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Determination of enantiomeric amphetamines as metabolites of illicit amphetamines and selegiline in urine by capillary electrophoresis using modified β-cyclodextrin

Yoo Jeong Heo[a], Yoon Sung Whang[a], Moon Kyo In[b], Kong-Joo Lee[a,*]

[a]*Center for Cell Signaling Research and College of Pharmacy, Ewha Womans University, Seoul 120-750, South Korea*
[b]*Abused Drugs Laboratory, Supreme Public Prosecutor's Office, Seoul 137-741, South Korea*

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Abstract

The determination of enantiomeric amphetamine and methamphetamine in urine samples is important in order to distinguish use of the prescription drug selegiline (metabolized to *R*(−)-A and *R*(−)-MA) from the illicit use of *S*(+)-A and *S*(+)-MA. For the analysis of enantiomeric amphetamine (A) and methamphetamine (MA) in biological samples, the optimization of analytical condition was performed by capillary electrophoresis using chiral selectors including β-cyclodextrin, carboxymethyl-β-cyclodextrin and 2-hydroxypropyl-β-cyclodextrin. We have examined the factors to obtain the best chiral resolutions, separation efficiency and sensitivity, and wide concentration linearity. Optimum resolutions were achieved using 100 m*M* phosphate buffer, pH 2.5, containing 10 m*M* of carboxymethyl-β-cyclodextrin. This method was applied for the quantitative determination of enantiomeric amphetamine and methamphetamine in urine samples obtained from patients taking illicit amphetamines or from rats and patients taking selegiline. Acceptable quantitative results in terms of resolution, precision, sensitivity and linearity were obtained from the real urine samples containing wide-ranging concentrations of A and MA by using two concentrations of internal standards, α(+)- (1 μg/ml) and β-phenylethylamine (50 μg/ml).

Keywords: Enantiomer separation; Amphetamine; Methamphetamine; Selegiline

1. Introduction

In pharmaceutical analysis, chiral separation is a rapidly growing area as enantiomers have varying pharmacological and toxicological effects on living bodies and also because the FDA has started to regulate chiral purity in drugs [1]. Liquid chromatography (LC), capillary gas chromatography (GC) and supercritical fluid chromatography (SFC) have been conventionally used for chiral separations. However, it is necessary to develop improved analytical methods for chiral separation of polar and involatile drugs because the conventional methods using the immobilized column chiral selector are expensive, need better resolution, sometimes require prederivatization for analysis and demand an extended analysis time.

Chiral discrimination of A and MA isomers is necessary to distinguish use of non-prescription nasal inhalants (*R*(−)-MA) or the prescription drug selegiline (metabolized to *R*(−)-A and *R*(−)-MA)

*Corresponding author. Tel.: +82-2-3277-3038; fax: +82-2-3277-3760.

E-mail address: kjl@mm.ewha.ac.kr (K.-J. Lee)

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from illicit use of *S*(+)-A and *S*(+)-MA. Definitive enantiodiscrimination requires the use of a chiral derivatization reagent to form diastereomers of *R*(−)- and *S*(+)-A and -MA, which can be resolved using conventional GC–MS. Capillary electrophoresis (CE) has been shown to be an attractive approach for the chiral separation using various additives (crown ethers [2], cyclodextrins [3–9], bile salt [10] etc.). Chiral selectors were added to the background electrolyte for the pseudostationary phase. Chiral separations have been possible for the different dynamic equilibrium forming diastereomeric complexes between the chiral analytes and chiral selectors [11–14]. Many studies present chiral separations of A, MA and related basic compounds by capillary electrophoresis (CE) using various chiral selectors including β-cyclodextrin, carboxymethyl-β-cyclodextrin and 2-hydroxypropyl-β-cyclodextrin [14–17]. Recently, these methods were applied to real analysis of urine and hair samples [16,17]. However, satisfactory separations for the real samples were not obtained with 2-hydroxypropyl-β-cyclodextrin and β-cyclodextrin. In this study, we attempted to refine the CE method using β-cyclodextrin derivatives for the analysis of enantiomeric A and MA in real urine samples. Real urine samples contain wide-ranging concentrations of A and MA, from 0.1 to 100 μg/ml. Therefore, the methods were optimized for high resolution and sensitivity, and wide linearity. Varied chiral selectors were used and the factors affecting the chiral separation and mechanism of interaction between chiral compounds and chiral selectors were examined. Two internal standards with a high and low concentration range were used to obtain wide linearity. The refined method was applied to the quantitative determination of enantiomeric A and MA in real urine samples obtained from patients taking illicit amphetamines or from rats and patients which had taken selegiline. The acceptable analytical results were obtained for real sample analysis.

2. Experimental procedures

2.1. Materials

Chemicals including phosphoric acid and sodium phosphate, α- and β-phenylethylamine, α- and β-cyclodextrin, racemic amphetamine and methamphetamine, came from Sigma (St. Louis, MO, USA) while β-cyclodextrin (BCD) derivatives, carboxymethyl-β-cyclodextrin (CMCD) and 2-hydroxypropyl-β-cyclodextrin (HPCD), were obtained from Cyclolab (Budapest, Hungary). The optically pure amphetamine derivatives were obtained from Alltech (Deerfield, IL, USA). Urine samples containing amphetamine derivatives were provided by the Korean Supreme Public Prosecutor's Office, and urine samples of mice and humans that had taken selegiline, were kindly provided by the National Institute of Scientific Investigation in Korea.

2.2. Capillary electrophoresis procedure

For the chiral separation, the untreated fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA), 65 cm long (56.5 cm to detector)×50 μm I.D., was used. The apparatus used for these studies was an automated HP^{3D}CE system (Hewlett-Packard, Palo Alto, CA, USA) with HP^{3D}CE Chemstation for control and data acquisition. Prior to each run, the capillary was rinsed with 0.1 *M* sodium hydroxide solution, distilled water and running buffer. The capillary was filled with low-pH running buffer, a 20–100 m*M* phosphate buffer (pH 2.5) containing various concentrations of CD derivatives. The samples were introduced by hydrodynamic injection (50 mbar for 1 min) and run by applying a voltage of 25 kV at 15°C. Detection was performed at 205 nm.

2.3. Sample preparations

Standard stock solutions of each racemic and *S*(+)-A and *S*(+)-MA (final concentration 3 mg/ml) were prepared in methanol spiked with α(+)- and β-phenylethylamine as internal standards (I.S.) (final concentration, 100 μg/ml and 1 mg/ml, respectively). Standard model mixtures of racemic A and MA (0.25–100.0 μg/ml) were prepared by diluting the stock solution with 0.1 *M* HCl and were then stored in the dark. Two different internal standards were

used for quantitative analysis of real samples with wide concentration range (0.5–100 μg/ml). Drug concentrations were determined in normal urine spiked with various concentrations of A and MA (final concentration, 1 μg/ml) and two internal standards (I.S.) with different concentrations, α(+)-(1 μg/ml) and β-phenylethylamine (50 μg/ml), and in patient urine containing A and MA spiked with two internal standards. Sample extractions were carried out using the modified liquid–liquid extraction method [18]. A 0.5 ml aliquot of standard solution, normal and patient urine spiked with I.S., was mixed with 100 μl of 1 *M* NaOH to raise the pH, at which point the deprotonated free bases could be extracted into the organic phase. Chloroform (1 ml) was used to extract these deprotonated amines. After vigorous vortex mixing and centrifugation at 12 500×*g* for 5 min, 0.5 ml of the organic layer was transferred to a conical glass tube. The organic layer was acidified by adding 100 μl of 0.1 *M* HCl and the protonated charged amines were reextracted into the aqueous phase. The collected aqueous phase was evaporated in a speed vacuum concentrator and reconstituted to double the volume with distilled water. This reconstituted sample was hydrodynamically injected into capillary electrophoresis. Urine samples from rats that had taken selegiline were treated in the same way as human urine samples.

3. Results and discussion

For the determination of enantiomeric A and MA in urine samples using capillary electrophoresis, factors affecting the chiral separation of A and MA and the separation mechanism were examined by using various cyclodextrin derivatives (BCD, HPCD, CMCD) as chiral selectors. Optimized separations were applied to analyze the metabolites of selegiline and methamphetamine in urine samples.

3.1. Optimization of chiral separations

To obtain the optimum separation, several factors were varied including chiral selector, buffer concentration and injection volume. Low pH running buffer (pH 2.5) was adopted for the separation of amphetamine derivatives, because the resolution of basic compounds could be improved by reducing the electroosmotic flow (EOF). Enantiomers were separated at the low temperature of 15°C, as a decrease in the capillary temperature worked to increase the separation efficiency [18].

3.1.1. Chiral selector

In order to select the best chiral selector for the quantitative analysis of enantiomeric A and MA, chiral selectivities of various β-cyclodextrin derivatives were compared as shown in Fig. 1. Satisfactory separation could not be obtained using 15 m*M* BCD, however, and additional chiral selector could not be used because of the low solubility of BCD. Instead, hydroxypropyl-β-cyclodextrin (HPCD) was used since it has higher solubility in an aqueous solution than BCD because of the hydroxyl residue. Using HPCD as the chiral selector, stable separation with high separation efficiency, resolution and peak symmetry was obtained by raising the concentration. Higher chiral selectivity using negatively charged carboxymethyl-β-cyclodextrin (CMCD) was obtained with lower concentration. In the case of a neutral chiral selector like BCD and HPCD combined with basic A and MA to form a complex, the positive charge density of A and MA was decreased due to an increase in the size of the complex. Therefore, the mobility of the complex toward the cathode was reduced and separation was accomplished according to the stability of the formation of the complex chiral compounds. On the other hand, the complex of negatively charged CMCD with positively charged A and MA could be formed by electrostatic interaction. The negative charge of CMCD and positive charge of A and MA resulted in electrophoretic mobility in opposite directions. The association of A in CMCD changes the electrophoretic mobility dramatically, producing an increase in the separation window and consequently, excellent separations [5]. As a result, the optimum chiral selector for the separation of enantiomeric A and MA was found to be 10 m*M* CMCD.

3.1.2. Effect of buffer concentration

Factors affecting the chiral separation with CMCD

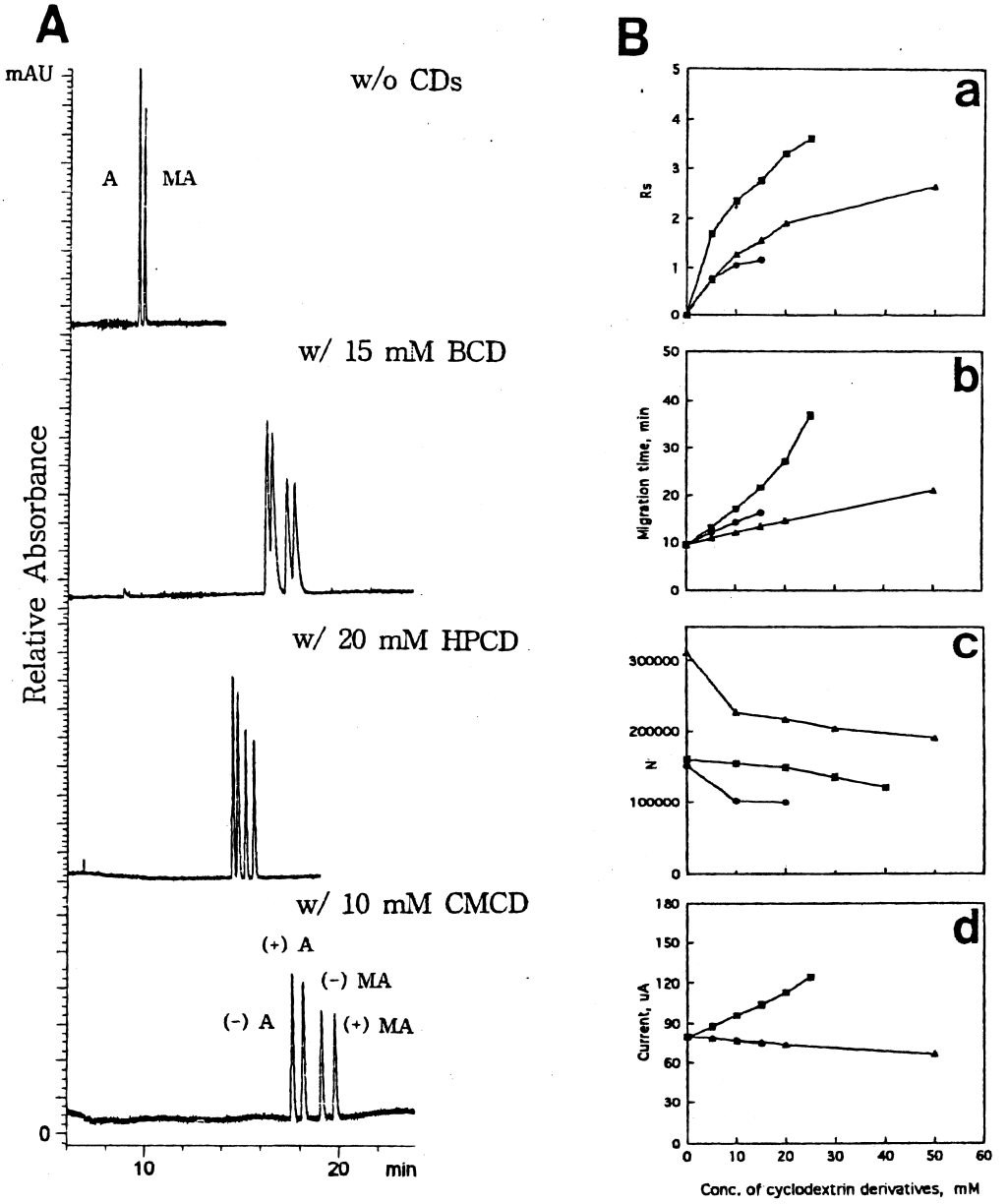

Fig. 1. (A) Electropherograms of enantiomeric A and MA obtained using 100 m*M* phosphate buffer (pH 2.5) containing (a) no chiral selector, (b) 15 m*M* BCD, (c) 20 m*M* HPCD and (d) 10 m*M* CMCD. (B) Effects of chiral selector concentrations (BCD, ●; HPCD, ▲; CMCD, ■) on enantiomeric separation of amphetamine in terms of (a) resolution, (b) migration time, (c) number of theoretical plates (N/m) and (d) running current. CE running conditions were as follows: a fused-silica capillary, 65 cm long (56.5 cm to detector)×50 μm I.D.; applied voltage, 20 kV; temperature, 15°C; sample injection, 50 mbar for 60 s by pressure; UV.

were examined by observing the dependence of migration time on the concentration of running buffer (sodium phosphate buffer, pH 2.5). Various concentrations of running buffer (20–200 m*M*) containing 15 m*M* chiral selector were used. Migration time of solutes with BCD and HPCD were not

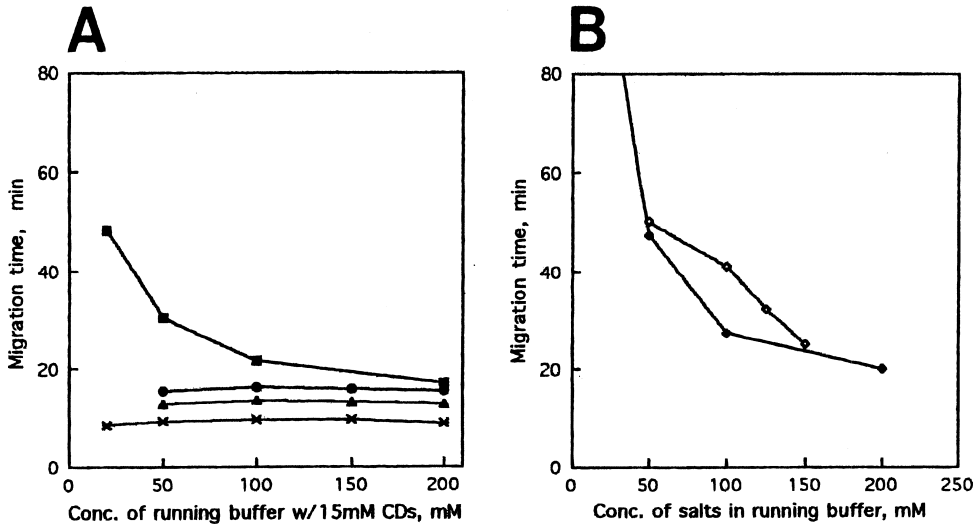

Fig. 2. Effects of salt concentration in running buffer on the migration time of *R*(−)-amphetamine. (A) Migration time was plotted against the concentration of running buffer (phosphate buffer, pH 2.5) containing no chiral selector (×), 15 m*M* of BCD (●), HPCD (▲), CMCD (■). (B) Migration time was plotted against the concentration of running buffer (♦) and total concentration of salts in the running buffer composed with various concentrations of NaCl, 50 m*M* sodium phosphate and 10 m*M* CMCD (◇). Other conditions are the same as in Fig. 1.

varied by increasing the buffer concentration, but were decreased with CMCD by raising the buffer concentration (Fig. 2). Generally, migration time increases with the increase in running buffer concentration because the higher ionic strength of the buffer acts to reduce electroosmotic mobility [19,20]. However, the electroosmotic flow at pH 2.5 is negligible, and the migration time of solutes is independent of the buffer concentration as shown in Fig. 2A. Therefore, the dependence of migration time on buffer concentration is due to the effect of ionic strength on electrostatic interaction between CMCD and A and MA. Raising the ionic strength of the buffer, resulted in decreased electrostatic interaction in the complex between CMCD and basic solute, more favorable equilibrium toward free A and MA from the complex and increased mobility of free A and MA toward the cathode. To confirm this mechanism, the same experiments were performed, except for varying NaCl concentrations (0–150 m*M*) in 50 m*M* sodium phosphate buffer, pH 2.5 (Fig. 2B). Almost identical results were obtained. It turned out that the electrostatic interaction between CMCD and basic solutes was a major factor affecting the separation. The results suggest that the separation of basic compounds using neutral chiral selectors, either BCD or HPCD, is based on the change in geometric structure between stereoisomers. When using CMCD, however, separation is based on the stability of the complex, which is affected by electrostatic interaction.

3.1.3. Effect of injection volume

For the real sample analysis, separation was examined by changing the sample injection volume (Fig. 3). The resolution of enantiomeric A and MA using 10 m*M* CMCD or 20 m*M* HPCD in 100 m*M* sodium phosphate buffer (pH 2.5) was examined by varying the injection volume (4.7–117.5 nl). This volume was 0.37–9.2% of total capillary volume (1.27 μl). In both cases, raising the sample injection volume decreased the resolution and separation efficiency. The separation efficiency was dramatically decreased, and the resolution and peak shape found unsatisfactory (R_s<1) for over 60 nl HPCD, but suitable separation was obtained with 90 nl CMCD. Therefore, the separation capacity of CMCD

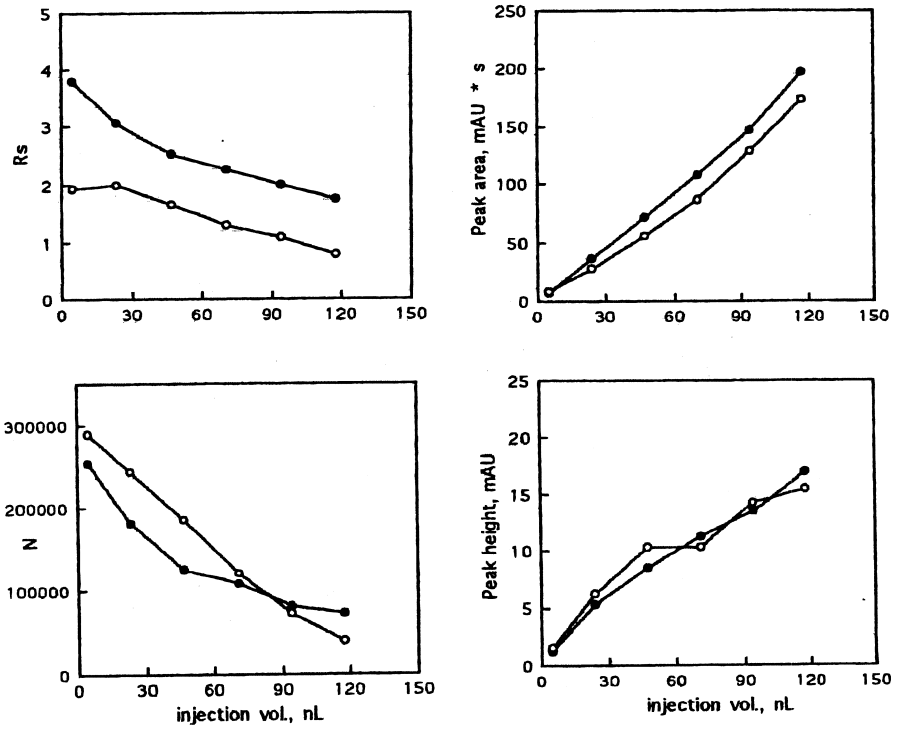

Fig. 3. Effects of sample injection volume on the chiral separation of amphetamine. Running buffer was a 100 m*M* phosphate buffer (pH 2.5) containing 10 m*M* CMCD (●) and 20 m*M* HPCD (○). Other conditions are the same as in Fig. 1.

proved to be superior to that of HPCD. The legal cut-off for the concentration of A and MA as illicit drugs is 0.5 μg/ml, and quantitative analysis should be achieved over this amount. Therefore, 70 nl sample injection, which maintains baseline separation, was adopted.

Table 1
Reproducibility of migration time and peak area of enantiomeric amphetamine (A) and methamphetamine (MA)[a]

Conc. (μg/ml)	Migration time				Peak Area			
	(−)-A	(+)-A	(−)-MA	(+)-MA	(−)-A	(+)-A	(−)-MA	(+)-MA
Within-run (n=5) C.V. %								
0.5	0.581	0.578	0.584	0.556	1.445	4.444	10.021	8.472
1.0	0.334	0.328	0.333	0.272	3.557	2.920	5.736	3.472
2.0	0.528	0.545	0.521	0.491	1.777	1.386	2.122	1.932
5.0	0.748	0.781	0.786	0.781	3.276	2.370	2.043	2.082
Between-run (n=5) C.V. %								
0.5	0.774	0.823	0.876	0.893	4.617	4.203	3.387	7.578
1.0	0.707	0.715	0.772	0.814	1.148	1.525	1.468	2.714
2.0	0.737	0.752	0.810	0.832	3.584	2.648	3.497	3.636
5.0	0.791	0.802	0.845	0.740	1.188	1.642	2.320	4.145

[a] Samples were spiked with internal standards (final concentration: 1 μg/ml α(+)-phenylethylamine and 25 μg/ml β-phenylethylamine). CE conditions were as follows: running buffer, 100 m*M* phosphate buffer (pH 2.5) containing 10 m*M* CMCD; applied voltage, 25 kV. Other conditions are the same as in Fig. 1.

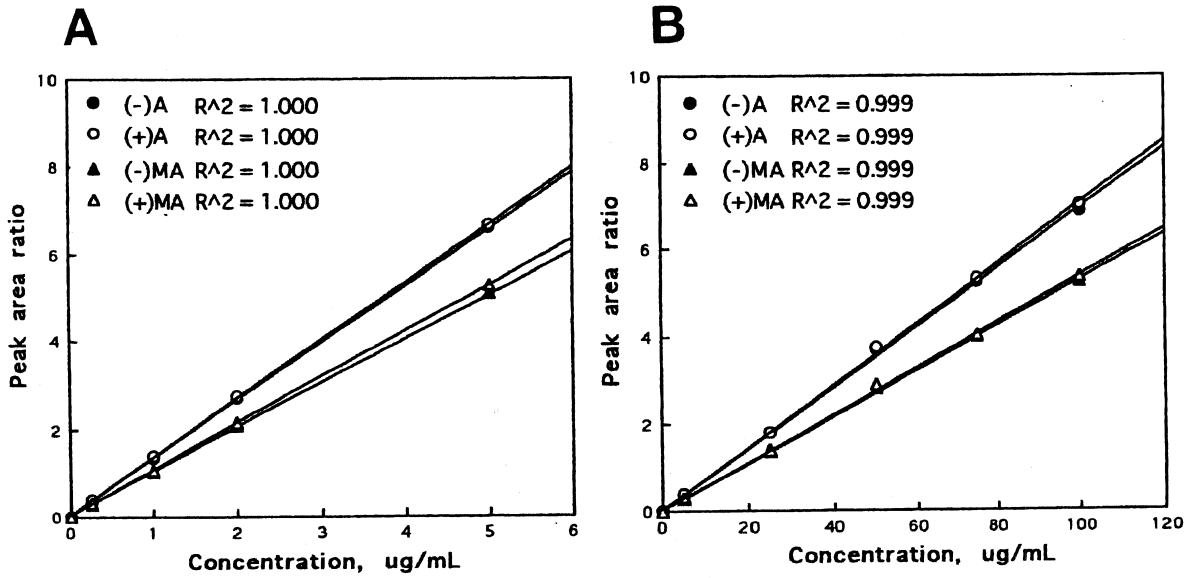

Fig. 4. Calibration curves for enantiomeric A and MA using a different concentration range of internal standards: (A) 1 μg/ml α(+)-phenylethylamine and (B) 25 μg/ml β-phenylethylamine. Other conditions are the same as in Table 1.

3.2. Analytical variables

3.2.1. Precision

We assessed the precision of the method by repeated analyses of blank urine specimens spiked with known concentrations of A, MA and I.S. As shown in Table 1, the coefficients of variations (C.V.) of retention time were less than 0.8% for the within-run precision, and less than 0.9% for the between-run precision. Those of the peak areas were less than 5%, for both within and between runs, except in the case of the 0.5 μg/ml samples.

3.2.2. Limit of detection (LOD)

The detection limits for quantitative determination were 50 ng/ml through the whole procedure, including the concentration process during extraction, at a signal-to-noise ratio of three. This detection limit was effective in the presence of a major chiral peak to 40 000 ng/ml.

3.2.3. Linearity

The concentration range of A and MA in forensic samples is 0.1–100 μg/ml. Two calibration curves were obtained from two standard mixtures (0.25–5.0

Table 2
Determination of enantiomeric A and MA in urine samples from patients taking illicit amphetamines, the analytical conditions are the same as in Table 1

Sample	Concentration found, μg/ml (C.V. %, $n=4$)			
	(−)-A	(+)-A	(−)-MA	(+)-MA
1	0.525 (1.964)	8.277 (4.241)	0.812 (3.312)	44.003 (3.861)
2	0.454 (0.502)	0.372 (3.526)	0.104 (2.973)	0.379 (4.850)
3		0.424 (2.893)		2.286 (3.452)
4		2.085 (1.275)		83.200 (0.463)
5	0.465 (0.366)	1.637 (0.200)		44.003 (2.932)
6		1.486 (4.807)		4.635 (4.824)
7		3.914 (2.668)		108.182 (0.299)
8		4.501 (0.780)		113.335 (0.291)
9		7.276 (4.019)		0.588 (1.644)

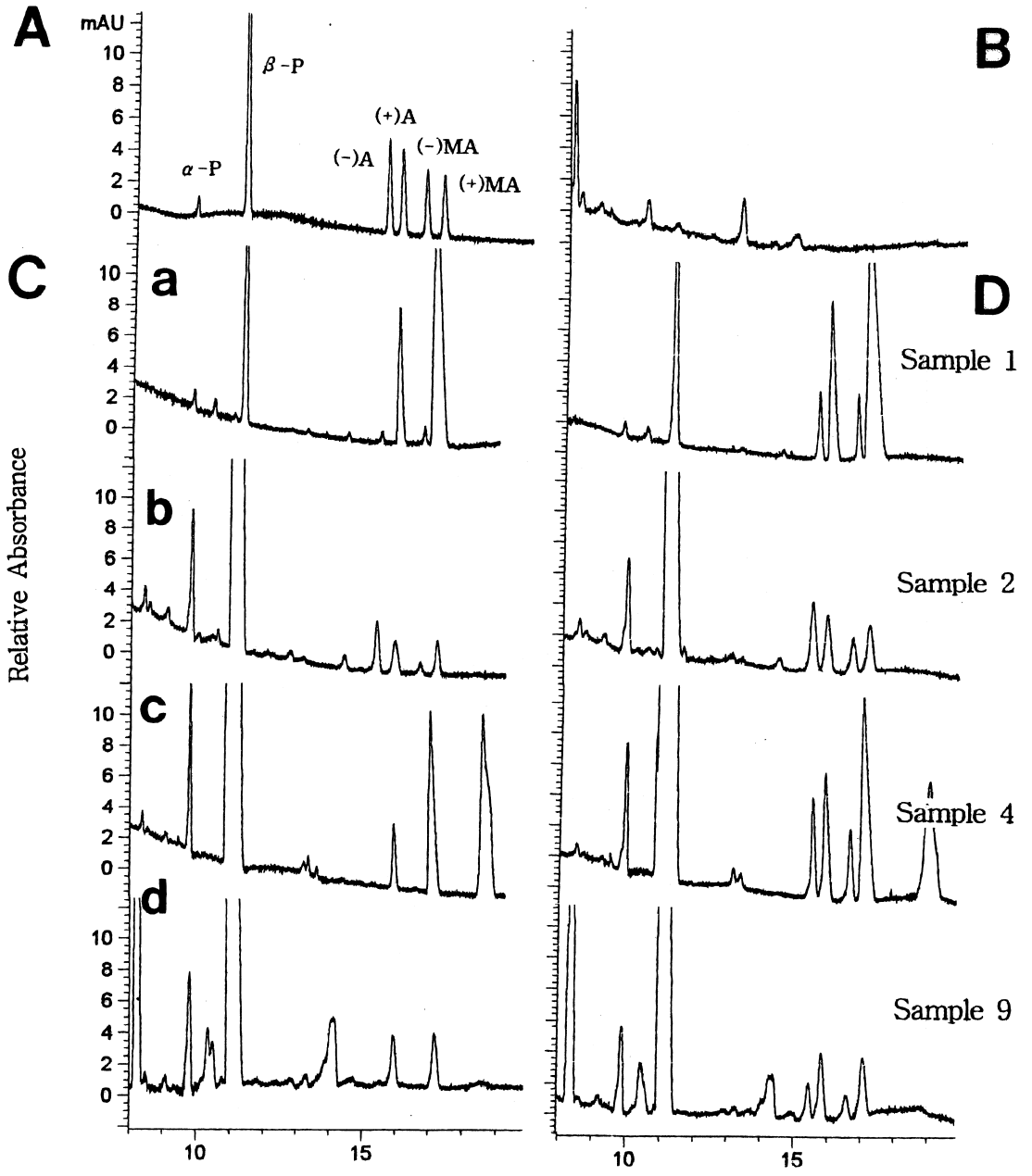

Fig. 5. Electropherograms of enantiomeric A and MA in illicit human urine samples: (A) blank urine spiked with standard mixture and two I.S, (B) blank urine, (C) real urine samples spiked with I.S.'s and (D) real urine spiked with I.S. and standard mixture. CE conditions are the same as in Table 1.

and 5–100 μg/ml) spiked with two different concentrations of I.S. (1 μg of α(+)-phenylethylamine and 25 μg/ml of β-phenylethylamine) as seen in Fig. 4. Linearities of calibration curves within the sample concentration range from 0.1 to 100 μg/ml were acceptable as $\gamma > 0.999$.

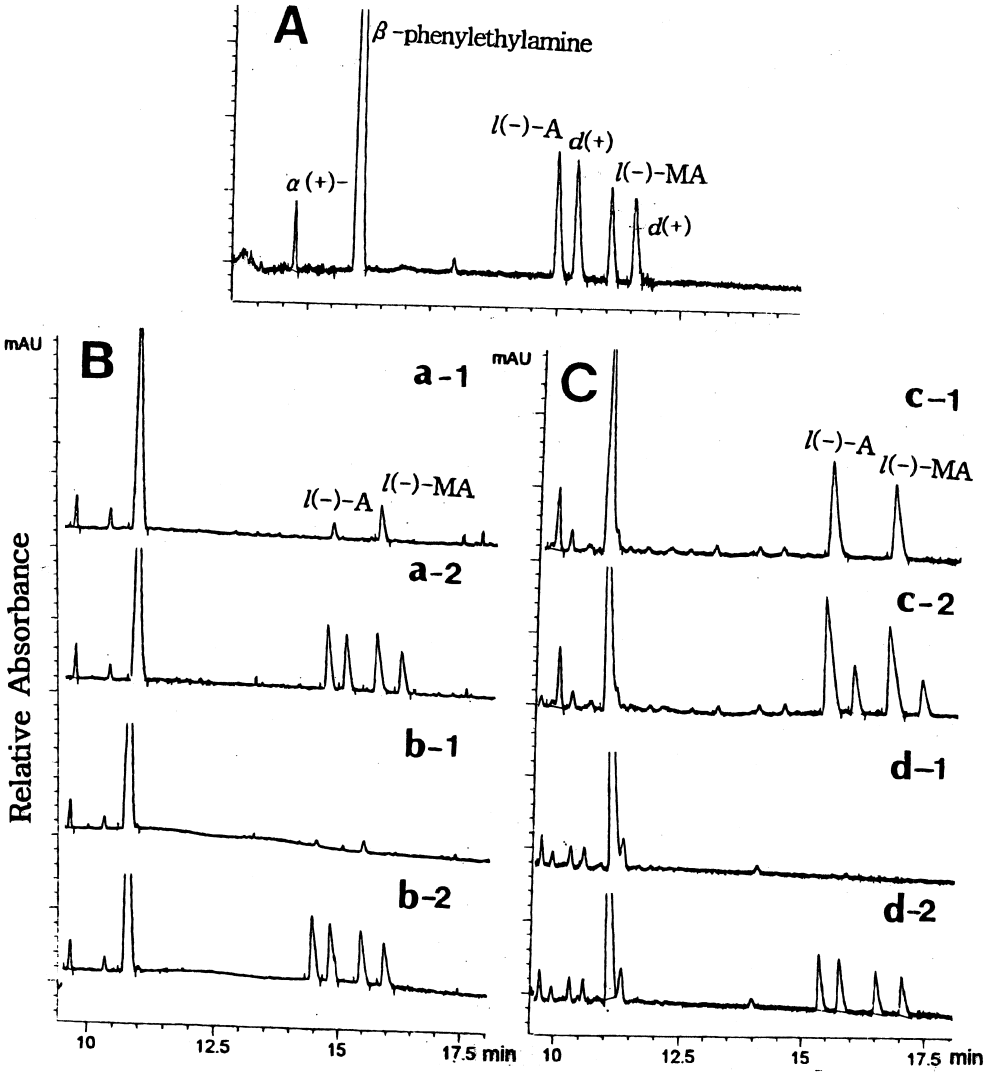

Fig. 6. Electropherograms of enantiomeric A and MA as metabolites of selegiline. Urine samples obtained from humans and rats, which had taken selegiline, were added to the internal standard and extracted as described in the Section 2. (A) Blank urine spiked with standard mixture and two I.S., (B) human real urine samples spiked with I.S. (a-1 and b-1) and standard mixture (a-2 and b-2) and (C) rat real urine spiked with I.S. (c-1 and d-1) and standard mixture (c-2 and d-2). CE conditions are the same as in Table 1.

3.3. Analysis of real urine samples

Chiral discrimination of A and MA is necessary in order to distinguish use of the prescription drug selegiline (metabolized to *R*(−)-methamphetamine) from the illicit use of *S*(+)-methamphetamine. Selegiline is a therapeutic medicine as a monoamine oxidase B inhibitor and metabolized and excreted into urine as *R*(−)-A and *R*(−)-MA. *S*(+)-A and *S*(+)-MA have a stronger effect on stimulating the central nervous system than the *R*(−)-form and are used as illicit drugs.

The optimum separation of enantiomeric A and MA in terms of resolution and separation efficiency was achieved with 100 m*M* phosphate buffer (pH 2.5) containing 10 m*M* CMCD at 15°C and an

applied voltage of 25 kV. Under these conditions, no interfering peak was observed when the blank urine was extracted as described in the Section 2 (Fig. 5B). When urine samples were spiked with the standard racemic A and MA (1 μg/ml each), including α(+)- (1 μg/ml) and β-phenylethylamine (25 μg/ml) as internal standards, and extracted, sound resolution was achieved as shown in Fig. 5A. The electropherograms of real urine samples from patients taking illicit amphetamines are shown in Fig. 5C. Also, each isomer was confirmed by adding the racemic standard mixture to the urine sample and by observing the overlapping peak and change of peak height (Fig. 5D). Quantitative analysis was performed in ten urine samples and the results are shown in Table 2. The use of two different internal standards containing different concentrations made possible quantitative analysis of urine samples having a wide concentration range of A and MA (0.1–115 μg/ml). The ratios of *R*(−) to *S*(+) and A to MA in samples were various, but *S*(+)-A and *S*(+)-MA were detected in all samples. The racemic ratio indicates the origin of illicit methamphetamine because that of European origin is mainly in the racemic form while that of Korean origin is *S*(+). This simple and routine analysis of enantiomeric A and MA using capillary electrophoresis gives important information on illicit methamphetamine seizures on the purchasing path.

The metabolites of the therapeutic drug selegiline in rat and human urine were analyzed in the same way as shown in Fig. 6. Only *R*(−)-A and *R*(−)-MA were detected in urine samples. This analytical method is reliable for determining the chiral metabolites of illicit methamphetamine and the therapeutic drug selegiline in urine samples.

4. Conclusion

Optimization of the enantiomeric separation of A and MA and quantitative analysis has been demonstrated by examining the effects of chiral selectors, buffer concentrations, sample injection volume, and two internal standard concentrations. The proposed method provides relevant linearity, sensitivity and reproducibility for *R*(−)- and *S*(+)-A and -MA using two concentrations of internal standards. These quantitative data prove the feasibility of the method for monitoring enantiomeric A and MA in urine and distinguishing illicit amphetamine seizures from legal prescription drug intake.

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