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Analysis of amphetamine-type stimulants and their metabolites in plasma, urine and bile by liquid chromatography with a strong cation-exchange column-tandem mass spectrometry

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ABSTRACT

The aim of this work was to develop and validate a method for analysing amphetamine-type stimulants (ATSs) and their metabolites in plasma, urine and bile by liquid chromatography with a strong cation-exchange column-tandem mass spectrometry, and to apply it to the pharmacokinetic study of ATSs. 3,4-Methylenedioxymethamphetamine, methamphetamine, ketamine and their main metabolites, 4-hydroxy-3-methoxymethamphetamine, 3,4-methylenedioxyamphetamine, *p*-hydroxymethamphetamine, amphetamine and norketamine, were simultaneously quantified by the new method (50–5000 ng/ml). The coefficients of variation and the percent deviations for the eight compounds were in the range of 0.2 to 5.3% and −9.4 to +12.8%, respectively. The recoveries were over 90% in all biological samples tested. This method was effective for the separation and the identification of ATSs and their main metabolites having amine moieties in plasma, urine and bile, and was applicable to pharmacokinetic analysis of methamphetamine, ketamine and their main metabolites in biological samples. This analytical method should be useful for the pharmacokinetic analysis of ATSs.

1. Introduction

The amphetamine-type stimulants (ATSs) include methamphetamine (MA), 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyamphetamine (MDA), and 3,4-methylenedioxyethylamphetamine (MDEA). ATSs have similar pharmacological and toxicological effects, which they exert by affecting the central nervous system. The abuse of ATS tablets has become a serious social problem throughout the world, including in Japan, where there has been a considerable increase in the number of seizures of ATS tablets in recent years [1]. ATS tablets typically contain one or more active ingredients, such as ketamine (KA), caffeine, or other components having hallucinogenic and/or stimulant effects [2–8]. The various components in these tablets can interact within the body, leading to serious toxic symptoms. Therefore, investigations into the pharmacokinetics and drug–drug interactions of ATSs and their related compounds are important for public health and forensic toxicology.

Numerous methods have been developed for the separation and identification of ATSs, including those using a liquid chromatograph (LC) with a diode array detector [9], an electrochemical detector [10], a fluorescence detector [11], and a mass spectrometer (MS) [11–15], and those using a gas chromatograph (GC) with either a nitrogen phosphorus detector [12] or an MS [9,16]. Because ATSs are similar to each other in their chemical structures, which possess aliphatic amine moieties, it is indispensable to adequately separate the compounds using a chromatographic technique. Although GC analyses have the advantage of peak separation, they require complicated preparations such as liquid–liquid extraction and solid phase extraction from biological samples. In addition, the derivatization of amine and hydroxyl moieties is necessary to prevent tailing peaks and improve the sensitivity. On the other hand, the samples for LC analyses can be prepared rapidly because deproteinization and filtration is simple. In addition, compounds with aliphatic amine moieties can be effectively separated and identified by means of an LC with a strong cation-exchange column-tandem MS (LC/SCX-MS/MS). An SCX column has the advantage of good retention of compounds with aliphatic amine moieties such as ATSs [11,17] and has a low affinity for compounds without aliphatic amine moieties. This enables the elimination of unnecessary matrices and compounds without aliphatic amine moieties, which are eluted at early retention times by changing the valve from an MS

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line to a waste line. Consequently, the pollution of the MS is minimized and its performance is maintained. Moreover, an MS/MS is useful for identifying ATSs because it gives reliable information about chemical structures by selecting characteristic *m*/*z* values for each targeted compound.

When ATSs are taken into the body, biological samples such as plasma, urine and bile may contain not only the ATSs themselves but also their metabolites. Plasma and urine are generally used to verify whether a suspect has taken drugs [14]. On the other hand, bile is not generally used for forensic analysis. It is, however, very important for the study of pharmacokinetics and toxicology. Biliary excretion, which occurs along a pathway from the liver to the duodenum, and urinary excretion are the main excretory routes of many drugs [18–20]. Because most drugs and their metabolites excreted in bile or urine are in forms that are conjugated with hydrophilic compounds such as glucuronide and sulfate [21], it is necessary to hydrolyze the conjugated compounds in samples before the samples can be analysed [9]. Most ATSs are metabolized through *N*-demethylation and hydroxylation of their aromatic moieties, while their amine moieties are preserved. It has reported that 4-hydroxy-3-methoxymethamphetamine (HMMA) and MDA from MDMA [9,22], and *p*-hydroxymethamphetamine (OHMA) and amphetamine (AP) from MA [23] were the main metabolites in humans and rats. Although KA, which possesses an aliphatic amine moieties, is not itself an ATS, ATS tablets often contain KA. Norketamine (NK) is the main metabolite of KA [24,25]. In our preliminary experiments, we administered MDMA, MA, and KA to rats, and detected large peaks corresponding to the above metabolites in plasma, urine, and bile. Therefore, these eight compounds were targeted in this study (Fig. 1).

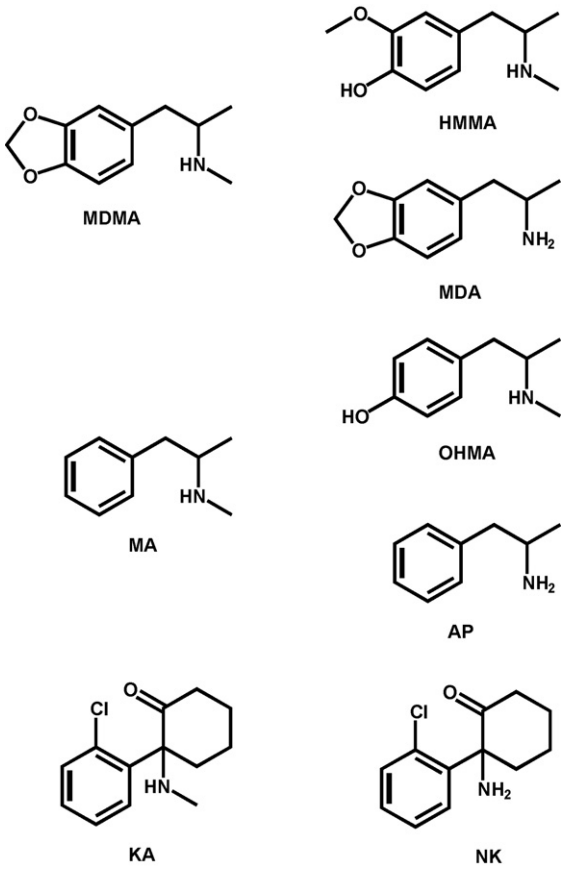

Fig. 1. Chemical structures of MDMA, MA, KA and their metabolites targeted in this study.

In this study, we developed and validated a method for analysing ATSs and their metabolites in plasma, urine and bile using LC/SCX-MS/MS. Moreover, the time courses of the plasma, urine, and bile concentrations of MA, KA, and their main metabolites, OHMA, AP, and NK, were examined after oral administration of MA and KA to rats.

2. Experimental

2.1. Materials

d-MA hydrochloride (MA·HCl) and *d*-OHMA·HCl were purchased from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan) and Sigma–Aldrich Co. (St. Louis, MO), respectively. *dl*-HMMA and *dl*-NK·HCl were purchased from Cerilliant Co. (Austin, TX) as methanol solutions. *dl*-MDMA·HCl, *dl*-MDA·HCl and *dl*-AP sulfate were synthesized as reported previously [26,27]. All other reagents and solvents were purchased from Wako Pure Chemical Co. (Osaka, Japan). Acetonitrile was of LC–MS grade.

Male *Wistar* rats (approximately 200–250 g body weight) were purchased from the Biomaterial Center of Japan Inc. (Tokyo, Japan). A polyethylene tube for cannulation was purchased from Natsume Seisakusho Co. (Tokyo, Japan). A Bollman cage was purchased from Sugiyamagen Co. (Tokyo, Japan).

2.2. Animal experiments

In the experiments involving drug administration, MA·HCl and KA·HCl were dissolved in distilled water. MA (10 mg/kg) and KA (50 mg/kg) was orally administrated to *Wistar* rats (male, 200–250 g body weight) using a cannula. Blood (about 0.2 ml) from the tail vein was periodically collected in a microtube containing heparin (0.1, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, and 24 h) and immediately centrifuged at 2000 × *g* for 10 min. The plasma was transferred to another tube. Urine was collected at from 0 to 10 h and 10 to 24 h on a tray under the cage. For biliary excretion, a polyethylene tube (0.61 mm o.d.) was inserted into a rat bile duct under anesthetization by inhalation of diethyl ether. After drugs were administered, the rat was fixed in a Bollman cage. Bile was collected in a microtube via a polyethylene tube inserted into the bile duct every determined time periods up to 24 h (0–0.5, 0.5–1, 1–1.5, 1.5–2, 2–3, 3–4, 4–6, 6–8, 8–10, and 10–24 h). Drug-free rat plasma, urine and bile were also collected. The plasma, urine and bile samples were stored at −30 °C until use.

2.3. Preparations of analytical samples from plasma, urine and bile

Plasma, urine and bile samples stored at −30 °C were thawed and then the urine was diluted 50-fold with distilled water. Fifty microliters of a sample (plasma, diluted urine or bile) was added to a 1.5-ml microtube, 100 μl of 3 M hydrochloric acid was added, and the tube was placed in a heat block at 120 °C for 30 min for hydrolysis of the conjugated compounds. Five hundred microliters of acetonitrile containing 0.2 μg/ml dibenzylamine (DBA) as an internal standard was added. After vortexing for 5 min and centrifuging at 15,000 × *g* for 10 min, the supernatant was transferred to another tube and was dried by nitrogen flow. The residue was dissolved in the mobile phase as described in Section 2.4. After filtration, 5 μl of the sample solution was injected into an LC/SCX-MS/MS.

2.4. LC/SCX-MS/MS conditions

A Shiseido Nanospace SI-2 (Tokyo, Japan) and a Thermo electron TSQ Quantum Ultra (San Jose, CA) were used as an LC–MS/MS.

Chromatographic separation was achieved using a polymer-coated strong cation-exchange column (Shiseido CAPCELL PAK SCX UG 80; 250 mm × 1.5 mm i.d.; Tokyo, Japan) at 40 °C. The mobile phase consisted of 25 mM ammonium acetate (pH 4.0) and acetonitrile (3:7). The flow rate was maintained at 0.2 ml/min. The switch valve was changed from a waste line to an MS line at 6.5 min. Ionization was performed by electrospray ionization (ESI) in the positive mode, and selected reaction monitoring (SRM) was used for the data acquisition. The MS parameters for the analysis were as follows: spray voltage, 5000 V; sheath gas pressure, 6 Pa; aux gas pressure, 0.5 Pa; capillary temperature, 300 °C; tube lens offset, 50 V; collision pressure, 0.2 Pa; collision energy, 15 eV. The precursor and product ions monitored were m/z 194 → 163 (MDMA), m/z 196 → 165 (HMMA), m/z 180 → 163 (MDA), m/z 150 → 119 (MA), m/z 166 → 135 (OHMA), m/z 136 → 91 (AP), m/z 238 → 179 (KA), m/z 224 → 207 (NK), and m/z 198 → 91 (DBA). Data acquisition and instrument control were performed using Xcalibur software (Thermo Electron Corp., San Jose, CA).

2.5. Validation procedures

Aqueous solutions (50 μg/ml as free bases) of the eight compounds (MDMA, HMMA, MDA, MA, OHMA, AP, KA, and NK) were individually prepared and then were combined to make up a standard mixture solution containing each compound at a concentration of 5 μg/ml. Standard solutions (0.05, 0.1, 0.5, 1, and 2.5 μg/ml) were prepared by diluting the standard mixture solution (5 μg/ml) with distilled water.

Rat plasma, urine and bile were obtained from drug-free rats as described above in Section 2.2. Fifty microliters of each sample (plasma, diluted urine, or bile) was added to a 1.5-ml micro tube, and 50 μl of the standard solution (0.05, 0.1, 0.5, 1, 2.5, and 5 μg/ml) and 50 μl of 6 M hydrochloric acid were added. The following procedure was the same as that described above.

Calibration curves were constructed by an internal standard method using DBA. The regression parameters for the slope and intercept were calculated by weighed ($1/x^2$) linear regression using Correlation2-2 freeware (http://homepage3.nifty.com/m_nw/j-frame.htm). The precision of the measurements was expressed by the coefficient of variation (CV) of the values from independent experiments. The accuracy of the quantifications was evaluated by the percent deviation (%DEV) from the nominal concentration using the formula:

$$\%\mathrm{DEV} = 100 \times \frac{(\text{measured concentration} - \text{added concentration})}{\text{added concentration}},$$

where the measured concentration is the concentration calculated using a calibration curve, and the added concentration is the concentration in the samples spiked with standard solutions.

To measure the recoveries of the eight compounds from plasma, urine and bile, 50 μl of each standard solution (0.1 μg/ml) was spiked into a blank sample (plasma, urine, or bile) before the hydrolysis or after the deproteinization with acetonitrile. The area ratios of the eight compounds obtained from the analytical samples were compared. The recovery from the whole sample preparation was represented using the ratios of the relative peak areas of the eight spiked compounds before the hydrolysis to those after the deproteinization.

To examine the ion suppression or enhancement of the eight compounds and DBA (internal standard) by plasma, urine, and bile, analytical samples prepared with water instead of plasma, diluted urine, and bile were measured. The ion suppression or enhancement was represented using the ratios of the absolute peak areas of the nine compounds spiked into plasma, urine, and bile to those spiked into water.

3. Results and discussion

3.1. Precision and accuracy of the analytical method

The analytical method of ATSs and their metabolites was validated using plasma, urine, and bile obtained from drug-free rats. After the standard solution containing the eight compounds was spiked into rat plasma, urine, and bile at different concentrations (50–5000 ng/ml as free base), the analytical samples were prepared as described in Section 2.3. Fig. 1 shows typical chromatograms of the eight targeted compounds and DBA used as the internal standard. The chromatogram obtained from drug-free rat plasma had no peaks that might have interfered with analysis of the targeted compounds. The targeted compounds were retained well by the SCX column, although they were not perfectly separated from each other. The SCX column enabled an acetonitrile-rich mobile phase, which had the advantage of sensitivity to MS. On the other hand, the metabolites having hydroxyl moieties, HMMA and OHMA, were hardly retained using an octadecylsilyl column even if the component of the mobile phase was water-rich (data not shown). To optimize the MS/MS conditions, automatic tuning of the tube lens offset and collision energy was performed for each targeted compound. The protonated molecules and the base peaks were selected as precursor ions and product ions, respectively. When the tube lens offset and the collision energy were approximately 15 V and 50 eV, respectively, the intensities of the product ions were maximal for each targeted compound. The SRM mode in MS/MS analysis enabled high sensitivity and good selective detection of ATSs and their metabolites.

The area ratios of the eight compounds were measured at each concentration in duplicate, and then a calibration curve was constructed for each compound. The calibration curves for all eight compounds were linear in the range of 50–5000 ng/ml, and the correlation coefficients were greater than 0.992. The concentrations of the eight compounds spiked into plasma, urine, and bile were measured using the calibration curves at two different concentrations added (100 and 1000 ng/ml). The CVs and DEVs were calculated using the concentrations measured in five experiments (Table 1). The CVs for the eight compounds were in the range of 0.4–4.4% for plasma, 0.4–5.3% for urine, and 0.2–3.8% for bile. The DEVs for the eight compounds were in the range of −7.5 to +4.7% for plasma, −9.4 to +8.9% for urine, and −5.3 to +12.8% for bile. The CVs and DEVs obtained were considered to be within the acceptable range according to the guidelines of the U.S. Food and Drug Administration [28]. At the preliminary study of drug administration to rats, it was confirmed that the concentrations of the eight compounds in rat plasma and bile were in the range of 50–5000 ng/ml. Because the concentrations of the eight compounds in rat urine were over 5000 ng/ml, the urine was diluted 50-fold with water as the first step in the sample preparation.

3.2. Recoveries and ion suppression of the targeted compounds in plasma, urine, and bile

The recoveries of the eight compounds in plasma, urine, and bile at 100 ng/ml were examined (Table 2). The recoveries of all eight compounds were over 90% in all three biological samples. The recovery from urine was the highest among the three biological samples. This may have been because the effects of matrices in urine were reduced in comparison with those in plasma and bile by diluting urine 50-fold with distilled water.

The ion suppression or enhancement by plasma, urine, and bile were evaluated by comparing the analytical samples obtained from water with those obtained from plasma, urine, and bile (Table 3). Most of the peak areas were not changed or were somewhat suppressed by plasma, urine, and bile, while the peak area of NK in

Table 1
Precision and accuracy evaluated using eight compounds spiked in rat plasma, urine and bile

Compounds	Added conc. (ng/ml)	Measured conc. (ng/ml) (mean ± S.D., $n = 5$)	CV (%)	%DEV
Plasma				
MDMA	100	97 ± 3.1	3.2	−3.0
	1000	1034 ± 11	1.0	3.4
HMMA	100	99 ± 2.6	2.7	−1.0
	1000	945 ± 17	1.8	−5.5
MDA	100	99 ± 4.3	4.4	−0.6
	1000	1047 ± 18	1.7	4.7
MA	100	96 ± 2.2	2.3	−4.5
	1000	1004 ± 11	1.1	0.4
OHMA	100	96 ± 2.3	2.4	−4.0
	1000	1029 ± 8	0.7	2.9
AP	100	97 ± 2.2	2.3	−2.8
	1000	1020 ± 4	0.4	2.0
KA	100	101 ± 1.3	1.3	0.7
	1000	925 ± 16	1.8	−7.5
NK	100	95 ± 2.5	2.6	−5.2
	1000	1011 ± 13	1.3	1.1
Urine				
MDMA	100	109 ± 3.2	3.0	8.9
	1000	1006 ± 43	0.4	0.6
HMMA	100	91 ± 1.4	1.6	−9.4
	1000	994 ± 20	2.0	−0.6
MDA	100	100 ± 2.1	2.1	−0.1
	1000	981 ± 18	1.9	−1.9
MA	100	100 ± 0.9	0.9	0.5
	1000	1007 ± 8	0.8	0.7
OHMA	100	96 ± 2.0	2.0	−3.6
	1000	993 ± 8	0.8	−0.7
AP	100	96 ± 5.1	5.3	−4.4
	1000	970 ± 9	1.0	−3.0
KA	100	98 ± 2.7	2.7	−1.6
	1000	1035 ± 5	0.5	3.5
NK	100	100 ± 2.5	2.5	0.2
	1000	1013 ± 41	4.0	1.3
Bile				
MDMA	100	106 ± 1.2	1.1	6.2
	1000	1047 ± 29	2.8	4.7
HMMA	100	104 ± 0.7	0.7	4.0
	1000	954 ± 36	3.8	−4.6
MDA	100	111 ± 2.3	2.1	10.7
	1000	1086 ± 22	2.1	8.6
MA	100	98 ± 1.1	1.2	−1.9
	1000	1028 ± 23	2.2	2.8
OHMA	100	104 ± 1.0	1.0	3.9
	1000	947 ± 20	2.1	−5.3
AP	100	100 ± 0.6	0.6	0.3
	1000	987 ± 21	2.2	−1.3
KA	100	108 ± 3.3	3.0	8.5
	1000	989 ± 20	2.0	−1.1
NK	100	96 ± 2.6	2.7	−4.4
	1000	1128 ± 27	0.2	12.8

Table 2
Recoveries of eight compounds spiked at 100 ng/ml in rat plasma, urine and bile

Compounds	Recovery (%) (mean ± S.D., $n = 5$)		
	Plasma	Urine	Bile
MDMA	91.3 ± 3.2	98.2 ± 2.8	93.2 ± 1.0
HMMA	95.4 ± 2.6	104.9 ± 1.2	97.3 ± 0.6
MDA	92.8 ± 4.0	103.7 ± 1.8	95.0 ± 1.9
MA	94.9 ± 2.1	101.0 ± 0.8	95.3 ± 0.9
OHMA	90.8 ± 2.2	104.9 ± 1.9	98.7 ± 1.0
AP	94.0 ± 2.2	102.6 ± 4.5	91.4 ± 0.6
KA	98.4 ± 1.6	99.0 ± 2.7	99.4 ± 1.3
NK	95.4 ± 2.6	95.4 ± 2.1	98.0 ± 0.6

Table 3
Ion suppression or enhancement of nine compounds in rat plasma, urine and bile

Compounds	Ion suppression or enhancement (%)[a] (mean ± S.D., $n = 4$)		
	Plasma	Urine	Bile
MDMA	73.9 ± 8.4	91.6 ± 3.7	47.4 ± 1.9
HMMA	74.4 ± 6.7	56.3 ± 6.3	57.6 ± 2.2
MDA	92.8 ± 3.7	74.4 ± 6.3	81.3 ± 2.4
MA	82.7 ± 5.7	83.0 ± 1.6	71.2 ± 2.3
OHMA	85.8 ± 6.5	72.3 ± 2.1	81.9 ± 1.9
AP	86.4 ± 5.0	71.8 ± 2.8	82.5 ± 2.9
KA	87.0 ± 7.7	97.9 ± 1.1	95.8 ± 5.8
NK	105.1 ± 4.6	92.3 ± 1.6	242.5 ± 4.9
DBA	106.3 ± 6.5	99.0 ± 4.2	72.6 ± 1.0

[a] The value represents the ratios of absolute peak areas of nine compounds spiked to plasma, urine and bile to those spiked to water.

bile was greatly enhanced. Because NK was eluted earliest of the nine compounds (Fig. 2), components in bile retained weakly by the SCX column might have enhanced the corresponding precursor ion and/or product ion of NK. Regardless of the ion suppression or enhancement, the linearity and the recovery of the eight compounds were both very good, as indicated above.

3.3. Hydrolysis of conjugated compounds

Almost all OHMA in plasma and urine is in the form conjugated with glucuronide and sulfate [21]. Because conjugated compounds are excessively hydrophilic, it is generally difficult to retain them

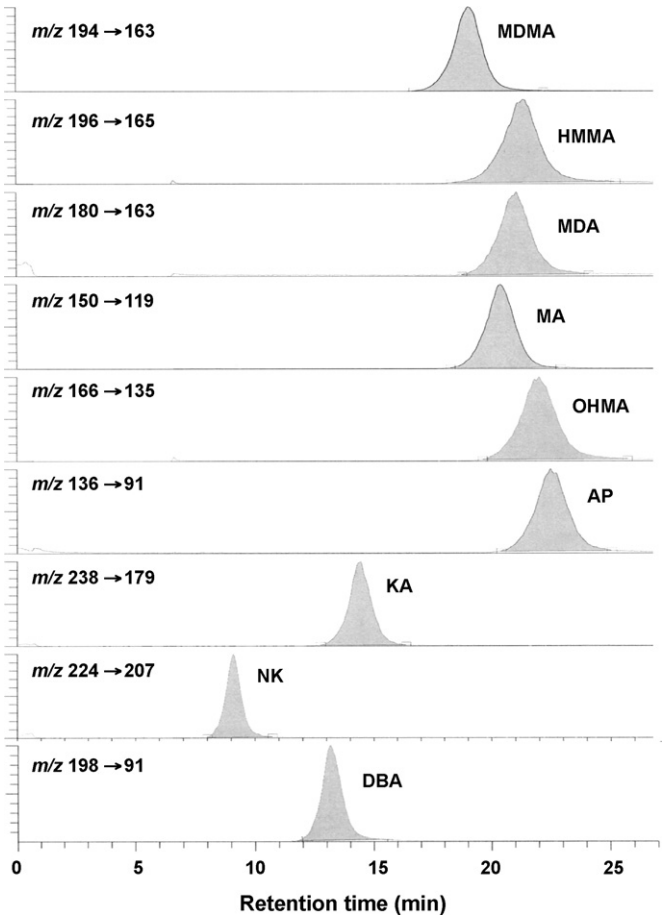

Fig. 2. Typical chromatograms of the eight targeted compounds and DBA used as the internal standard. A standard solution containing the eight compounds (1000 ng/ml) was spiked into rat plasma. Details of the analytical conditions are described in the text.

using a reverse phase column or even using an SCX column. Thus, we hydrolyzed the conjugated compounds. The reaction conditions (2 M hydrochloric acid at 120 °C for 15 min) previously used for the acidic hydrolysis of plasma and urine after MDMA administration [9] were modified for hydrolysis of plasma, urine, and bile after administration of MA and KA. The relative peak areas of MA, KA, and their main metabolites at different reaction times were compared to examine the optimal reaction time for hydrolysis. The relative peak areas of OHMA in plasma, urine, and bile at a reaction time of 15 min were significantly higher than those at 0 min. This indicates that the hydrolysis of conjugated OHMA was necessary for the analysis of OHMA in this method. Moreover, the area ratio of OHMA in plasma increased up to 30 min, which implied that the hydrolysis proceeded further. On the other hand, some compounds in plasma and urine decreased at the reaction time of 60 min. These compounds may have been decomposed by the longer-term reaction. Because a reaction time of 30 min was found to hydrolyze conjugated OHMA with minimum decomposition, this duration was used for the hydrolysis of conjugated compounds in this study.

3.4. Application of the analytical method to pharmacokinetic study of ATSs in rats

The time courses of the concentrations of MA, KA, and their metabolites in rat plasma were examined. After MA (10 mg/kg) and KA (50 mg/kg) were orally administrated to rats, the plasma concentrations of MA, KA, and their metabolites, OHMA, AP, and NK, were measured in the range of 0.5–24 h. The concentration of MA reached the maximum at 3 h (Fig. 3). The concentration of OHMA changed more slowly than that of AP. The concentration of KA was much lower than that of NK even immediately after administration of KA (Fig. 4). NK increased sharply in the range of 0.5–1.5 h and then decreased gradually. Most of the KA was found to be immediately metabolized to NK.

Urinary and biliary excretion of MA and its main metabolites, OHMA and AP, were examined. Because the volumes and time points of urinary excretion in the early stage after drug administration vary widely among individuals, the collection of urine was divided into two periods (0–10 and 10–24 h). The amounts of MA, OHMA, and AP excreted in urine were measured (Table 4). Although OHMA was excreted more slowly than AP, the amount of OHMA excreted in urine over 24 h was greater than the amounts of MA and AP. The total amount was approximately 20% of MA administered to rats.

Because bile was excreted continuously at a steady rate, the cumulative amounts of MA, OHMA, and AP excreted in bile were monitored periodically for up to 24 h (Fig. 5). MA, OHMA, and AP were steadily excreted in bile for up to 8 h and reached plateaus. The total amount of MA, OHMA, and AP excreted in bile was less than that excreted in urine.

Although there have been numerous pharmacokinetic studies of ATSs [29–31] and studies analysing ATSs using LC–MS [29,32–36], there have been far fewer studies analysing metabolites with hydroxyl moieties, such as HMMA and OHMA. HMMA and OHMA are the main metabolites of MDMA and MA, respectively, in humans as well as rats. It is difficult to simultaneously analyse ATSs and their metabolites having hydroxyl moieties using simple preparations from biological samples [37]. In this study, we have demonstrated

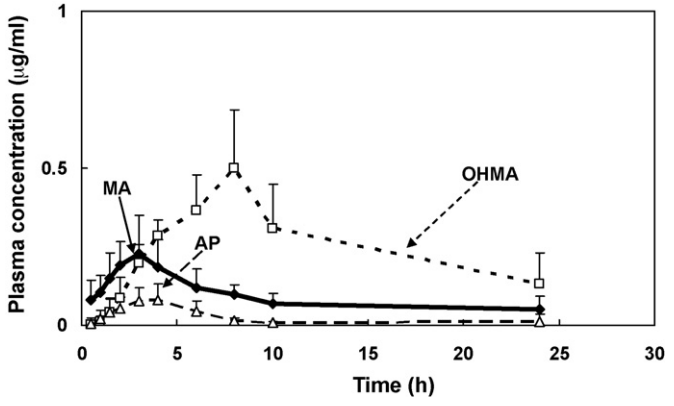

Fig. 3. Plasma concentration–time curves of MA, OHMA, and AP after oral administration of MA to rats. After administration of MA, plasma concentrations of MA, OHMA, and AP were measured in the range of 0.5–24 h. Each point represents the mean ± S.D. (n = 5).

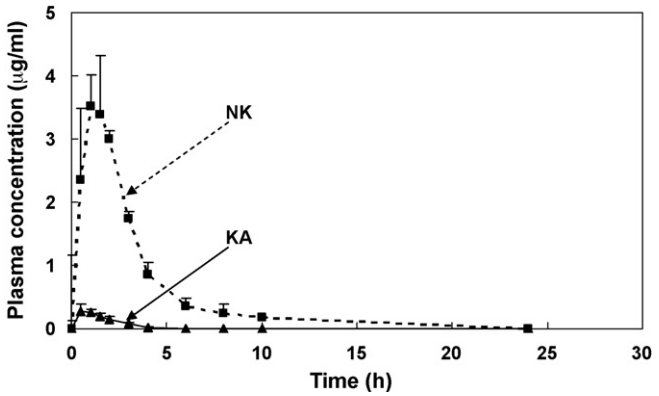

Fig. 4. Plasma concentration–time curves of KA and NK after oral administration of KA to rats. After administration of KA, plasma concentrations of KA and NK were measured in the range of 0.5–24 h. Each point represents the mean ± S.D. (n = 4).

Table 4
Amounts of MA, OHMA and AP excreted in urine after oral administration of MA to rats

Periods after MA administration (h)	Ratios of amounts excreted in urine (%)			
	MA	OHMA	AP	Total
0–10	4.4 ± 0.79	3.3 ± 1.1	1.6 ± 0.45	9.3 ± 1.1
0–24	6.4 ± 2.4	11 ± 2.6	2.6 ± 1.3	20 ± 6.4

The ratios (%) of amounts of MA, OHMA, and AP excreted in urine to MA administered were calculated after conversion to values in molar quantity. Total represents the sum of MA, OHMA and AP. Each value represents the mean ± S.D. (n = 4).

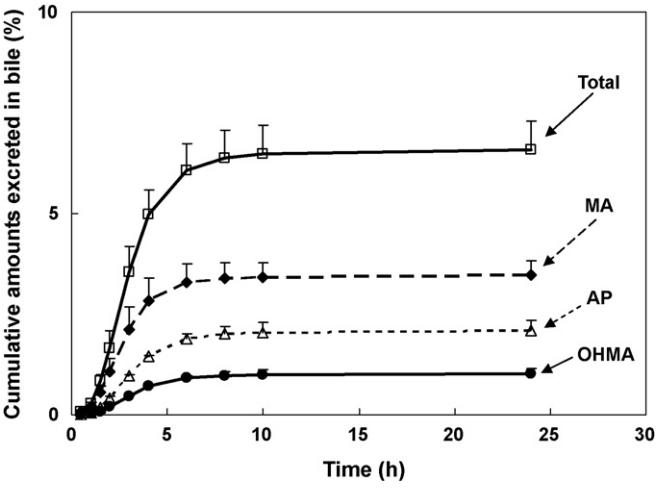

Fig. 5. Cumulative amounts excreted in bile–time curves of MA, OHMA, and AP after oral administration of MA to rats. The ratios (%) of cumulative amounts of MA, OHMA, and AP excreted in bile to MA administered were calculated after conversion to values in molar quantity. Total represents the sum of MA, OHMA, and AP. Each point represents the mean ± S.D. (n = 4).

the simultaneous analysis of not only ATSs but also their demethylated and hydroxylated metabolites in plasma, urine, and bile.

In our previous study, the MA plasma concentration was decreased by oral administration of MA with KA to rats [38]. The results implied that the interaction of MA and KA in the absorption process might occur in humans as well as rats. Because many factors in a living body affect pharmacokinetics, including absorption, distribution, metabolism and excretion, it is necessary to investigate drug–drug interactions in the various processes in order to interpret effects in the whole body. The details of the pharmacokinetic interaction of co-administered MA and KA will be reported in another paper. Although the metabolites of MA excreted in urine qualitatively and quantitatively differ between rats and humans, as reported by Shima et al. [39], findings obtained using laboratory animals will continue to be helpful in understanding the pharmacokinetics of ATSs as long as we can not perform *in vivo* experiments in humans.

4. Conclusions

A method for analysing ATSs and their metabolites in plasma, urine, and bile by LC/SCX-MS/MS was developed and validated. MDMA, MA, KA, and their main metabolites, HMMA, MDA, OHMA, AP, and NK, in each biological sample were simultaneously quantified by the analytical method. The results showed that the method was applicable to the pharmacokinetic analysis of MA, KA, and their main metabolites, OHMA, AP, and NK in rat plasma, urine, and bile. This analytical method should be useful for the pharmacokinetic analysis of ATSs.

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