Simultaneous Chiral Analysis of Methamphetamine and its Metabolites by Capillary Electrophoresis/Mass Spectrometry with Direct Injection of Urine

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We describe a rapid and simple method for the analysis of the *d*- and *l*-isomers of seven methamphetamine-related compounds and the *d*-isomer of pseudoephedrine (pseudoEP) by capillary electrophoresis/mass spectrometry (CE/MS) with direct injection of urine. The compounds were methamphetamine (MA), amphetamine (AP), dimethylamphetamine, ephedrine, norephedrine, methylephedrine, *p*-hydroxymethamphetamine (*p*OHMA). The electrolyte was 1 M formic acid/1 M ammonium formate (10/0.2, v/v) (pH 2.0) containing 1.5 mM heptakis-(2,6-diacetyl-6-sulfato)-β-cyclodextrin. The 14 enantiomers and *d*-pseudoEP were completely separated within 30 min. A urine sample was mixed with an equal volume of internal standard solution, filtered with a 0.45 μm filter and then injected into the CE/MS system. In an analysis of urine sample from a healthy person spiked with racemic MA and AP, the reproducibilities (n = 6) of the migration times and peak areas after correction by an internal standard were under 0.08% and under 3.6%, respectively. The detection limits using selected ion monitoring were 0.01 μg/ml (which corresponds to 0.02 μg/ml urine) for the enantiomers of MA, AP and *p*OHMA. The detection yields of the enantiomers of MA, AP and *p*OHMA from urine were in the range of 97.7–108.8%. The proposed method was successfully used for the chiral analysis of urine samples from MA and dimethylamphetamine (DMA) addicts and patients under selegiline pharmacotherapy.

Key words ——— methamphetamine, chiral separation, urine, capillary electrophoresis, mass spectrometry, direct injection

INTRODUCTION

Amphetamines [which include methamphetamine (MA) and amphetamine (AP)] are used by an estimated 30 million people.[1)] Worldwide, use of amphetamines is more widespread than use of opiates (15 million people) or use of cocaine (13 million people).[1)] In Japan, where abuse of MA is a serious social problem, analysis of MA is important in forensic science. The presence of both MA and its metabolites in urine is generally considered proof of use of MA. There is a need for a rapid analysis of MA and its metabolites in urine, because the police should turn over a suspected person to the public prosecutor's office as soon as possible. In Japan, the maximum time that is allowed for several legal procedures from arrest to transference to the prosecutor (including collection of a urine sample and confirmatory analysis of the urine sample) is 48 hr. Sometimes absolute analytical results are required within a period of an interrogation a suspected person. In such cases, rapidity of responses (within one or two hours) is expected.

MA has an asymmetric carbon in its structure and thus exists as two enantiomers. Until several years ago, almost all MA used by abusers in Japan was in the form of the *d*-isomer.[2)] However, recently, mixtures of *d*- and *l*-isomers have been observed.[3)] The chiral information is useful for tracing complex routes of illicit manufacture and sale. In addition, selegiline (*l*-deprenyl) is a medicine used for Parkinson's disease, which is metabolized to the *l*-isomers of MA and AP.[4)] Therefore, chiral informa-

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tion is needed to discriminate MA abuse from selegiline use.

Several gas chromatography and liquid chromatography (LC) methods are available for the chiral analysis of MA in urine. These methods usually use chiral columns[2,4,5] or require derivatization using chiral regents.[6,7] A disadvantage of these methods is that the analytes must first be extracted from urine, which increases the labor and time needed for these methods. Recently, microextraction methods such as solid-phase microextraction have been reported to increase the extraction rate and to automate the extraction procedures.[8–10] These methods are easy to automate, but suffer from the possibility of cross-contamination (carryover).[11] Another method, chiral analysis by column-switching LC/mass spectrometry (MS),[5] does not require an extraction procedure, but it also has a carryover problem. Carryover is often observed in chromatography techniques and is caused by residual amounts of analytes left in columns, lines and/or column-switching valves.[12]

Capillary electrophoresis (CE) is a powerful technique for chiral analysis because of its high resolution. A number of CE methods have been utilized for the chiral separation of amphetamines in seized drugs *etc*.[13–18] and in urine.[19–24] However, these methods for amphetamines in urine require a procedure of extraction from urine and are unsatisfactory with respect to the need for rapid analyses. We developed a method for the chiral analysis of MA and its metabolites by CE/diode-array detection with direct injection of urine.[25] This method is rapid and simple because it requires only that the urine be diluted and filtered, and does not show any carryover. Recently, we have reported highly sensitive methods for MA and its metabolites by CE/MS.[26,27] However, these methods require a procedure of extraction from urine. Therefore, the purpose of this study was to develop a rapid, simple and highly sensitive CE/MS with direct injection of urine for quantifying chiral analysis of seven methamphetamine-related compounds. The compounds were MA, AP, *p*-hydroxymethamphetamine (*p*OHMA) (a metabolite of MA), and four compounds used as raw materials for MA [dimethylamphetamine (DMA), ephedrine (EP), norephedrine (NE) and methylephedrine (ME)]. The method distinguished MA addicts from DMA addicts and patients under selegiline pharmacotherapy, and also assayed *d*-pseudoephedrine (pseudoEP), which is one of the isomers of ephedrine, and has recently been used as an over-the-counter (OTC) drug in Japan.

MATERIALS AND METHODS

Chemicals —— *d*-MA hydrochloride and *l*-, *dl*-EP hydrochlorides were obtained from Dainippon Pharmaceutical Co. (Osaka, Japan). *l*-MA hydrochloride and *d*-AP sulfate were donated by the Ministry of Health, Labour and Welfare, Japan. Both *d*- and *l*-NE hydrochlorides were obtained from Wako Pure Chemical Industries (Osaka, Japan). *dl*-ME hydrochloride was obtained from Hoei Pharmaceutical Co. (Osaka, Japan). *dl*-AP sulfate, *l*-ME hydrochloride and *d*-, *dl*-DMA hydrochlorides and desmethylselegiline (DM-SG) were donated by the National Research Institute of Police Science (Kashiwa, Japan). *d*-DMA-*N*-oxide was donated by the Forensic Science Laboratory of Osaka Prefectural Police Headquarters (Osaka, Japan). *dl-p*OHMA were obtained from Sigma Chemical (St. Louis, MO, U.S.A.). *d*-PseudoEP hydrochloride was isolated from an OTC drug (ANETON ALMEDI NASAL TABLET, containing 20 mg per tablet) in our laboratory. *l*-1-Phenylethylamine (*l*-1-PEA, internal standard) was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). The structures of the analytes and the internal standard are shown in Fig. 1.

Formic acid, ammonium formate and ammonium acetate were from Wako Pure Chemical Industries. Heptakis-(2,6-diacetyl-6-sulfato)-β-cyclodextrin (DAS-β-CD) was from REGIS Technologies (Morton Grove, IL, U.S.A.). All other reagents used were of analytical reagent grade. Ultrapure water provided by a Milli-RX12α and Milli-Q SP system (Millipore, Bedford, MA, U.S.A.) was used for all procedures.

Apparatus and Optimized Conditions —— Experiments were carried out using an Agilent CE (Agilent Technologies, Waldbronn, Germany) with an Agilent 1100 series MSD mass spectrometer (Agilent Technologies, Palo Alto, CA, U.S.A.). Samples were injected by applying a pressure of 50 mbar for 12 sec. Analytes were separated in an uncoated fused silica capillary of 50 μm i.d. $\times$ 100 cm (GL science, Tokyo, Japan). The applied voltage was +30 kV, and the capillary temperature was maintained at 20°C. The electrolyte was 1 M formic acid/1 M ammonium formate (10/0.2, v/v) (pH 2.0) containing 1.5 mM DAS-β-CD. The running electrolyte was filtered with a 0.45 μm filter (Millipore, Bedford, MA, U.S.A.) before use. Electrospray ionization/MS was conducted in the positive ion mode, and the capillary voltage was set at 4.0 kV. The fragmentor was set at 80 V. Dry nitrogen gas was heated to 250°C

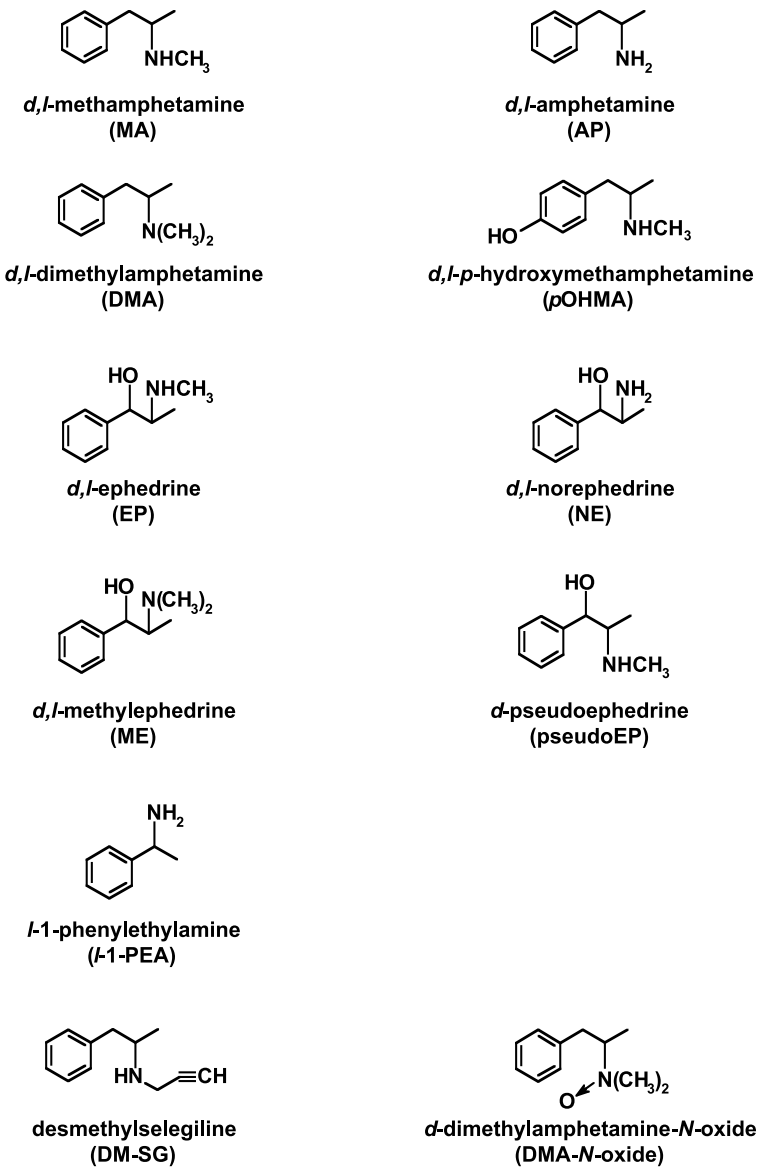

Fig. 1. Structures of Analytes and the Internal Standard

and delivered at a flow rate of 7 l/min. The pressure of nebulizing nitrogen gas was set at 10 psi. The sheath liquid was 10 mM ammonium acetate/methanol (50/50, v/v) and was maintained at 4 μl/min. The spectrometer was scanned from *m/z*: 100 to 250 at 0.85 sec/scan. Other conditions were the same as those used by Soga and Heiger.[28)]

Urine Samples —— The urine samples were taken from MA addicts arrested by the Ishikawa Prefectural Police under "Voluntary Presentation." Control urine samples were collected from healthy persons. Urine samples were stored at +4°C for several days until analysis. For an analysis, 100 μl of urine sample was mixed with the equal volume of 10 μg/ml *l*-1-PEA as an internal standard, and this mixture was passed through a 0.45 μm filter.

RESULTS AND DISCUSSION

Optimization of Electrolyte

We previously reported that DAS-β-CD was suitable as a chiral selector for the chiral CE/MS method because of its high chiral resolution ability for methamphetamine-related compounds.[27)] Using this DAS-β-CD (background electrolyte; 1 M formic acid, pH 1.7), a control urine sample, which was spiked with a mixture of analytes and passed through a 0.45 μm filter, was analyzed by CE/MS with direct injection of urine. The mixture of analytes consisted

of racemic MA ([M+H]$^+$ ion is *m*/*z*: 150), AP ([M+H]$^+$ ion is *m*/*z*: 136), DMA ([M+H]$^+$ ion is *m*/*z*: 164), EP ([M+H]$^+$ ion is *m*/*z*: 166), NE ([M+H]$^+$ ion is *m*/*z*: 152), ME ([M+H]$^+$ ion is *m*/*z*: 180), *p*OHMA ([M+H]$^+$ ion is *m*/*z*: 166) and *d*-pseudoEP ([M+H]$^+$ ion is *m*/*z*: 166). No obstructive peaks appeared at the migration times of enantiomers of MA, AP, DMA, NE, ME and *d*-pseudoEP. At *m*/*z*: 166, two urine matrix peaks were detected. These peaks were not observed in the analysis of urine extracts. The urine first matrix peak (*m*/*z*: 166) did not obstruct the detection of enantiomers of EP, *p*OHMA and *d*-pseudoEP, but the urine second matrix peak (*m*/*z*: 166) overlapped a peak of *l*-*p*OHMA (data not shown). Moreover, the peak of *d*-*p*OHMA overlapped the peak of *d*-EP (data not shown). *p*OHMA is a metabolite of MA and needs to be detected for proof of MA use.

We examined CE/MS with different electrolytes to separate the *l*-*p*OHMA peak from the urine second matrix peak (*m*/*z*: 166) and the *d*-*p*OHMA peak from the *d*-EP peak. The pH of electrolyte (1 M formic acid and 1.0 mM DAS-β-CD) was raised from 1.7 by adding 1 M ammonium formate. At pH values over 2.3, and at a voltage of +30 kV, the electric current exceeded 50 μA. This value is the upper limit for the apparatus. Therefore, the electrolyte was examined in the pH range 1.7–2.2. The resolutions are shown in Fig. 2a. The resolution of *l*-*p*OHMA and the urine second matrix peak (*m*/*z*: 166) dramatically increased with increasing pH. In the pH range 1.85–2.2, the resolution was 4.0–18.4. The cause of this dramatic increase was that the migration time of the urine second matrix peak (*m*/*z*: 166) became longer as pH increased. We estimated that the pK_a of this urine matrix was around 1.7–2.2, which would make the migration time very sensitive to pH in this range. The resolution of *d*-EP and *d*-*p*OHMA also increased with increasing pH. However, at pH values over 2.0, the resolution hardly increased. Thus, in the pH range 1.85–2.2, *l*-*p*OHMA could be separated from the urine second matrix peak (*m*/*z*: 166), and *d*-*p*OHMA could be separated from *d*-EP. On the other hand, the chiral resolutions of the seven analytes generally decreased with increasing pH (Fig. 2b). Accordingly, we decided that the optimum background electrolyte was 1 M formic acid/1 M ammonium formate (10/0.2, v/v) (pH 2.0).

Using the electrolyte 1 M formic acid/1 M ammonium formate (10/0.2, v/v) (pH 2.0) containing 1.0 mM DAS-β-CD, all enantiomers were separated, respectively, but the enantiomers of NE were not baseline resolved (Fig. 2b). Increasing the concentration of DAS-β-CD from 1.0 to 2.0 mM increased chiral resolution. However, as the concentration of DAS-β-CD increased, the migration times became longer. We decided that the optimum concentration of DAS-β-CD was 1.5 mM. This value gave sufficient chiral resolution, and kept the analysis time within 30 min. Using the conventional method of analysis of MA and its metabolites in urine (liquid-liquid extraction and derivatization in forensic science laboratory followed by GC/MS[29]), pretreatment takes more than 90 min, so that the total analysis time (from pretreatment to equipment analysis) is more than 2 hr. However, in this proposed method, the total analysis time is only *ca*. 30 min.

A mass pherogram of a control urine spiked with

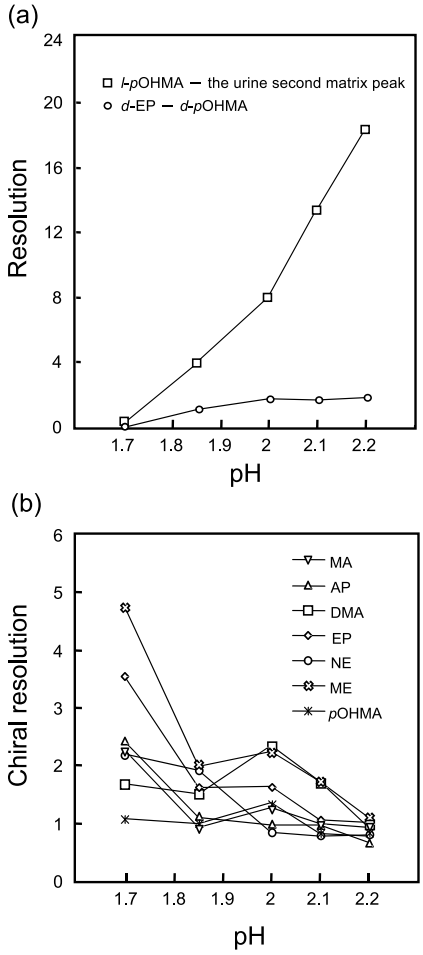

Fig. 2. Effect of pH of Background Electrolyte on (a) the Resolutions of *l*-*p*OHMA — the Urine Second Matrix Peak and *d*-EP — *d*-*p*OHMA, (b) the Chiral Resolutions of MA, AP, DMA, EP, NE, ME and *p*OHMA

Conditions: capillary, 50 μm i.d. × 100 cm uncoated fused silica; applied voltage, +30 kV; temperature, 20°C; sample injection, 50 mbar for 12 sec.

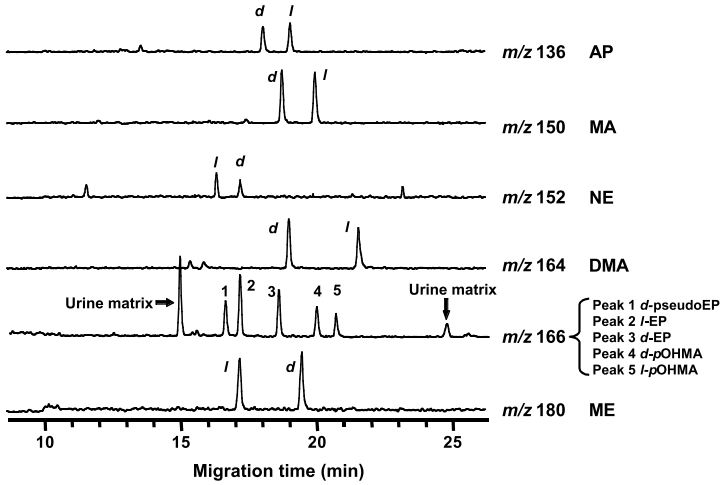

Fig. 3. Mass (Scan) Pherogram of a Control Urine Sample Spiked with Racemic MA, AP, DMA, EP, NE, ME, *p*OHMA and *d*-pseudoEP (each 1 μg/ml Urine) Using 1 M Formic Acid/1 M Ammonium Formate (10/0.2, v/v) (pH 2.0) Containing 1.5 mM DAS-β-CD Other CE/MS conditions were as described in Fig. 2.

a mixture of racemic MA, AP, DMA, EP, NE, ME, *p*OHMA and *d*-pseudoEP (each 1 μg/ml urine) using the above optimized electrolyte is shown in Fig. 3. All compounds were clearly detected. At *m*/*z*: 166, high resolution of *d*-pseudoEP, *d*-EP, *l*-EP, *d*-*p*OHMA and *l*-*p*OHMA were achieved. The urine matrix peak (*m*/*z*: 166), which overlapped a peak of *l*-*p*OHMA in the analysis using the electrolyte of our previous report,[27] was separated from a peak of *l*-*p*OHMA. This urine matrix peak (*m*/*z*: 166) was detected at 24.7 min. And the other urine matrix peak was detected at 14.9 min. Several dozen control urine samples did not shown any peaks at the migration times of the enantiomers of MA, AP, DMA, EP, NE, ME, *p*OHMA, or *d*-pseudoEP (data not shown). This result suggested that filtering the samples through a 0.45 μm filter was enough to allow detection of all compounds.

Validation of the Proposed Method and Verification of Removing Carryover

In CE/MS analysis, the injection volume is influenced by the viscosity of the sample, because the sample is injected by applying pressure at the injection end of the capillary. For correcting the injection volume, we found that *l*-1-PEA (Fig. 1) was suitable as an internal standard. *l*-1-PEA is not usually present in normal human urine. We also used the internal standard for correcting the migration times of the analytes peaks. One hundred μl of 10 μg/ml aqueous internal standard solution was mixed with an equal volume of urine sample, and then the mixture was filtered. This volume was large enough to accurately determine the injection volume. For each urine sample, the time-axis of the mass pherogram was automatically moved by the ChemStation software in order to adjust the migration time of the internal standard to the same migration time observed in the standard spiked urine sample. The amount of an analyte was expressed as the ratio of its peak area to the peak area of the internal standard.

The reproducibilities of migration times and peak areas for within-run (n = 6) and between-run assays (n = 6) were measured with a single control urine sample that had been spiked with a mixture of racemic MA and AP (each 2 μg/ml urine) (Table 1). In the within-run assays, the relative standard deviation (RSD) values of the migration times and peak areas were 0.4 and 2.1–4.0%, respectively. However, in the between-run assays, the RSDs of the migration times and peak areas were large (over 1.5% and over 8.4%, respectively). The RSDs of the migration times and peak areas corrected by the internal standard in the within-run assays were under 0.08% and under 3.6%, respectively, while the values for the between-run assays were under 0.4% and under 5.3%, respectively. Use of the internal standard gave good reproducibility even for peak areas in the between-run assays. The reproducibilities of the relative peak areas for the urine samples from the 10 healthy persons, after correction by the internal standard, were no more than 4.9%. Therefore, using the internal standard made it possible to determine

Table 1. Reproducibilities of Within-Run and Between-Run Assays for Migration Time (MT) and Peak Area (PA) of a Urine Sample[a)] by Use of Internal Standard (IS)

Compound	Within-run (RSD,[b)] %)				Between-run (RSD,[b)] %)			
	Without IS		With IS		Without IS		With IS	
	MT	PA	MT	PA	MT	PA	MT	PA
d-MA	0.4	2.1	0.08	3.6	1.5	11.7	0.3	2.7
l-MA	0.4	2.8	0.08	2.3	1.7	9.3	0.4	2.0
d-AP	0.4	3.5	0.04	3.5	1.5	12.5	0.2	4.3
l-AP	0.4	4.0	0.07	2.7	1.5	8.4	0.3	5.3

a) Healthy person's urine sample spiked with racemic MA and AP (each 2 μg/ml urine) and the sample was mixed with the same volume of 10 μg/ml *l*-1-PEA solution. *b*) $n = 6$.

the quantities of analytes for urine samples from different persons.

The control urine sample was spiked with various concentrations of the enantiomers of MA, AP and *p*OHMA to obtain calibration curves. The calibration curves were based on the ratio of the peak area using selected ion monitoring (SIM) of the target compounds' [M+H] ions. The calibration curves were linear in the range of 0.025–5 μg/ml, which corresponds to 0.05–10 μg/ml in urine ($r^2 = 0.998$–0.999). The detection limit was defined as the concentration that produced a signal equal to 3–4 times the background noise level using SIM. The detection limits for the enantiomers of MA, AP and *p*OHMA were all 0.01 μg/ml (which corresponds to 0.02 μg/ml urine). The detection limit of *d*-MA using the Triage® (immunoassay technique) is 1 μg/ml.[30] The cut-off levels of MA by EMIT® (immunoassay technique) was 0.5 μg/ml,[31] and the detection limit of *d*-MA by column-switching LC/MS with direct injection of urine was 0.5 ng/ml.[5] The column-switching LC/MS technique has high sensitivity but it also has a carryover problem.[12] On the other hand, there is rarely carryover in the CE/MS method, because CE/MS apparatus has no columns, lines or column-switching valves. Even after an analysis of 200 μg/ml *d*-MA, no peaks appeared at the migration times of *d*-MA in the blank analysis. Concentrations of MA in urine samples of MA addicts are typically in the range 3–10 μg/ml,[32] but in chronic MA addicts they can be as high as 100 μg/ml. The cut-off levels of MA in urine are 0.3 μg/ml according to the National Institute of Justice (U.S.A.)[33] and the Australian Institute of Criminology (Australia),[34] and 0.5 μg/ml according to the Research Development and Statistics Directorate, Home Office (U.K.).[35] Thus, our method has sufficient sensitivity and can be successfully applied to the analysis of urine samples of MA addicts.

To evaluate the detection yields of analytes in the urine sample, samples from five healthy persons were spiked with a mixture of racemic MA, AP and *p*OHMA (each 1 μg/ml urine). The detection yields were in the range of 97.7–108.8% with RSDs of 2.6–5.7%.

Chiral Information of Analytes in Forensic Urine Samples

The present method can distinguish *d*-MA addicts from addicts who use a mixture of *d*- and *l*-MA. Figure 4a shows a mass pherogram of a urine sample from a *d*-MA addict. Unchanged *d*-MA, the *d*-MA metabolites *d*-AP and *d*-*p*OHMA were detected. Figure 4b shows a mass pherogram of a urine sample from an addict who used a mixture of *d*- and *l*-MA. Unchanged *d*- and *l*-MA, the MA metabolites *d*-, *l*-AP and *d*-, *l*-*p*OHMA were detected.

The present method can also distinguish MA addicts from users who use compounds that are metabolized to MA and excreted in the urine. *d*-DMA, whose abuse has increased significantly in Japan in the past few years,[36] is metabolized to *d*-isomers of MA, AP, *p*OHMA, and a *d*-DMA specific metabolite *d*-DMA-*N*-oxide ($[M+H]^+$ ion is *m*/*z*: 180). Each of these metabolites plus unchanged *d*-DMA are excreted into the urine. Selegiline, a medicine used for Parkinson's disease, is metabolized to *l*-isomers of MA, AP, *p*OHMA, and a selegiline specific metabolite DM-SG ($[M+H]^+$ ion is *m*/*z*: 174). Each of these metabolites are excreted in the urine, while unchanged selegiline is not excreted.[4] A urine sample was spiked with a mixture of *d*-DMA-*N*-oxide and DM-SG and racemic MA, AP, *p*OHMA and DMA (each 0.5 μg/ml, corresponding to 1 μg/ml urine) and analyzed by the proposed method. All compounds were clearly detected (Fig. 5a). Using SIM, the detection limits of *d*-DMA-*N*-oxide and DM-SG spiked in the urine were 0.01

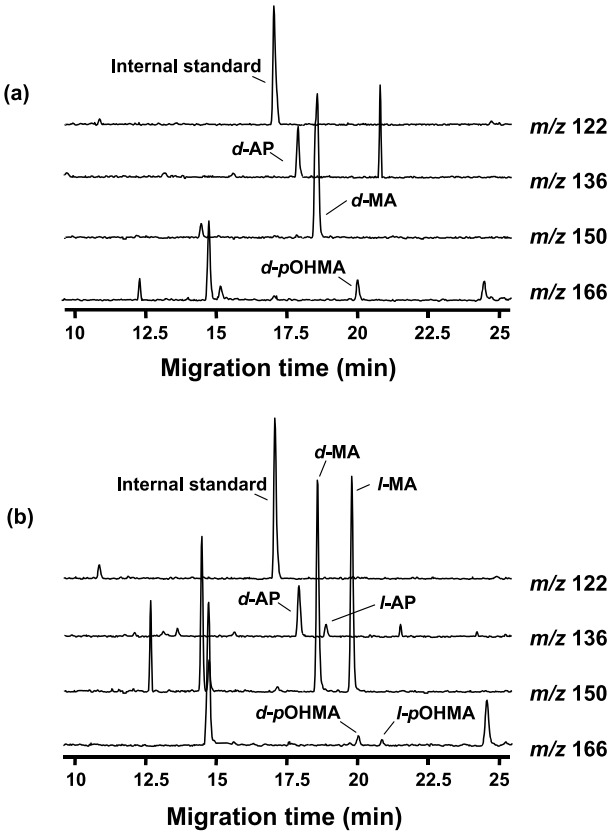

Fig. 4. Mass (Scan) Pherograms of Typical Urine Samples (a) from a *d*-MA Addict, and (b) from an Addict who Used a Mixture of *d*- and *l*-MA

CE/MS conditions were as described in Figs. 2 and 3.

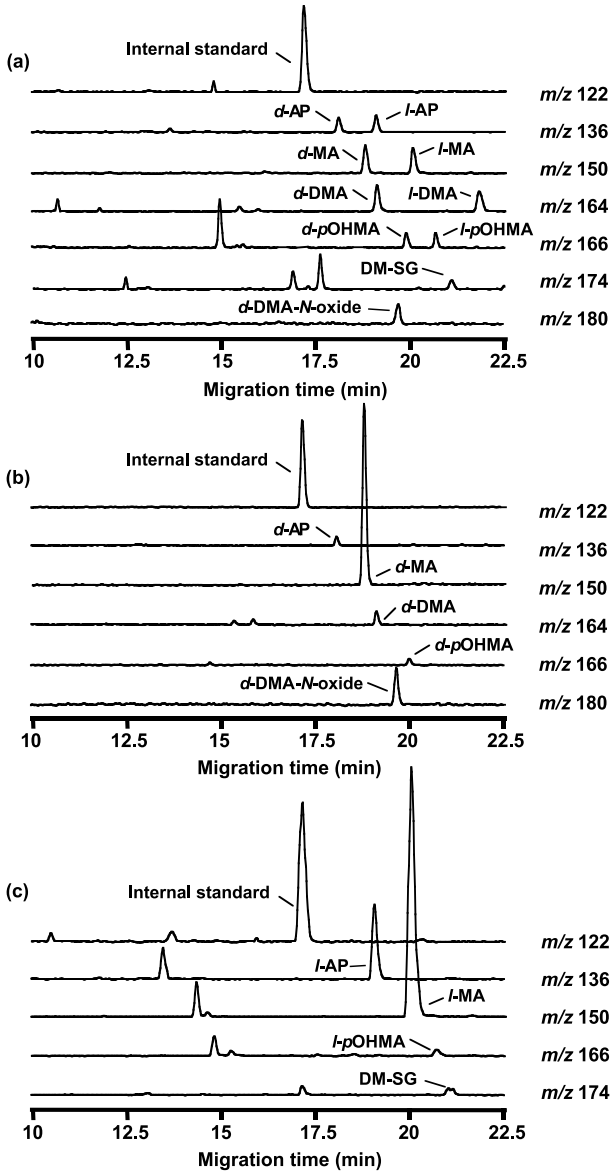

Fig. 5. Mass (Scan) Pherograms of (a) a Control Urine Sample Spiked with Racemic MA, AP, DMA, *p*OHMA, and DM-SG, *d*-DMA-*N*-Oxide (each 0.5 μg/ml, which Corresponds to 1 μg/ml Urine), (b) a Typical Urine Sample from an Addict who Used a Mixture of *d*-MA and *d*-DMA, (c) a Typical Urine Sample from a Patient under Selegiline Pharmacotherapy

CE/MS conditions were as described in Figs. 2 and 3.

and 0.03 μg/ml, respectively (which corresponds to 0.02 and 0.06 μg/ml urine). A mass pherogram of a urine sample from an addict who used a mixture of *d*-MA and *d*-DMA revealed unchanged *d*-MA and *d*-DMA, *d*-MA (a metabolite of *d*-DMA), *d*-AP and *d*-*p*OHMA (metabolites of *d*-MA and/or *d*-DMA) and *d*-DMA-*N*-oxide (Fig. 5b). Figure 5c shows a mass pherogram of a urine sample from a patient under selegiline pharmacotherapy. This mass pherogram was obtained after concentration, because the concentration of DM-SG in urine was under the limit of detection. Metabolites *l*-MA, *l*-AP and *l*-*p*OHMA were detected, and DM-SG was also detected. These results suggest that in the analysis of a urine sample with direct injection of urine, if the *l*-isomers of MA, AP and *p*OHMA were detected and the *d*-isomers of these compounds and specific metabolites such as DM-SG were not detected, it is necessary to concentrate the urine sample and analyze it again. As methods of the concentration of urine, techniques such as liquid-liquid or solid phase extraction can be available in order to identify the presence of specific metabolites.

In conclusion, the present CE/MS method using *l*-1-PEA as an internal standard enables reliable analyses of the enantiomers of MA, AP, DMA, EP, NE, ME, *p*OHMA and *d*-pseudoEP in urine. This method is rapid, simple, highly sensitive, and free from errors because it requires that urine samples

be only filtered, does not show any carryover, and can successfully assay for analytes in urine samples from MA and DMA addicts and patients under selegiline pharmacotherapy.

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