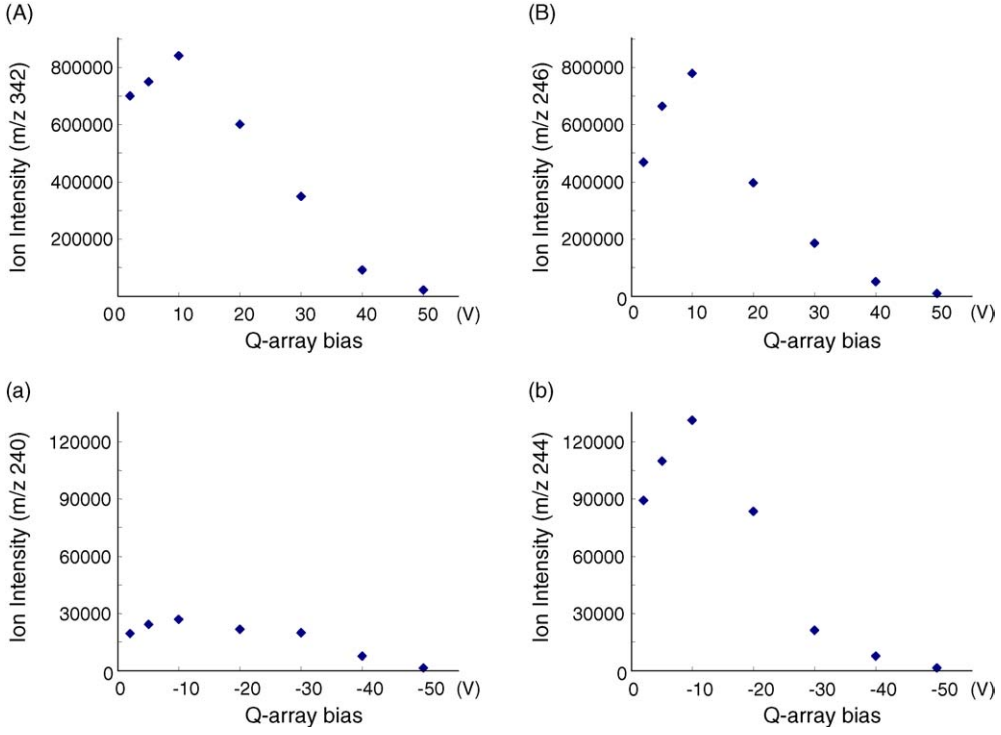

Fig. 2. Effect of Q-array voltage on ion intensity obtained from *p*-OHMA-Glu in the positive (A) and the negative (a) modes, and *p*-OHMA-Sul in the positive (B) and the negative (b) modes.

Table 1
Validation data for the established procedure

Analyte	POHMA-Glu	POHMA-Sul	POHMA	MA	AP
Recovery (%)[a]	82	85	90	92	92
Estimated limits of detection (ng/ml)					
Full-scan mode	50	50	30	10	10
SIM mode	5	5	2	1	1
Linearity range (μg/ml)	0.01–5	0.01–20	0.01–5	0.05–100	0.01–5
RSD (%)	4.2	6.3	7.2	8.2	6.9
Accuracy (at 1.0 μg/ml)[a]	1.02 ± 0.03	1.09 ± 0.05	0.97 ± 0.07	1.02 ± 0.09	0.99 ± 0.07
Pecision (%)					
Within-day[a]	5.3	6.5	7.0	7.9	7.0
Between-day[a]	7.2	8.1	8.0	9.2	8.1

[a] Evaluated using a drug-free urine sample spiked with the analytes at 1.0 μg/ml each ($n = 5$).

3.2.2.1. Concentrations of organic solvent. Upon utilizing acetonitrile as the organic solvent, even at the acetonitrile concentration of 5% or less, *p*-OHMA-Glu was hardly retained and thus co-eluted with the urinary components that would disturb the reliable and sensitive mass spectrometric measurement. When methanol was used at a 5% concentration as the organic solvent, *p*-OHMA-Glu was somewhat retained with sufficient separation from the urinary components, which led to an excellent spectral measurement. However, more than 30 min was required for the elution of AP and MA under the isocratic condition with 5% methanol. For improvement of the retention characteristics, gradient eluent systems with methanol were investigated. Consequently, the linear gradient eluent program (5–40% methanol in 20 min) provided successful elutions of MA and its four metabolites along with IS within 20 min.

3.2.2.2. pH of the mobile phase. To optimize the pH of the eluent, the pH was varied between 3.5 and 7.0 where the conjugates would be stable, and the retention characteristics and peak shapes were examined. As the buffer solution, 10 mM ammonium formate and ammonium acetate buffers were employed throughout the pH range from 3.5 to 5.0 and 5.0 to 7.0, respectively. The higher pH led to a wider peak and worse separation for all the analytes. At a pH of 3.5, all the analytes eluted within 20 min and very excellent peak shapes were achieved.

Although we tested a hydrophilic methacrylate polymer-type analytical column, Shodex DE-213 (2.0 mm i.d. × 150 mm, Showadenko, Tokyo, Japan) for further improvement, co-elution of *p*-OHMA-Glu and the urinary components were observed, resulting in a less sensitive measurement of its mass spectrum. Based on this comparison, we finally chose the system that consisted of the ODS-type analytical column utilizing the linear gradient eluent of 10 mM ammonium formate buffer (adjusted to pH 3.5 with formic acid) and methanol (5–40% methanol in 20 min) in the present study.

3.2.3. Extraction process

In order to simultaneously extract MA and its four metabolites including the *p*-OHMA-conjugates, the solid-phase extraction (SPE) and deproteinization with methanol were compared. SPE with the Oasis HLB (Waters, Milford, MA, USA) and Bond Elut Certify (Varian, Harbor City, CA, USA) was performed according to the methods described in previous reports [16,17]. Neither of the conjugates was retained on the cartridge.

On the other hand, all of the five analytes showed high recoveries by deproteinization with methanol (Table 1). In addition, the technique is extremely simple and rapid, and allowed us to analyze without any significant disturbance by the urinary components. We then chose deproteinization with methanol as the sample preparation method in the present study.

3.3. Method validation

Table 1 lists the validation data evaluated by analyzing the spiked urine samples and diluted standard solutions at known concentrations. Quantitative recoveries were achieved for all of the analytes in urine at 1.0 μg/ml. The limits of detection were defined as the detection limits of the target ion peaks on each mass chromatogram in the SIM mode (S/N ≧ 3). As listed in Table 1, the validation data guaranteed the quantitative efficiency of the procedure.

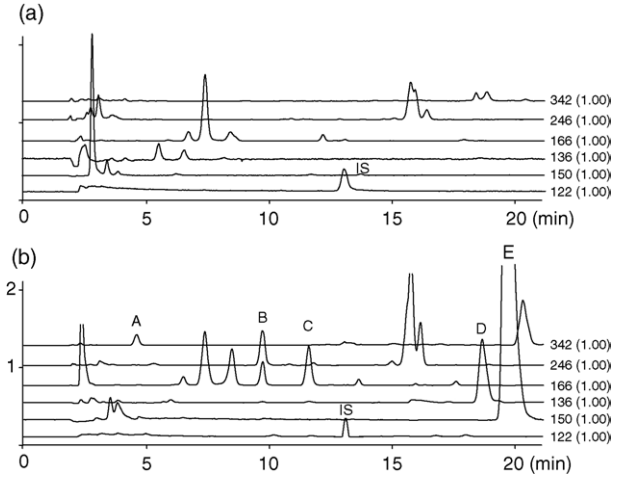

Fig. 3. Extracted mass chromatograms obtained from (a) a drug-free and (b) an MA user's (Subject 1) urine sample. The samples were prepared and analyzed as described in Section 2. Concentrations estimated are 1.1 μg/ml for *p*-OHMA-Glu, 6.0 μg/ml for *p*-OHMA-Sul, 1.0 μg/ml for *p*-OHMA, 1.8 μg/ml for AP and 18 μg/ml for MA. Peaks: (A) *p*-OHMA-Glu; (B) *p*-OHMA-Sul; (C) *p*-OHMA; (D) AP; (E) MA.

Table 2
Concentrations of MA and its metabolites in the urine samples from three MA users

Subject	POHMA-Glu (μg/ml)	POHMA-Sul (μg/ml)	POHMA (μg/ml)	MA (μg/ml)	AP (μg/ml)
1	1.1	6.0	1.0	18	1.8
2	0.19	1.8	0.32	5.3	0.17
3	1.0	5.8	0.60	60	1.5

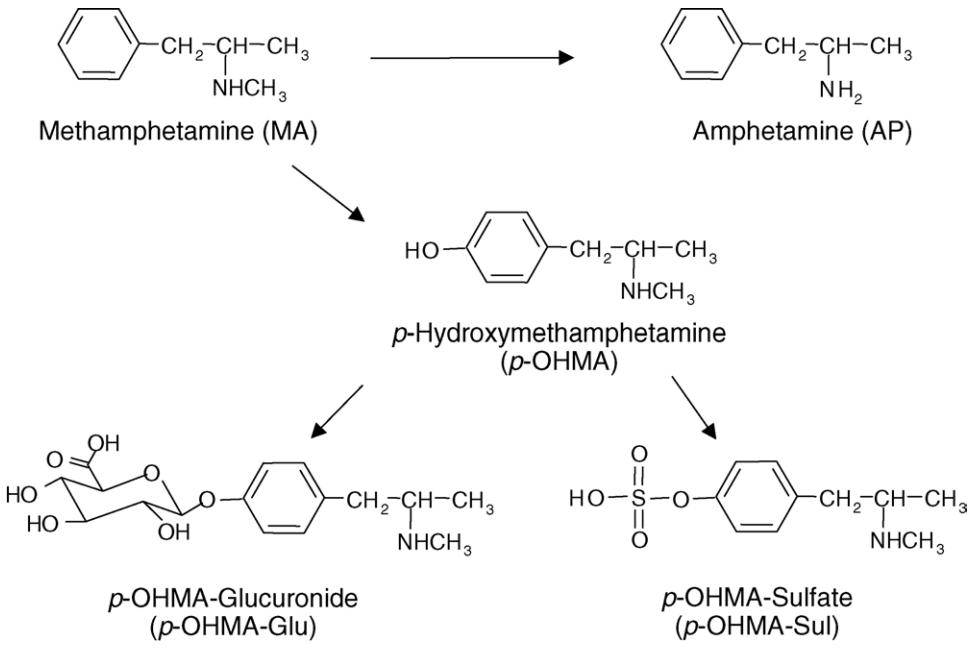

Fig. 4. Proposed principal metabolic pathways of MA.

3.4. Determination of p-OHMA-Glu and p-OHMA-Sul in urine from MA users

In order to survey the urinary levels of MA and its four metabolites, the present method was applied to urine samples from three MA users. Fig. 3 shows the extracted mass chromatograms obtained from an MA user's (Subject 1) urine sample and Table 2 lists the urinary levels in the three samples. Not only *p*-OHMA, but also *p*-OHMA-Glu and *p*-OHMA-Sul were detected in all of the samples. The level of *p*-OHMA-Sul reached approximately 5.5–9.7 times those of *p*-OHMA-Glu and *p*-OHMA in the samples. The levels ranged from 0.19 to 1.1 μg/ml for *p*-OHMA-Glu, from 1.8 to 6.0 μg/ml for *p*-OHMA-Sul, and from 0.32 to 1.0 μg/ml for *p*-OHMA.

In a previous paper [3], Caldwell et al. reported that the transformation of MA into *p*-OHMA was the main metabolic pathway in humans. The present data have indicated that the glucuronidation and sulfation are responsible for the following phase II conjugation of *p*-OHMA (Fig. 4). Based on the urinary levels of the conjugates, the sulfation may be quite superior to the glucuronidation in humans, which has been regarded as the main conjugation of *p*-OHMA. On the other hand, a high level of *p*-OHMA-Glu was detected, but no *p*-OHMA-Sul was detected in rats' urine in another study (data not shown). These results demonstrate that interspecies variation between humans and rats would exist in the conjugation of *p*-OHMA.

Based on the urinary levels of *p*-OHMA and its conjugates in three users' urine samples, it was indicated that more than 85% of the *p*-OHMA was conjugated and excreted in the urine. This suggests that complete hydrolysis will induce an increase in the free *p*-OHMA to produce five times or higher levels of *p*-OHMA, therefore, hydrolysis would be indispensable for the sensitive analysis of *p*-OHMA as an indicator of MA use. In a previous paper [4], Shimosato et al. reported that hydrolysis with acid and β-glucuronidase gave almost the same increases in the *p*-OHMA levels. This is probably because the β-glucuronidase used by them was from abalone which not only had the gulucuronidase activity but also sulfatase activity.

4. Conclusion

This is the first report of the direct detection of *p*-OHMA-Glu and *p*-OHMA-Sul in human urine as well as their synthesis. In the present study, the conjugates of *p*-OHMA, *p*-OHMA-Glu and *p*-OHMA-Sul, have been directly identified in MA users' urine by utilizing LC–MS–MS. In addition, following the synthesis of these conjugates, a sensitive LC–ESI-MS procedure with a simple pretreatment was established for the simultaneous determination of MA and its four metabolites, AP, *p*-OHMA, *p*-OHMA-Glu and *p*-OHMA-Sul, in urine.

Based on the urinary levels of the conjugates in three MA users, sulfation may be superior to glucuronidation in humans.

No significant differences in the ratios of the urinary conjugate levels were observed between three samples employed in this study. Interindividual variation may, however, exist in the phase II metabolism of *p*-OHMA in humans. Further studies utilizing much more urine samples from various ages and races of MA users are now in progress.

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