Yu-San Huang[1]
Ju-Tsung Liu[2]
Li-Chang Lin[1]
Cheng-Huang Lin[1]

[1]Department of Chemistry, National Taiwan Normal University, Taipei, Taiwan
[2]Military Police School, Command of the Army Force of Military Police, Department of Defense, Wuku, Taipei, Taiwan

Chiral separation of 3,4-methylenedioxymethamphetamine and related compounds in clandestine tablets and urine samples by capillary electrophoresis/fluorescence spectroscopy

The *R*-(−)- and *S*-(+)-isomers of 3,4-methylenedioxymethamphetamine (MDMA) and its metabolite 3,4-methylenedioxyamphetamine (MDA) were prepared, identified by gas chromatography/mass spectrometry (GC/MS) and then used as standards in a series of capillary electrophoresis (CE) experiments. Using these *R*-(−)- and *S*-(+)-isomers, the distribution of (*RS*)-MDA and (*RS*)-MDMA stereoisomers in clandestine tablets and suspect urine samples were identified. Several electrophoretic parameters, such as the concentration of β-cyclodextrin used in the electrophoretic separation and the amount of organic solvents required for the separation, were optimized.

Keywords: Capillary electrophoresis / Chiral separation / Clandestine tablet / Designer drug / Gas chromatography-mass spectrometry / Urine EL 5350

1 Introduction

The increasing distribution of 3,4-methylenedioxymethamphetamine (MDMA) derivatives in the illicit market has became a serious social problem. 3,4-Methylenedioxyamphetamine (MDA), which is the major metabolite of MDMA, along with *N*,*N*-dimethyl-3,4-methylenedioxyamphetamine (DMMDA), 1-(1,3-benzodioxol)-5-yl-2-butylamine (BDB) and *N*-methyl-1-(1,3-benzodioxol-5-yl)-2-butylamine (MBDB) are often referred to as "designer drugs" (Fig. 1). All of these compounds contain a chiral center and, as a result, can exist in *R*- and *S*-forms. Because of the different pharmacological activity of enantiomers of these compounds [1, 2], we became interested in examining the distribution of these enantiomers and in suspect urine samples in clandestine tablets. The former would permit us to better understand the metabolism of these drugs and the latter would be useful in forensic analysis in order to identify the synthetic pathways used in their synthesis [3]. Furthermore, the chiral separation and identification of enantiomers would be helpful in clinical and forensic analysis because different enantiomers have different pharmacological activities. Hence, it would be desirable to develop an assay that is capable of rapidly separating and identifying these enantiomers in clandestine tablets and in biological fluids such as urine or blood more accurately. The separation methods that are currently used for the enantiomers are either chromatographic, such as gas chromatography (GC) [4–11], high-performance liquid chromatography (HPLC) [12–15], thin-layer chromatography (TLC), supercritical fluid chromatography (SFC) or electrophoretic such as capillary electrophoresis (CE) [10, 16–24]. Each method has unique advantages and disadvantages with respect to

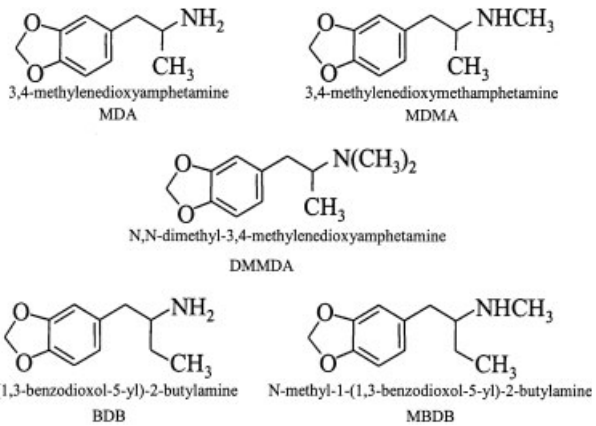

Figure 1. Molecular structures of MDMAs and abbreviations used.

Correspondence: Dr. Cheng-Huang Lin, Department of Chemistry, National Taiwan Normal University, 88 Sec. 4, Tingchow Road, Taipei, Taiwan
E-mail: chenglin@cc.ntnu.edu.tw
Fax: +886-2-2932-4249

Abbreviations: BDB, 1-(1,3-benzodioxol)-5-yl-2-butylamine; **DMMDA**, *N*,*N*-dimethyl-3,4-methylenedioxyamphetamine; **MBDB**, *N*-methyl-1-(1,3-benzodioxol-5-yl)-2-butylamine; **MDA**, 3,4-methylenedioxyamphetamine; **MDMA**, 3,4-methylenedioxymethamphetamine; ***S*-TPC**, (*S*)-(−)-*N*-(trifluoroacetyl)prolyl chloride; **TIC**, total ion chromatogram

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efficiency, precision, and simplicity of use. Although GC/MS is widely used for drug analysis, for the chiral separation of abused drugs, the derivatization steps and additional sample handling are frequently required.

In this study, we describe a simple method for preparing standard samples of *R*- and *S*-isomers by the reaction of (*RS*)-isomers and (*S*)-(−)-*N*-(trifluoroacetyl)prolyl chloride (*S*-TPC). The individual *R*- and *S*-form isomers can be obtained after appropriate separation. Using these standards, the optimum conditions for the chiral separation of a model mixture of MDA, MDMA, DMMDA, BDB and MBDB (Fig. 1) were examined by CE. The quantitation and differential distribution of the enantiomers of MDMA and its physiologically active metabolite MDA in clandestine tablets and suspect urine samples were investigated.

2 Materials and methods

2.1 Chemicals

MDA and MDMA were obtained from Radian International (Austin, TX, USA). Sodium cholate (SC) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Acetonitrile and methanol (99.8%) were obtained from Acros (Belgium). β-Cyclodextrin and sodium phosphate were obtained from Sigma (St. Louis, MO, USA). Ethyl acetate was acquired from Santoku Chemical (Tokyo, Japan). Urea and phosphoric acid were obtained from Sihmakyu (Osaka, Japan) and J. T. Baker (Phillipsburg, NJ, USA), respectively. All of the other standards, including BDB, MBDB and DMMDA, tablets and suspect urine samples were generously donated by Command of the Army Force of Military Police, Forensic Science Center, Taiwan.

2.2 Preparation of *S*-MDA and *R*-MDMA

(*RS*)-MDA and (*RS*)-MDMA can be purchased from Radian International, but single enantiomers of MDA and MDMA are not commercially available. To prepare the individual *R*- and *S*-isomers, we first synthesized an *S*-TPC reagent. The synthetic pathway is shown in scheme I of Fig. 2. Briefly, 1.1 g of anhydrous *l*-proline was added to 1.6 mL of trifluoroacetic anhydride and the mixture reacted for ~20 min. After evaporating the excess solvent, 10 mL thionyl chloride was then added for a further 10 min reaction with warming. On completion of the reaction, nitrogen gas was used to evaporate the excess thionyl chloride, to give *S*-TPC. This reagent (*S*-TPC, 10.0 mM) is reacted (at 100°C refluxing for ~20 min) with racemic 2.0 mM (*RS*)-MDA (or MDMA) in 20 mL of toluene (see scheme II in Fig. 2) to give *R*-MDA-*S*-TPC and *S*-MDA-*S*-TPC (or *R*-MDMA-*S*-TPC and *S*-MDMA-*S*-TPC) derivatives. The resulting derivatives were separated on a silica gel column with a mixture of ethyl acetate/hexane (1:5 v/v). The fractions were repurified by HPLC (Spherisorb® S 10 W, 20 × 250 mm; Waters, Milford, MA, USA) with the same solvent (ethyl acetate/hexane 1:5 v/v) at a flow rate of 3.0 mL/min and

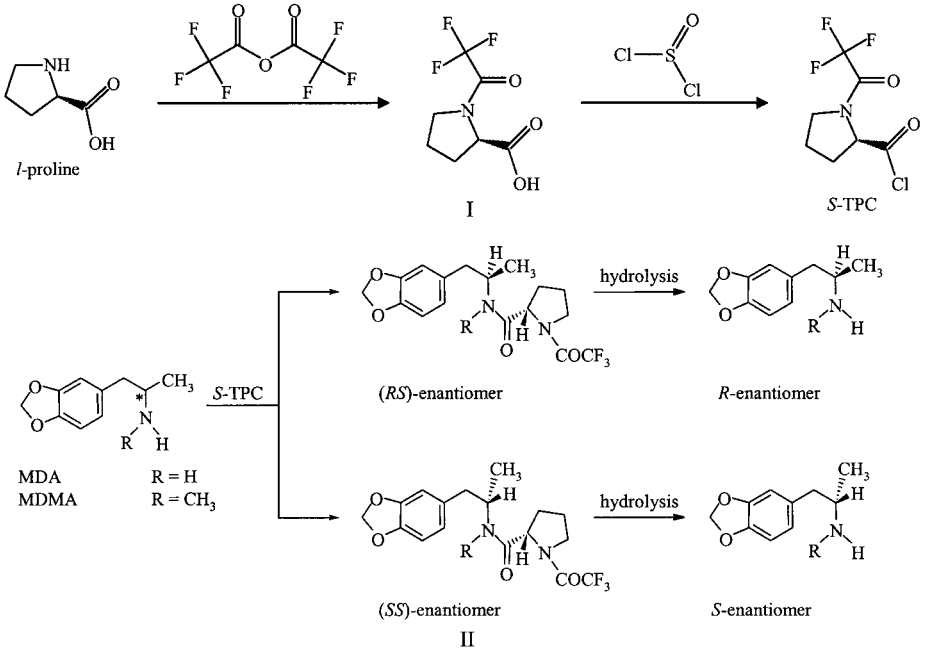

Figure 2. Synthetic pathway for the preparation of the S-TPC reagent (scheme I) and reaction of S-TPC and MDA (scheme II).

then identified by GC/MS. After confirmation by GC/MS, the *R*- or *S*-isomer was hydrolyzed by treatment with 6 M KOH for 4 h to give the *R*- or *S*-MDA, respectively, as shown in scheme II of Fig. 2. The optical rotation was checked by polarimetry (model DIP-1000; Jasco, Japan). *R*- or *S*-MDMA can also be obtained using the same procedures. Without these derivatization steps, it is difficult to obtain the individual *R*- or/and *S*-forms for use in CE separation studies. These isomers were used in the subsequent identifications.

2.3 CE apparatus

The CE setup is laboratory-made and is similar to that described previously [25–27]. Briefly, a reversible high-voltage power supply (Model RR30-2R, 0–30 kV, 0–2 mA; Gamma, FL, USA) was used to drive the electrophoresis and a 50 μm ID fused-silica capillary column (J&W Scientific, Folsom, CA, USA) was used for the separation. The excitation source was selected by a monochromator (Acton Research Corporation; Model SP-150) connected to a Xe lamp (Müller Elektronik Optik, SVX/LAX 1450). Fluorescence data were collected at a right angle to the light source and dispersed by a second monochromator (ARC Model SP-300i), followed by detection by means of a photomultiplier tube (ARC Model P2-R928). Electropherograms were collected with a data acquisition system (ARC's Spectra-Sense NCL package), connected to a personal computer.

2.4 GC/MS apparatus and methods

A gas chromatograph (Hewlett-Packard 6890 GC; Palo Alto, CA, USA) equipped with a mass spectrometer (Hewlett-Packard 5973 mass selective detector) and an autoinjector (Model 7683) was used for the separation. A capillary column (30 m × 0.32 μm ID) with an HP-5 MS (5% diphenyl and 95% dimethylpolysiloxane) bonded stationary phase film 0.25 μm thickness (Agilent Technologies, USA) was used. The temperatures of the inlet, quadrupole, injector and interface were maintained at 230, 150, 250 and 280°C, respectively. The temperature program for the column oven was as follows: 70°C for 1 min, a linear ramp to 200°C at 15°C/min and a 2 min hold. Finally, the temperature was ramped linearly to 260°C at 20°C/min with a 12.3 min hold, to give a total analysis time of 27 min. Helium carrier gas was used at a constant flow rate of 1.0 mL/min (at splitless mode). Data were collected using the Hewlett-Packard Chem-Station software. The mass conditions were as follows: ionization energy, 70 eV; ion source temperature, 230°C; full-scan, 40–450 amu at 1.84 scans per second.

2.5 Liquid-liquid extraction procedure

Tablet: Tablets were grounded into a powder and approximately 30 mg was dissolved in 3.0 mL 0.2 N KOH solutions by shaking for 5 min. The solution was extracted with 3.0 mL ethyl acetate (containing diphenylamine at 0.5 mg/mL as the internal standard) by shaking for 5 min. The mixture was centrifuged for 5 min at 3000 rpm and a 2.0 mL aliquot of the organic layer was transferred to an autosampler vial. The sample was analyzed (see GC/MS procedure above) on the day of extraction. For CE experiments, the tablet power (1 mg) was extracted with methanol (1 mL). After 2 min of sonication and a 2 min centrifugation at 5000 rpm at room temperature, the upper layer was collected and was used directly. Urine: One mL of urine sample was made alkaline by the addition of an excess of K_2CO_3. The free bases were then extracted into 2 mL of a hexane/CH_2Cl_2 solution (3:1 v/v) by mixing for 1 min. After centrifugation, the upper layer was collected and this organic phase was then evaporated to dryness. The residue was dissolved in 10 μL of methanol for the subsequent CE separation.

3 Results and discussion

3.1 Identification of *S*-(+)/(*R*)-(−)-MDA and *S*-(+)/(*R*)-(−)-MDMA

Figure 3A shows the total ion chromatogram (TIC) of a mixture of *R*-MDA-*S*-TPC and *S*-MDA-*S*-TPC derivatives (upper). The detected peak, with a retention time of 21.7 min permitted the specific characterization of MDA-TPC and its mass fragmentation spectrum is shown at the bottom. Another detected peak, with a retention time of 22.2 also permitted the specific characterization of MDA-TPC (data not shown). The derivative was sufficiently pure to be used for the subsequent column separation and hydrolysis to give the *R*-(−)- or *S*-(+)-MDA, as described in Section 2.2. Figure 3B shows the TIC of *S*-(+)-MDA (upper) and the mass fragmentation spectrum (bottom). The presence of specific fragments, such as *m/z* 44 and 136, permitted this characterization. Following this, the product was checked by polarimetry to confirm the optical rotation.

Figure 4A shows typical fluorescence ($\lambda_{ex}/\lambda_{em}$ = 280/320 nm) electropherograms for (*RS*)-MDA before (electropherogram a) and after spiking with *S*-(+)-MDA (electropherogram b). The CE buffer used was an aqueous buffer that contains 50 mM of β-CD, 50 mM phosphate and 3 M of urea. The total length of the capillary was 85 cm (effective length: 80 cm) and the applied voltage was 25 kV (~ 20 μA). As a result, the separation was complete. The peak that appeared at 29 min corresponded to *S*-(+)-MDA (arrow);

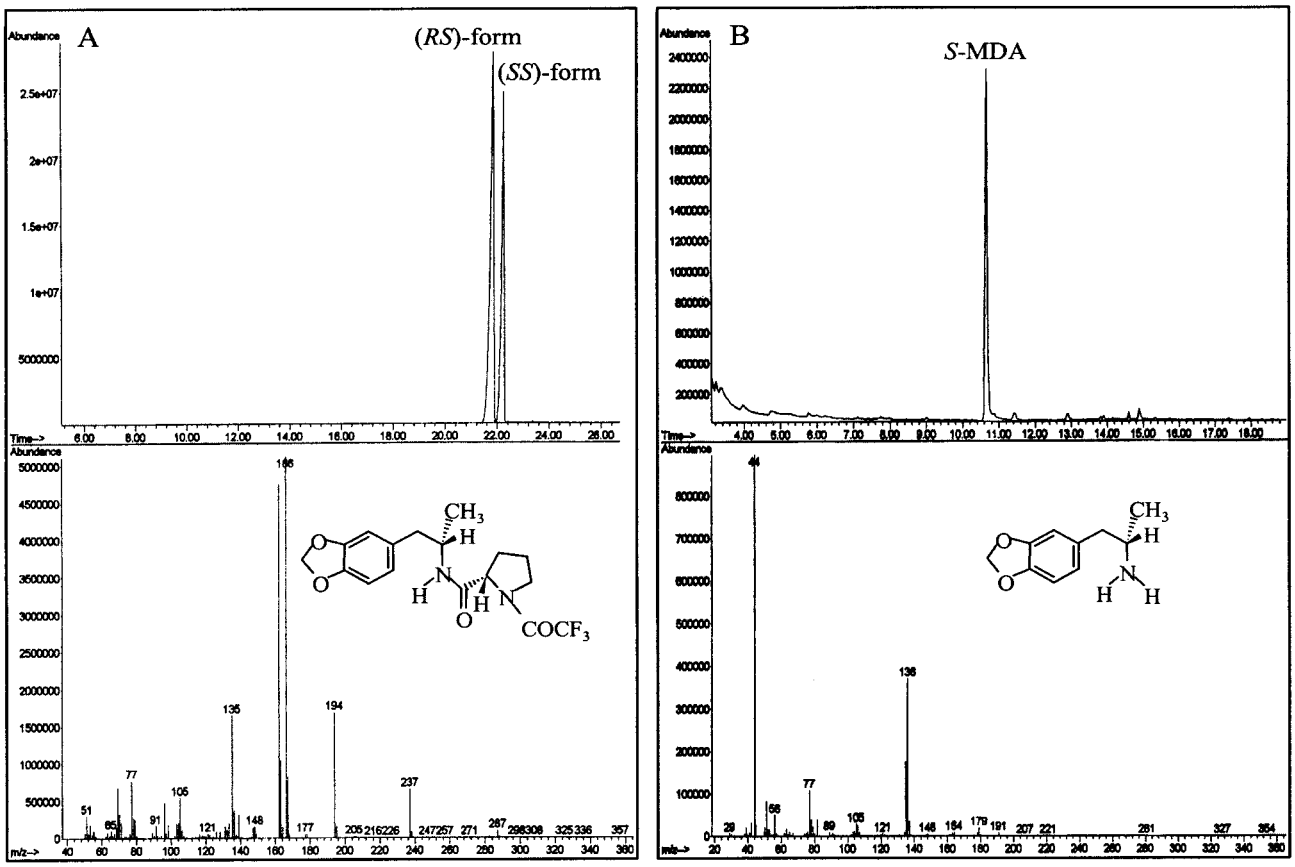

Figure 3. (A) TIC of a mixture of *R*-MDA-*S*-TPC and *S*-MDA-*S*-TPC derivatives (upper). (B) TIC of *S*-(+)-MDA (upper) and the mass fragmentation spectrum (bottom).

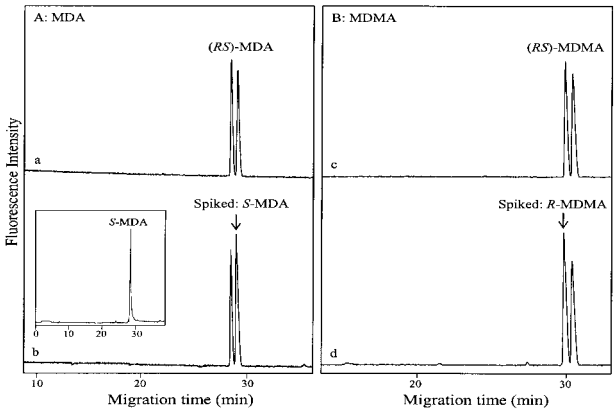

Figure 4. (A) Typical fluorescence ($\lambda_{ex}/\lambda_{em}$ = 280/320 nm) electropherograms of (*RS*)-MDA before (electropherogram a) and after spiking with (*S*)-MDA (electropherogram b). (B) Typical fluorescence ($\lambda_{ex}/\lambda_{em}$ = 280/320 nm) electropherograms of (*R,S*)-MDMA before (electropherogram c) and after spiking with (*S*)-MDMA (electropherogram d). CE buffer for both runs: an aqueous 50 mM phosphate buffer (pH 3.0) including 50 mM of β-CD and 3 M of urea. Total length of the capillary, 85 cm (effective length, 80 cm); applied voltage, 25 kV (~ 20 μA); sample concentrations, 100 ppm.

whereas the peak at 28.5 min corresponded to *R*-(−)-MDA. The inset shows the CE electropherogram of *S*-(+)-MDA under the same CE conditions. One major peak appeared and this also provided additional proof that this *S*-(+)-MDA was pure. Similarly, Fig. 4B shows the fluorescence electropherograms of (*RS*)-MDMA before (electropherogram c) and after spiking with *R*-(−)-MDMA (electropherogram d), respectively. The peak corresponding to *R*-(−)-MDMA appeared (arrow) verifying that pure *R*-MDMA was also successful prepared. Thus, we conclude that the *S*-(+)/(*R*)-(−)-MDA and *S*-(+)/(*R*)-(−)-MDMA were synthesized successfully and were sufficiently pure to be used as standards in the experiments described below.

3.2 Optimization of the separation of (*RS*)-MDMA and related designer drugs

In the case of the CE separation, β-CD is the most commonly used chiral additive. However, its solubility in water (1.8 g/100 mL at 25°C) is low [28], and highly sulfated CDs have been developed by Beckman to improve solubility.

Various chiral selectors of derivatized β-CDs have also been reported by Rizzi and Schmitt *et al.* [29, 30]. Although these modified β-CDs provide unique advantages for routine analyses, the use of the native β-CD is convenient, if an optimized CE buffer and separation conditions can be found. For this purpose, we investigated the optimum conditions for the separation by investigating several parameters, such as the concentration of β-CDs, the length of the capillary, the concentration of surfactant used and the organic solvents. The typical CE chromatograms of the model mixture of MDMA and related designer drugs (Fig. 1) are shown in Fig. 5. In frame A (without β-CD), the CE buffer was a mixture solution of methanol-acetonitrile-water (M:A:W = 14:4:82 v/v/v) which contained 50 mM phosphate buffer (pH 3.0). Herein, a capillary 85 cm in length (effective length: 80 cm) was used. The migration order was: MDA < DMMDA < MDMA < BDB < MBDB. The compounds basically migrated in the order of mass per charge. DMMDA is a tertiary amine which has a much different molecular structure than the others, making it difficult predict and explain its migration order. As shown in frame B (with 50 mM β-CD), these enantiomers can be completely separated when a longer capillary (total: 97 cm; effective length: 92 cm) was used and with β-CD added to the buffer (CE buffer: 50 mM of β-CD, 10 mM of SC, 50 mM phosphate buffer, 3 M of urea

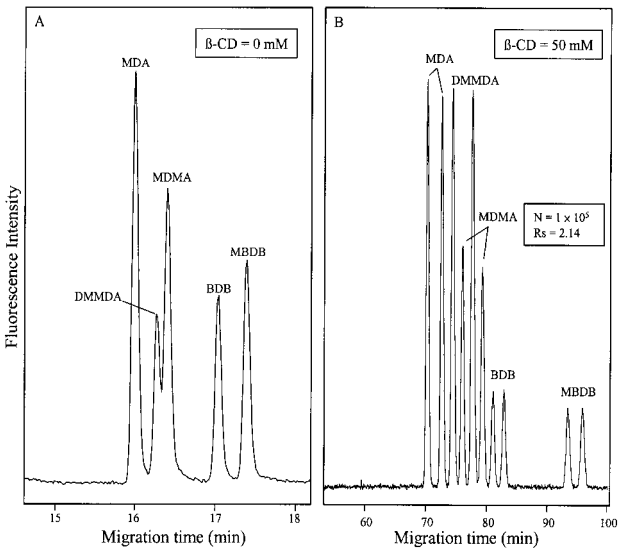

Figure 5. Typical CE chromatograms of a model mixture of MDA, DMMDA, MDMA, BDB and MBDB. (A) The CE buffer was a mixture solution of methanol-acetonitrile-water (M:A:W = 14:4:82 v/v/v) which contained 50 mM phosphate (pH 3.0); capillary length, 85 cm (effective length, 80 cm). (B) The CE buffer was a mixture solution which contained 50 mM of β-CD, 10 mM of SC, 50 mM phosphate, 3 M of urea in the same solution of M:A:W (14:4:82 v/v/v); pH 2.3; capillary length, 97 cm (effective length, 92 cm); applied voltage, 25 kV.

in the same solution of M:A:W; pH 2.3). The applied voltage was 25 kV (current, 6–7 μA). In order to investigate the effects of organic solvents, various M:A:W solutions (such as M:A:W = 16:4:80; 14:4:82; 10:4:86; 8:4:88) were examined, but in all cases the separation was poorer. The other experimental data show that 50 mM of β-CD was better than 20 mM. The SC surfactant (10 mM) and a longer capillary (total 92 cm in length) were necessary for complete separation. These results are useful for the analysis of related designer drugs in future studies.

3.3 Separation and identification of MDA and MDMA in clandestine tablets

Table 1 shows the results of analyses of 427 clandestine tablets obtained by GC/MS, which were seized from the Taiwan illicit market during 2001. The experimental conditions and methods are described in Section 2.4. Most of these clandestine tablets contained either MDMA or MDA. Some contain both MDA and MDMA; only a few samples contained DMMDA. For a comparison of GC/MS and CE, we selected three samples and the TICs of these, obtained by GC/MS, are shown in Fig. 6A (chromatograms a–c). The peaks having retention times of 8.9 and 9.4 min were assigned as MDA and MDMA based on their mass spectra. We also found that some nonfluorescent compounds such as caffeine and even amphetamines may be present. However, our focus was on identifying the enantiomeric distribution of these drugs, which is difficult by GC/MS. The three tablet extracts were separated by CE and the results are shown in Fig. 6B (electropherograms d–f). The CE conditions are similar to those stated in Fig. 4. The peaks on the left and right represent the *R*-(−)- and *S*-(+)-isomers, respectively. The ratio (in peak area) of *R*-(−)- and *S*-(+)-isomers in tablets were found to be ~1. It thus appears that they are naturally present in equal amounts. Thus, the method of β-CD modified CE separation provides a simple and rapid alternative to GC/MS.

Table 1. Distribution of MDMA and related compounds by GC/MS in 427 tablets seized from the Taiwan illicit market during 2001

MDA	MDMA	DMMDA	BDB	MBDB	*n*	Distribution (%)
+					27	5.78
	+				420	89.94
+	+				15	3.21
+	+	+			4	0.86
	+	+			1	0.22

+, detected

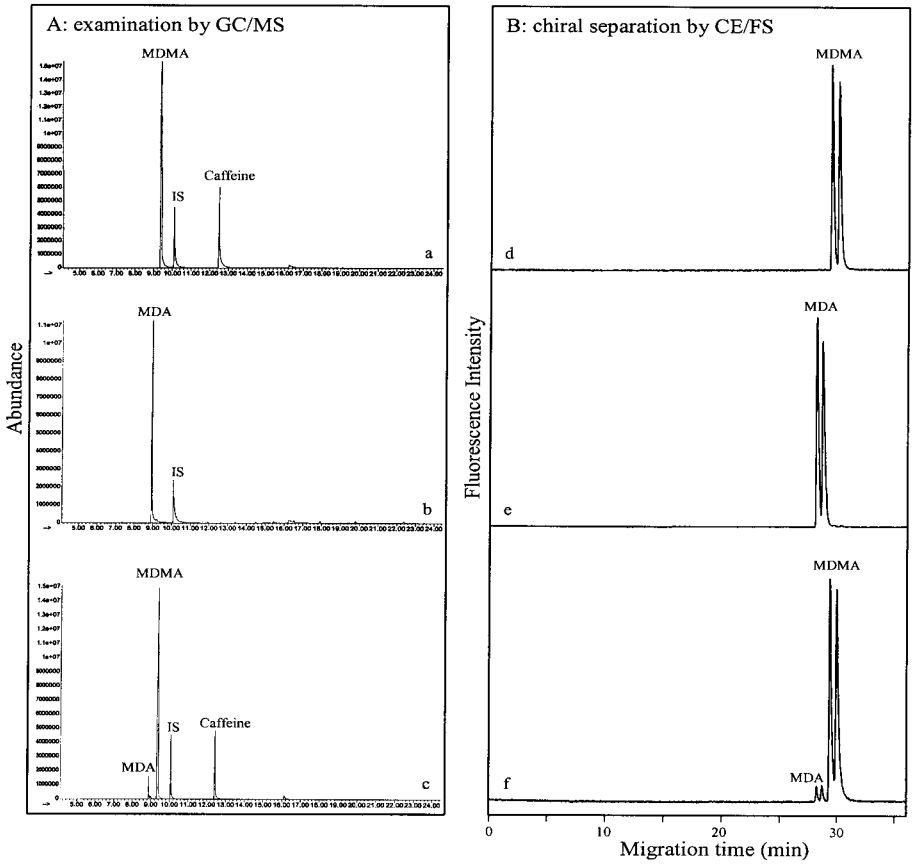

Figure 6. (A) GC/MS chromatogram of three selected tablet extracts. Internal standard: diphenylamine hydrochloride. (B) CE electropherogram of the same tablet extracts. The CE buffer was a mixture solution of methanol-acetonitrile-water (M:A:W = 14:4:82, v/v/v) which contained 50 mM phosphate (pH 3.0), 50 mM of β-CD and 3 M of urea; capillary length, 85 cm (effective length, 80 cm).

3.4 Separation and identification of MDA and MDMA in suspect urine samples

Studies using animal models have demonstrated the stereoselective pharmacokinetics and neurotoxicity of MDMA and related compounds [4, 31, 32]. The distribution of *R*-(−)- and *S*-(+)-MDMA and its metabolite MDA in a fatal poisoning also has been reported by Poklis *et al.* [2] by means of GC/MS. In their report the concentration of *R*-(−)-MDMA was slightly higher than that of *S*-(+)-MDMA (302 *vs*. 227 mg/L, in urine). In our research, we separated a urine extract from one of the suspects (No. III in Table 2) by CE and the results are shown in Fig. 7 (frame A: without β-CD; frame B: with 50 mM β-CD).

Table 2. MDMA and MDA concentrations (mg/L) in the urine samples examined

No.	MDMA				MDA			
	R-	*S*-	Total	*R*-form (%)	*R*-	*S*-	Total	*R*-form (%)
I	9.4	7.5	16.9	125	ND	ND	–	–
II	17.8	13.2	31.0	135	0.3	3.8	4.1	7.9
III	34.4	26.5	60.9	130	0.8	2.0	2.8	38.0
IV	14.4	12.0	26.4	120	0.1	0.5	0.6	19.0
V	28.2	18.7	46.9	151	0.4	3.8	4.2	10.5
VI	6.5	4.7	11.2	138	0.7	0.4	1.1	175.0
VII	10.2	7.0	17.2	145	0.2	0.5	0.7	32.0
VIII	8.8	5.8	14.6	151	1.4	0.5	1.9	287.0
IX	11.5	8.3	19.8	138	4.2	1.6	5.8	269.0
X	28.8	24.0	52.8	120	ND	ND	–	–

ND, not detected

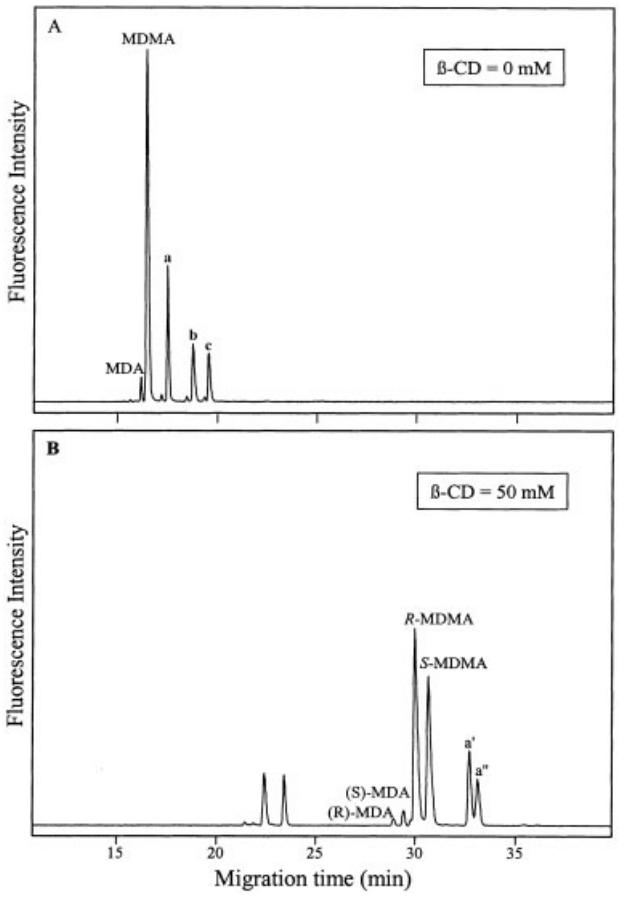

Figure 7. Electropherograms of a urine sample extract from No. III suspect listed in Table 2. (A) Without β-CD; (B) with 50 mM β-CD. CE conditions are as in Fig. 6B.

The MDA and MDMA peaks are marked on the figure by spiking with the standards, whereas peaks a–c represent unknown compounds. Comparing frames A and B, peak is split into two peaks (a′ and a″) after the addition of β-CD. This indicates the presence of enantiomers. Spiking with the MBDB standard shows that the peak was superimposed and, thus, could be MBDB. However, it has not been identified it by GC/MS at this time. On the other hand, it is clear that in typical human urine samples, only a few native fluorescent compounds are present which fluoresce in the wavelength range of 320 ± 2 nm. With this fluorescence detection, the electropherogram was much more simple than UV detection (data not shown) due to the UV-absorption of numerous organic compounds in urine sample. This approach also provides a simple way to observe non-natural compounds in urine, such as MDMAs.

Using the same procedures, ten suspect urine samples were investigated and the results are shown in Table 2. *R*-(−)-MDMA was found in higher concentrations than the *S*-(+)-MDMA for all specimens analyzed, by about 1.2 ~ 1.5-fold. These data are in agreement with the report by Poklis *et al.* [2], who reported a ratio for *R*-(−)-/*S*-(+)-MDMA of was 1.33. Although the concentration of *R*-(−)-MDA in urine was lower than *S*-(+)-MDA, as reported by Poklis *et al.*, in which the ratio of *R*-(−)-/*S*-(+)-MDA was 0.44, we found that there are certain discrepancies between the two isomers of the MDA, as shown in Table 2 for ten forensic cases. This is due to the reabsorption of MDA by body, indicating that the ratio can not remain constant.

4 Concluding remarks

R- and *S*-MDMA and *R*- and *S*-MDA were prepared for the first time. These isomers were first identified by GC/MS and were then used in the analysis of the *R*- and *S*-MDMA and *R*- and *S*-MDA distributions in clandestine tablets and suspect urine samples by capillary electrophoresis/fluorescence spectroscopy. Although CE with fluorescence detection will not be able to replace GC/MS in forensic analysis, as the fluorescence spectrum lacks the specificity of MS that is unequivocally required in forensic analysis in order to hold up in court, this sensitive and rapid method could serve as a reliable complementary method to GC/MS for routine use.

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