Metabolism of the recently encountered designer drug, methylone, in humans and rats

H. T. KAMATA, N. SHIMA, K. ZAITSU, T. KAMATA, A. MIKI, M. NISHIKAWA, M. KATAGI, & H. TSUCHIHASHI

Forensic Science Laboratory, Osaka Prefectural Police HQ, Osaka, Japan

(Received 23 March 2006; accepted 1 May 2006)

Abstract
The urinary metabolites of methylone in humans and rats were investigated by analysing urine specimens from its abuser and after administrating to rats with gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-electrospray ionization mass spectrometry (LC-ESI MS), using authentic standards. The time-course excretion profiles of methylone and its three metabolites in rats were further investigated after a single intraperitoneal dosing of 5 mg kg\(^{-1}\) methylone hydrochloride. Two major metabolic pathways were revealed for both humans and rats as follows: (1) side-chain degradation by N-demethylation to the corresponding primary amine methylenedioxycathinone (MDC), partly conjugated; and (2) demethylation followed by O-methylation of either a 3- or 4-OH group on the benzene ring to produce 4-hydroxy-3-methoxymethcathinone (HMMC) or 3-hydroxy-4-methoxymethcathinone (3-OH-4-MeO-MC), respectively, mostly conjugated. Of these metabolites, HMMC was the most abundant in humans and rats. The cumulative amount of urinary HMMC excreted within the first 48 h in rats was approximately 26% of the dose, and the amount of the parent methylone was not more than 3%. These results demonstrate that the analysis of HMMC will be indispensable for proof of the use of methylone in forensic urinalysis.

Keywords: Methylone, N-demethylation, O-methylation, demethylenation

Introduction
Methylone [2-methylamino-1-(3,4-methylenedioxyphenyl)propan-1-one; Figure 1], the \(\beta\)-ketone analogue of 3,4-methylenedioxymethamphetamine (MDMA), increases the concentrations of monoamine neurotransmitters such as serotonin, dopamine and...
noradrenaline in the human synaptic cleft through inhibition of plasma membrane monoamine reuptake transporters (Cozzi et al. 1998, 1999). This increase results in stimulation of the central nervous system and hallucinogenic effects (Dal Cason et al. 1997).

Methylone was first synthesized as an anti-depressant and an anti-Parkinsonism agent in 1996 (Jacob et al. 1996), and its amphetamine-like and MDMA-like character had also been reported (Dal Cason et al. 1997). More recently, it has been encountered in the Japanese drug market as well as in Europe under the name of ‘Explosion’ (Bossong et al. 2005) and has been increasingly abused as a new designer drug. Thereby, intoxication due to its overdosing has been occasionally encountered. However, this drug is not currently classified as an illegal drug, and nothing prevents its sale and abuse; consequently there is thus much apprehension regarding its popularity in Japan and Europe.

With regard to its pharmacological properties, metabolism and toxicity, very little is known (Dal Cason et al. 1997, Cozzi et al. 1998, 1999, Bossong et al. 2005). However, its structure is very similar to that of MDMA, and health risks common to MDMA would be expected. Thus, there is an obvious need to develop analytical methodologies for both the parent compound and potential metabolites in both forensic toxicology and clinical chemistry.

In the present study, we have synthesized authentic standards of methylone metabolites that were predicted based on previous studies on MDMA (Maurer et al. 2000, Green et al. 2003, Shima et al. 2005). Utilizing optimized gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-electrospray ionization mass spectrometry (LC-ESI MS) procedures in conjunction with the authentic standards, the urinary metabolites of methylone in rats were identified, and the urinary excretion profiles in the rat have been investigated after its administration. In addition, a urine specimen from a methylone abuser was analysed by both GC-MS and LC-ESI/MS. The excretion profiles are presented, and the metabolic pathways of methylone in humans and rats are discussed.

Figure 1. Chemical structures of methylone (a), 3,4-methylenedioxycathinone (b), 4-hydroxy-3-methoxy-methcathinone (c) and 3-hydroxy-4-methoxymethcathinone (d).
Materials and methods

Methylone hydrochloride was synthesized in our laboratory according to previously published methods (Jacob et al. 1996) with partial modification, and 3,4-methylenedioxy-cathinone [MDC, 2-amino-1-(3,4-methylenedioxyphenyl)propan-1-one] hydrochloride, 4-hydroxy-3-methoxymethcathinone [HMMC, 2-methylamino-1-(4-hydroxy-3-methoxy-phenyl)propan-1-one], 3-hydroxy-4-methoxymethcathinone [3-OH-4-MeO-MC, 2-methylamino-1-(3-hydroxy-4-methoxyphenyl)propan-1-one] were all synthesized in our laboratory, as detailed in the subsequent section (chemical structures can be seen in Figure 1). Every synthesized compound was >98% pure based on LC-MS analysis by a flow-injection method. Stock standard solutions of these four compounds were prepared in distilled water (1 mg ml\(^{-1}\) each), and these solutions were then diluted to appropriate concentrations with distilled water or control human urine, immediately prior to use. The internal standard (IS) \(\beta\)-phenethylamine (PEA) was purchased from Tokyo Kasei (Tokyo, Japan), and an IS solution (1 \(\mu\)g ml\(^{-1}\)) was prepared in the initial mobile phase for LC-MS analysis. Methanol was of HPLC-grade, and other chemicals used were of analytical grade. Trifluoroacetic anhydride (TFAA) used for trifluoroacetyl (TFA) derivatization was purchased from Wako (Osaka, Japan).

Chemical synthesis

**Methylone (I).** I was synthesized according to the procedures of Jacob et al. (1996) with modifications as follows: methylenedioxybenzene and 1.05 equivalents of propionic anhydride were placed in an ice-cooled round-bottom flask under an argon atmosphere and with continuous stirring. Small portions of iodine (0.2–0.5 equivalent) were carefully added, and the mixture was slowly heated at 20°C for 30 min. The reaction mixture was dissolved in a threefold volume of diethyl ether, and the ether solution was successively washed with 10% (w/v) \(\text{Na}_2\text{CO}_3\) aqueous solution, 10% (w/v) \(\text{Na}_2\text{S}_2\text{O}_3\) aqueous solution, and brine. The organic extract was dried over anhydrous \(\text{Na}_2\text{SO}_4\), and evaporated to obtain crude 3,4-methylenedioxypropiophenone. This product was purified by silica gel column chromatography (Wakogel\textsuperscript{®}, Wako Pure Chemical Industries, Osaka, Japan), using hexane/ethyl acetate (6:1, v/v) as an eluent.

To a solution of 3,4-methylenedioxypropiophenone in \(\text{CH}_2\text{Cl}_2\) were added 1.1 equivalents of \(\text{CuBr}_2\) and 0.1 equivalent KBr. The mixture was refluxed with stirring overnight and then cooled to room temperature. The resulting inorganic precipitate was removed through filtration, and the organic extracts were concentrated under vacuum to give crude 2-bromo-3′,4′-methylenedioxypropiophenone. This product was purified by silica gel column chromatography, using hexane/ethyl acetate (6:1, v/v) as an eluent.

To a solution of 2-bromo-3′,4′-methylenedioxypropiophenone in tetrahydrofuran (THF) was added dropwise 2 M methylamine in THF (Aldrich, St Louis, MO, USA). The mixture was stirred at room temperature overnight. The reaction mixture was treated with 10% HCl aqueous solution to make it acidic and was then washed with diethyl ether. The aqueous layer was then made basic with 10% \(\text{Na}_2\text{CO}_3\) and extracted with ethyl acetate. The organic extract was washed with brine, dried over anhydrous \(\text{Na}_2\text{SO}_4\), and evaporated to give crude methylone (I) as a yellow oil. Finally, 10% HCl methanol solution was added dropwise to the oil. After removal of the solvent, the crude hydrochloride salt was purified by recrystallization from chloroform/methanol.
3,4-Methylenedioxyxcatinone (MDC) (II). II was synthesized according to the above-mentioned procedure, with a slight modification: Instead of 2 M methylamine in THF, 6 M ammonia in methanol (Aldrich) was added to 2-bromo-3,4'-methylenedioxypropio-phenone, and the resultant product 1-(3,4-methylenedioxy)cathinone (II) was purified as its hydrochloride by recrystallization from chloroform/methanol.

4-Hydroxy-3-methoxymethcathinone (HMMC) (III). To a solution of 4-benzyloxy-3-methoxybenzaldehyde in anhydrous THF, stirred and cooled in an external ice bath, 1.1 equivalents of ethyl magnesium bromide in THF was added, dropwise over the course of 5 min. The mixture was stirred for an additional 30 min. The reaction was quenched by the addition of saturated NH₄Cl solution, and was extracted with ethylacetate. The organic extract was washed with brine, dried over anhydrous Na₂SO₄, and evaporated to obtain crude 1-(4-benzyloxy-3-methoxyphenyl)propane-1-ol, which was used without further purification.

To a solution of 1-(4-benzyloxy-3-methoxyphenyl)propane-1-ol in a solvent mixture of n-hexane/diethyl ether (1:1, v/v), activated MnO₂ was added portionwise, and the mixture was stirred at room temperature for 24 h. The solids were removed by filtration, the filter cake washed with ethylacetate, and the filtrate and washings pooled. The combined organic extract was concentrated under vacuum to give 4-benzyloxy-3-methoxypropiophenone.

According to the above-mentioned procedure for methylone, 4-benzyloxy-3-methoxypropiophenone was reacted with CuBr₂ in the presence of KBr followed by treatment of the resulting 2-bromo-4'-benzyloxy-3'-methoxypropiophenone with 2 M methylamine in THF. The resulting product was purified by silica gel column chromatography using a solvent mixture of chloroform/methanol (1:2, v/v) to give 4-benzyloxy-3-methoxymethcathinone.

The benzyl group was removed by hydrogenation of a solution of 4-benzyloxy-3-methoxymethcathinone in anhydrous ethanol containing a catalytic amount of 10% Pd/C catalyst. The mixture was left under a hydrogen atmosphere and stirred for 24 h at room temperature. The solution was filtered through Celite®, and the filter cake was washed with ethanol. The filtrate and washings were combined, and the solvent was removed under vacuum to give crude 4-hydroxy-3-methoxymethcathinone (III). This product was purified by silica gel column chromatography using a solvent mixture of chloroform/methanol (2:1, v/v) as an eluent.

3-Hydroxy-4-methoxymethcathinone (3-OH-4-MeO-MC) (IV). IV was synthesized according to the above-mentioned procedure, with a slight modification: Instead of 4-benzyloxy-3-methoxybenzaldehyde, 3-benzyloxy-4-methoxybenzaldehyde was used, and the resulting 1-(3-hydroxy-4-methoxy)methcathinone (IV) was purified according to the above-mentioned procedure.

Unambiguous structural assignments and confirmation of the above synthesized standards were done on the basis of chemical shifts and mass spectra employing ¹H-NMR (Varian, Polo Alto, CA, USA), GC-MS and LC-MS.

Instrumentation

GC-MS was carried out on a GCMS QP-2010 (Shimadzu, Kyoto, Japan). A fused-silica capillary column DB-5MS (30 m × 0.25 mm i.d.; 0.25 μm; J&W Scientific, Rancho Cordova, CA, USA) was used for separation. Injections were effected automatically in the
splitless mode at 260°C. The column oven temperature was maintained at 80°C for 2 min and then raised at 15°C min⁻¹ to 320°C. The transfer line temperature was set at 250°C. High purity helium, at a flow rate of 3 ml min⁻¹, was used as the carrier gas. The electron ionization (EI) operating parameters were as follows: source temperature, 200°C; electron energy, 70 eV; ion multiplier gain, 1.2 kV. Data were collected from 40 to 600 at a scan rate of 0.5 s scan⁻¹.

LC-ESI/MS was performed on 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 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, 1.51 min⁻¹; curved desolvation line (CDL) temperature, 200°C; Q-array DC, 10 V; Q-array RF, 150 V. The analytical column used was an L-column ODS semi-micro column (1.5 mm i.d. × 150 mm, Chemicals Evaluation and Research Institute, Tokyo, Japan). Each 30-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 three ions for the analytes (m/z 208 for methylone, m/z 194 for MDC, m/z 210 for 3-OH-4-MeO-MC and HMMC, and m/z 122 for PEA) in the selected-ion monitoring (SIM) mode.

**Human urine sample**

A urine specimen from a methylone user (a 19-year-old Japanese male) included in this study had been submitted to our laboratory for forensic analysis. He orally used methylone hydrochloride powder (dosage being unclear) and was taken to the emergency department with strong dementia. His urine specimen was collected at approximately 36 h post-intake. The sample was immediately frozen and stored at −20°C until analysis.

**Rat urine**

Three male Wistar rats (7 weeks of age) from Japan SLC (Hamamatsu, Japan) weighing 210 ± 15 g were housed in an air-conditioned room at 24 ± 2°C for 1 week before use and administered a single intraperitoneal dose of 5 mg kg⁻¹ methylone hydrochloride. The animals were kept singly in suitable metabolism cages, and urine was collected for 24 h in a vessel. The collected urine samples were adjusted to approximately pH 4–5 with acetic acid and stored at −20°C until analysis.

**Sample preparation**

*Acid hydrolysis.* To 1 ml of a urine specimen, 1 ml of 1.2 N HCl aqueous solution was added, and the mixture was heated at 100°C for 1 h. The reaction mixture was cooled and subsequently neutralized with 28% ammonium hydroxide. The resultant solution was subjected to liquid–liquid extraction as described below.

*Extraction and TFA derivatization for GC-MS.* A urine specimen (1 ml) before or after acid hydrolysis (with 0.6 N HCl at 100°C for 1 h) was adjusted to pH 9 with 28% aqueous ammonia and saturated with NaCl. The solution was extracted three times with 1 ml of a
chloroform-2-propanol mixture (3:1, v/v), and the organic layer was separated after centrifugation at 1500 g. The combined extract was dried with anhydrous Na$_2$SO$_4$, transferred into a screw-capped glass tube, and dried under a gentle stream of nitrogen. The residue was derivatized by adding 200 μl of trifluoroacetic anhydride and 200 μl of ethylacetate at 80°C for 1 h. The reaction mixture was carefully evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 100 μl of ethylacetate, and a 1-μl aliquot was then injected into the GC-MS system.

**Extraction of free-form metabolites for LC-MS.** A urine specimen (1 ml) before or after acid hydrolysis (with 0.6N HCl at 100°C for 1 h) was adjusted to pH 9 with 28% aqueous ammonia and saturated with NaCl. The solution was extracted three times with 1 ml of a chloroform-2-propanol mixture (3:1, v/v), and the organic layer was separated after centrifugation at 1500 g. The combined extract was dried under a gentle stream of nitrogen. The residue was dissolved in 100 μl of IS solution. This was then filtered through a 0.22 μm membrane filter, and an aliquot of 5 μl was automatically injected into the LC-MS system.

**Extraction of conjugates for LC-MS.** To 100 μl of a urine sample, 100 μl of a β-phenethylamine (PEA, IS) aqueous solution (1 μg ml$^{-1}$) 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, filtered through a 0.22 μm membrane filter, and an aliquot of 5 μl was automatically injected into the LC-MS systems.

**Validation of the LC-MS procedure.** In order to quantify methylone and its metabolites in urine, the optimized LC-MS procedure was validated. A 1-ml drug-free urine spiked with the synthesized standards at 500 ng ml$^{-1}$ each was processed as described in the Experimental section. The obtained residue was dissolved in 100 μl of IS (PEA) solution. A 5 μl aliquot was injected into the LC-MS system in the SIM mode, where the protonated molecule of each analyte was selected as the monitoring ion, and the peak area ratios to IS were calculated. The recoveries at 500 ng ml$^{-1}$ were 93, 95, 69 and 85% for methylone, MDC, HMMC, and 3-OH-4-MeO-MC, respectively ($n=3$). The detection limits were 2.5 ng ml$^{-1}$ for methylone, 25 ng ml$^{-1}$ for MDC, 10 ng ml$^{-1}$ for HMMC, and 5 ng ml$^{-1}$ for 3-OH-4-MeO-MC. Calibration curves constructed by the IS method showed good lineairties over the ranges from 25 to 500 ng ml$^{-1}$ for methylone, HMMC and 3-OH-4-MeO-MC, and from 50 to 1000 ng ml$^{-1}$ for MDC. The within-day relative standard deviations (evaluated at 500 ng ml$^{-1}$, $n=5$) ranged from 1.4 to 7.1% for all of the analytes. These results guaranteed the reliability of the present procedure for the analysis of urine specimens from a methylone user and rats.

**Results and discussion**

**Identification of metabolites by GC-MS and LC-MS**

The urinary metabolites of methylone have not been previously reported. However, in previous studies on the metabolism of MDMA that possesses almost the same structure as
methylone, several metabolic routes in humans including N-demethylation and demethylation followed by O-methylation of either a 3- or 4-OH group on the benzene ring have been reported (Maurer et al. 2000, Green et al. 2003, Shima et al. 2005). Based on these latter reports, MDC, HMMC and 3-OH-4-MeO-MC were expected as the major urinary metabolites, which retained the structural characteristics of the parent drug methylone. The authentic standards of these compounds were synthesized for the first time, and using them, the unequivocal identification of methylone metabolites in human urine was carried out by both GC-MS and LC-ESI MS.

In order to identify the metabolites in urine by GC-MS and LC-MS, the mass spectral characteristics of the authentic standards were first examined. The EI mass spectra of the authentic standards after TFA derivatization that usually provides higher sensitivity and clearer identification of amines and phenols, predominant ions due to the β-cleavage of benzene ring moieties (m/z 149 for methylone and MDC, and m/z 247 for HMMC and 3-OH-4-MeO-MC) and/or to the α-cleavage of amine moieties (m/z 154 for HMMC and 3-OH-4-MeO-MC), and some other ions including a very small molecular ion (m/z 303 for methylone, 289 for MDC and 401 for HMMC and 3-OH-4-MeO-MC) (Figure 2). Figure 3 shows the ESI mass spectra of the authentic standards by LC-MS. Contrary to the GC-MS spectra, these spectra were characterized by the predominant protonated molecules at m/z 208 for methylone, 194 for MDC, 210 for HMMC and 3-OH-4-MeO-MC.

The extracts of the hydrolysed urine specimens from rats administered a single intraperitoneal dose of 5 mg kg\(^{-1}\) methylone hydrochloride were first subjected to GC-MS analysis following TFA derivatization, and the retention times and mass spectra of the compounds detected were compared with those of the standards. As a result, TFA
derivatives of these four compounds were clearly detected from the derivatized extracts (Figure 4).

LC-ESI MS analysis was additionally performed for the confirmation of methylone, MDC, HMMC and 3-OH-4-MeO-MC detected by GC-MS. LC-ESI MS was carried out under the optimized conditions detailed in the experimental section, and the results were compared with those of the authentic standards. Extracted ion chromatograms obtained from rat urine are shown in Figure 5. The presence of methylone, MDC, HMMC and 3-OH-4-MeO-MC were confirmed.

In addition, Brenneisen et al. (1986) proposed that cathinone, which possesses a $\beta$-keto moiety, is partly metabolized by keto reduction to the corresponding aminoalcohols norephedrine and norpseudoephedrine, suggesting the possibility of reduction to 3,4-methylenedioxyephedrine (MDEP) in the metabolism of methylone. In another study by Staack et al. (2005) on the metabolism of aromatic ring substituted $\alpha$-pyrrolidinopropiophenone derivatives, reduction of the keto group to the corresponding secondary alcohol was not observed. In order to confirm whether the $\beta$-keto group in methylone is metabolically reduced to MDEP, methylone was chemically reduced by sodium borohydride (Springer et al. 2003), and the corresponding secondary alcohol, MDEP, was then analysed by GC-MS after TFA derivatization. However, no trace of MDEP was detected in any of the specimens examined. This suggests that methylone was not metabolized to MDEP in rats.

Based on these GC-MS and LC-MS analyses, methylone, MDC, HMMC and 3-OH-4-MeO-MC were unequivocally identified in the urine specimens from methylone-administered rats. In addition, no metabolic transformation of methylone to MDEP was indicated in the rat.
Urinary excretion of methylone and its metabolites in rats

In order to clarify the urinary excretion of methylone and its metabolites MDC, HMMC and 3-OH-4-MeO-MC in rats, the time-course of urinary levels of methylone and its three metabolites observed for the three rats following a single dose of 5 mg kg\(^{-1}\) methylone hydrochloride was examined by LC-ESI MS in combination with liquid–liquid extraction. In addition, the concentrations before and after acid hydrolysis (to determine the presence of conjugates) were carefully compared.

The resultant time-course urinary levels of methylone, MDC, HMMC and 3-OH-4-MeO-MC after hydrolysis are presented in Figure 6. The peak urinary level of methylone was attained at approximately 4 h post-dosing. The level was extremely low compared with those of the predominant metabolite HMMC, and it rapidly to below the detection limit (2.5 ng ml\(^{-1}\)) 36 h post-dosing. On the other hand, the level of HMMC reached maximum at approximately 4 h post-dosing, and the levels then rapidly dropped. However, HMMC was detectable even up to 48 h post-dosing.

Figure 4. Total ion chromatogram and extracted ion chromatograms obtained by GC-MS from a rat urine sample (4 h after dosing) taken after a single intraperitoneal dose of 5 mg kg\(^{-1}\) methylone \(·\) HCl (A), and a standard mixture solution of methylone and its three metabolites (B). The concentration of the standard solution was 1 \(\mu\)g ml\(^{-1}\) each. Peaks: 1, HMMC; 2, 3-OH-4-MeO-MC; 3, MDC; and 4, methylone.

**Urinary excretion of methylone and its metabolites in rats**

In order to clarify the urinary excretion of methylone and its metabolites MDC, HMMC and 3-OH-4-MeO-MC in rats, the time-course of urinary levels of methylone and its three metabolites observed for the three rats following a single dose of 5 mg kg\(^{-1}\) methylone hydrochloride was examined by LC-ESI MS in combination with liquid–liquid extraction. In addition, the concentrations before and after acid hydrolysis (to determine the presence of conjugates) were carefully compared.

The resultant time-course urinary levels of methylone, MDC, HMMC and 3-OH-4-MeO-MC after hydrolysis are presented in Figure 6. The peak urinary level of methylone was attained at approximately 4 h post-dosing. The level was extremely low compared with those of the predominant metabolite HMMC, and it rapidly to below the detection limit (2.5 ng ml\(^{-1}\)) 36 h post-dosing. On the other hand, the level of HMMC reached maximum at approximately 4 h post-dosing, and the levels then rapidly dropped. However, HMMC was detectable even up to 48 h post-dosing.
Figure 5. Extracted ion chromatograms obtained by LC-ESI/MS from a rat urine sample (4 h after dosing) taken after a single intraperitoneal dose of 5 mg kg\(^{-1}\) methylone·HCl (A), and a standard mixture solution of methylone and its three metabolites (B). The concentration of the standard solution was 1 \(\mu\)g ml\(^{-1}\) each. As the internal standard (IS), \(\beta\)-phenethylamine was used. Peaks: 1, HMMC; 2, 3-OH-4-MeO-MC; 3, MDC; and 4, methylone.

Figure 6. Time-course of urinary levels of methylone and its three metabolites after a single intraperitoneal dose of 5 mg kg\(^{-1}\) methylone·HCl. The levels were quantitated by LC-ESI/MS in the SIM mode after acid hydrolysis. ●, Methylone; ○, MDC; ■, HMMC; □, 3-OH-4-MeO-MC.
The cumulative amounts of methylone and its three metabolites excreted in urine as a percentage of the parent methylone dose administered are summarized in Table I. The cumulative amount of the most abundant characteristic metabolite HMMC excreted within the first 48 h was approximately 26% of the dose, most of which was excreted approximately within 12 h post-dosing. In addition, over 80% of the excreted urinary HMMC and 3-OH-4-MeO-MC were found to be conjugated. On the other hand, the amount of parent methylone was not more than 3% of the dose, and it was mostly excreted approximately within 12 h post-dosing. In addition, it is notable that methylone and its demethylated metabolite MDC were partly conjugated.

In our previous study on MDMA, 4-hydroxy-3-methoxy-methamphetamine (HMMA) glucuronide and sulfate were confirmed by LC-MS in human and rat urine (Shima et al. 2005). Also, for the metabolism of N-benzylpiperazine (BZP) and 1-(3-trifluoromethylphenyl)piperazine (TFMPP) in the rat, the urinary excretion of both glucuronides and sulfates of their main hydroxylated metabolites p-hydroxy-BZP (Tsutsumi et al. 2006) and p-hydroxy-TFMPP (Tsutsumi et al. 2005) was expected. In the present study, we monitored the ions corresponding to protonated molecules of the glucuronides and sulfates of methylone, MDC, HMMC and 3-OH-4-MeO-MC, after direct injection of rat urine into the LC-MS system, and relatively intense ions corresponding to the glucuronide and a fairly low intense ion corresponding to the sulfate of HMMC and 3-OH-4-MeO-MC were observed (data are not shown). These observations suggest that methylone is metabolically transformed to HMMC and 3-OH-4-MeO-MC followed mainly by conjugation as its glucuronide and, to small extent, by conjugation as its sulfate. On the other hand, no traces of methylone and MDC conjugates were detected. Cardwell et al. (1972) reported an increase in the urinary level of benzylmethylketone, the deamination metabolite of methamphetamine after acid hydrolysis, and suggested urinary excretion as its conjugates. The excretion of the glucuronides and sulfates following enolization were then also expected for both methylone and MDC based on their increased levels after acid hydrolysis. However, the existence of neither glucuronide nor sulfate was confirmed here, and other conjugation forms are then expected, and future studies on these subjects will be required to clarify the conjugation of methylone and MDC.

### Table I. Cumulative amount of methylone and its three metabolites excreted in rat urine within 48 h post-dosing.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Free (%)</th>
<th>Total (%)</th>
<th>Conjugate ratio (% of the total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylone</td>
<td>2.4 ± 1.5</td>
<td>2.7 ± 1.4</td>
<td>12.2</td>
</tr>
<tr>
<td>MDC</td>
<td>1.1 ± 0.41</td>
<td>1.7 ± 0.50</td>
<td>34.1</td>
</tr>
<tr>
<td>HMMC</td>
<td>4.6 ± 3.0</td>
<td>25.9 ± 4.0</td>
<td>81.0</td>
</tr>
<tr>
<td>3-OH-4-MeO-MC</td>
<td>0.56 ± 0.45</td>
<td>4.9 ± 1.1</td>
<td>87.6</td>
</tr>
<tr>
<td>Total</td>
<td>8.7 ± 4.8</td>
<td>35.1 ± 3.6</td>
<td>74.2</td>
</tr>
</tbody>
</table>

*a Values are expressed as mean ± standard deviation for three rats.

b Values are expressed as the mean of the percentage for three rats.
Urinary excretion of methylone and its metabolites in humans

Based on the results from rats, the urinary metabolites of methylone in a methylone abuser were examined. The extracts of the hydrolysed human urine specimen were analysed by both GC-MS following TFA derivatization and LC-ESI MS, and the retention times and mass spectra of the compounds detected were compared with those of the standards. As depicted in Figure 7, methylone, MDC, HMMC and 3-OH-4-MeO-MC were detected in the extracts. In addition, no trace of MDEP was detected, as was the case for rats. Also, the concentrations of methylone and its three metabolites identified in the human urine specimen were quantified by the validated LC-MS procedure, and the excretion profiles were investigated. The urinary levels were calculated using the calibration curves constructed, and the concentrations before and after acid hydrolysis were compared. The results are summarized in Table II and indicate that HMMC is an abundant characteristic metabolite and that MDC and 3-OH-4-MeO-MC are minor metabolites for methylone in humans.

In addition, acid hydrolysis significantly increased the concentrations of all four analytes. These facts suggest the conjugation including glucuronidation and sulfation of not only HMMC and 3-OH-4-MeO-MC but also for methylone and MDC. Also, in our previous studies on the metabolism of psilocin (Kamata et al. 2003) and 5-methoxy-N,N-diisopropyltryptamine (Kamata et al. 2006) in humans, glucuronides and/or sulfates of their hydroxylated metabolites have been successfully identified by LC-MS. The direct
analysis of urine specimens before hydrolysis was then attempted by LC-SIM-MS, and the ions corresponding to protonated molecules of glucuronides and sulfates of methylone, MDC, HMMC and 3-OH-4-MeO-MC were monitored. However, the existence of the conjugates was not confirmed. This is certainly because the total urinary levels of the four analytes were too low to determine by LC-ESI MS.

Table II. Levels of methylone and its three metabolites in a methylone abuser’s urine.

<table>
<thead>
<tr>
<th>Compound (ng ml⁻¹)</th>
<th>Before hydrolysis (free)</th>
<th>After hydrolysis (total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylone</td>
<td>n.q.ᵃ</td>
<td>48</td>
</tr>
<tr>
<td>MDC</td>
<td>n.q.</td>
<td>n.q.</td>
</tr>
<tr>
<td>HMMC</td>
<td>n.d.ᵇ</td>
<td>170</td>
</tr>
<tr>
<td>3-OH-4-MeO-MC</td>
<td>n.d.</td>
<td>74</td>
</tr>
</tbody>
</table>

ᵃn.q., Not quantitated. ᵇn.d., Not detected.

Figure 8. Proposed main metabolic pathways for methylone in the rats and humans.
In conclusion, the urinary metabolites of considerable quantitative significance for methylone in the rat and humans, which retain the structural characteristics of the parent drug, have been identified as MDC, HMMC and 3-OH-4-MeO-MC. In addition, the time-course urinary excretion profile of methylone and its three metabolites in the rats were clarified. Based on the present data, we propose the principal metabolic pathways for methylone illustrated in Figure 8. Of these characteristic metabolites, HMMC was the major species identified and was mostly excreted as its conjugates.

As expected in the present study, the conjugation of HMMC and its isomer 3-OH-4-MeO-MC to its glucuronide would be a major pathway for their phase II metabolism in the rats. On the other hand, our previous studies reported that sulfation is more predominant than glucuronidation in conjugation of the MDMA metabolite, HMMA (Shima et al. 2005), and the methamphetamine metabolite, p-hydroxymethamphetamine, in humans (Shima et al. 2006a, 2006b). Thus, sulfation of HMMC may be a major phase II metabolic pathway in humans. However, further studies on the metabolism of methylone in humans would be required for application in forensic toxicology and clinical pharmacology. Nevertheless, the identification of characteristic urinary metabolites of methylone reported here will be of great importance in forensic and clinical urine analysis. In addition, the present study will provide useful information in the urine analysis for unknown β-keto phenethylamine-type drugs that may be encountered in the future.

Acknowledgements

The valuable suggestions and skilful technical assistance of Associate Professor Dr T. Iwamura (Gifu Pharmaceutical University) in synthesizing methylone is gratefully acknowledged.

References

Metabolism of methylone in human and rats


