Abstract

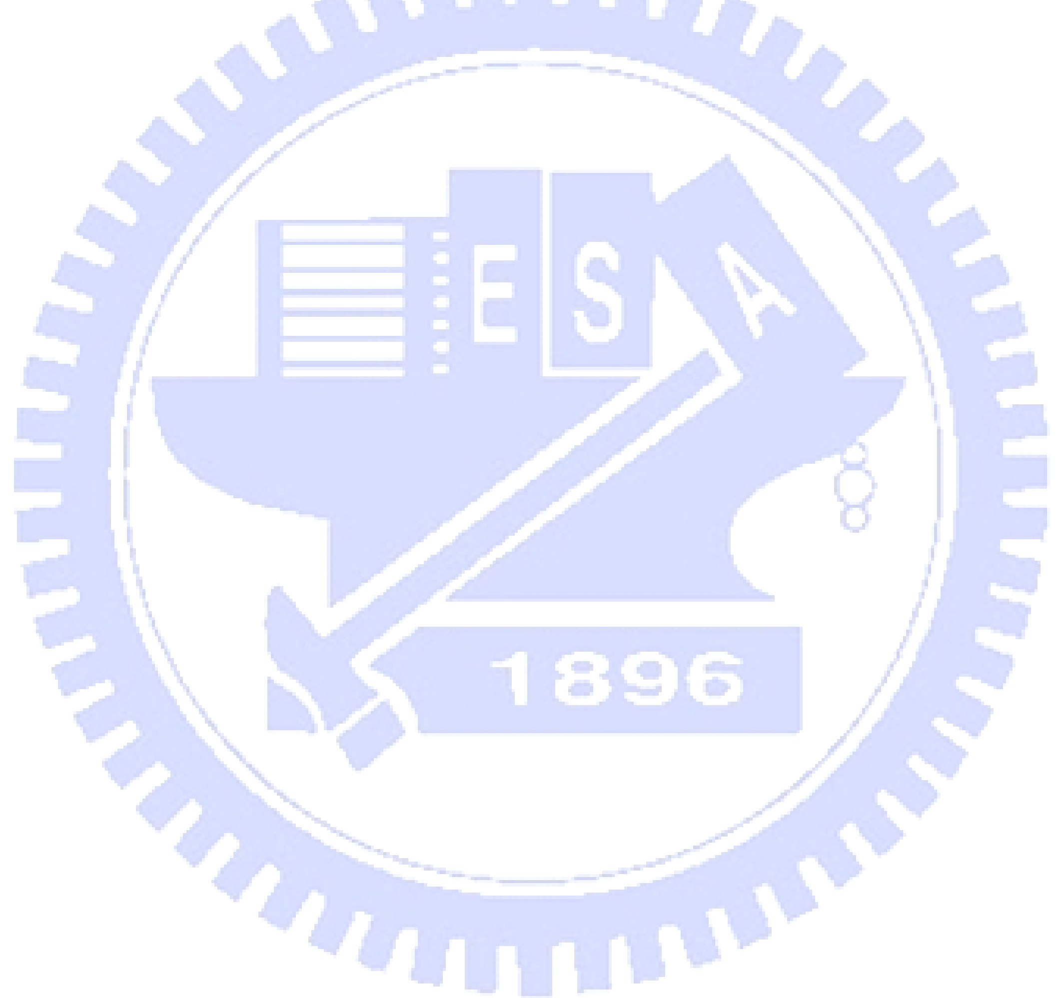
Illicit and abused drugs are often known by street names that vary from area to area. A call to a local police station, or animal or human poison control center, can be extremely helpful in identifying the illicit substance. Most human hospitals, emergency clinics, or veterinary diagnostic laboratories have illicit drug screens available and can check for the presence of illicit drugs or their metabolites in different body fluids. The presence of a parent drug or its metabolites in blood or urine may help confirm the exposure in suspect cases. Veterinarians should contact these laboratories for the types of samples needed and time required for completion.

Commonly available over-the-counter drug test kits may be helpful in ruling out a suspected case of illicit drug toxicosis. These test kits are inexpensive, efficient, and easy to use. They are designed to detect drug metabolites in the urine and can detect most commonly available illicit or recreational drugs such as amphetamines, cocaine, marijuana, opiates, and barbiturates. The sensitivities and specificities of these test kits may vary. The instructions provided with each kit should be followed carefully for best results.

In this work, first, we have simultaneously determinated and quantified ketamine and its major metabolites, norketamine, 5,6-dehydronorketamine, and deaminonorketamine, in human urine and hair using liquid–liquid extraction (LLE) and solid phase extraction (SPE) in combination with gas chromatography/mass spectrometry (GC/MS) (Chapter 2).

The next, we also have investigated a rapid, simple, and highly efficient on-line preconcentration method using in micellar electrokinetic chromatography (MEKC) for the analysis of abused drugs including ketamine (Chapter 3), flunitrazepam (Chapter 4), cocaine, heroine, opiates (Chapter 5), and their major metabolites. The optimized sweeping method was also used to examine a urine sample. We conclude that sweeping with micellar electrokinetic chromatography has considerable potential use in clinical and forensic analyses of flunitrazepam and its metabolites.

Finally, we have devised a rapid and highly efficient separation method for the separation and analysis of amphetamine, methamphetamine, and ephedrine using micellar electrokinetic chromatography (MEKC) and dry-film-based microchip capillary electrophoresis (DFB-MCE) with electrochemical detection. These analytes were separated in a plastic microchip capillary electrophoresis with electrochemical detection. The capillary electrophoresis-based methods are extremely complementary to GC/MS-based forensic analyses (Chapter 6).



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Chapter 1
Introduction

1.1. Introduction

Illicit drug abuse has continued to draw attention from the public and represents a worldwide problem. Drug screening is an effective tool for monitoring and screening for illicit drug consumption. It has played a key role in saving costs yet still providing a picture of drug use for different audiences. Currently, several immunoassays are available in the market for this purpose. Syva's enzymes multiplied immunoassay technique (EMIT) II screening assay has been the most popular one for many years for the detection of drugs and/or their metabolites in urine [1]. Because each immunoassay was developed uniquely based on the specific techniques and the properties of the target drug(s) and/or metabolites, immunoassays normally display different properties in detecting different types of drugs, at different concentrations and at different cut-off levels. For this reason, research had been conducted in this area in an attempt to explore the similarities and differences among different types of assays in detecting chemicals and/or drugs [2–18]. For this reason, it is usually just only employed to screen the suspect specimens, initially.

Gas chromatography-mass spectrometry (GC-MS) is a method that combines the features of gas-liquid chromatography and mass spectrometry to identify different substances within a test sample. Applications of GC/MS include drug detection, fire investigation, environmental analysis, and explosives investigation. GC/MS can also be used in airport security to detect substances in luggage or on human beings. Additionally, it can identify trace elements in materials that were previously thought to have disintegrated beyond identification. From the perspective of qualitative analysis, GC/MS also provides additional spectral information as well as excellent sensitivity. Although GC/MS can obtain a good data for the result and as a scientific proof and accepted in the court, the derivatization step is necessary because of

similar fragmentation patterns and poor diagnostic ion in the mass spectrum [19]. It often results in running time too long. Especially, when we analyze hundreds of samples, the method is not available.

In recent years, capillary electrophoresis (CE) has expanded its scope and range in both instrumentation and applications [20]. Capillary electrophoresis (CE) encompasses a family of related separation techniques that use narrow-bore fused-silica capillaries to separate a complex array of large and small molecules. High electric field strengths are used to separate molecules based on differences in charge, size and hydrophobicity. Although CE shows important advantages (small quantity of injected sample, high speed and resolution and low expenditure of chemicals) for the analysis of many compounds in a great variety of samples [21], the concentration LODs obtained with UV-absorption detection are still, in many cases, unsatisfactory. They can be improved by hyphenation of CE with more sensitive detectors, such as LIF [22] or electrochemical detection [23]. However, the cheapest and the most popular one is the UV-absorption detector. Although the short diameter of the capillary (25–100 μm) does not allow to measure absorbance of some compounds which do not possess good chromospheres, the use of special detection windows (e. g. bubble cells, zeta cells) [24, 25] may slightly improve sensitivity with optical detection.

In addition, CE offers a great possibility for online sample preconcentration and enables the automatisation of the process, which always is desirable in analytical chemistry, as well as in other fields. Sample stacking is an inherent and exclusive feature of CE [26], taking place when the sample compounds encounter isotachophoretic concentration at the interface between sample zone and buffer (isotachophoretic sample stacking, ITPSS) [27, 28] or when the conductivity of the sample is smaller than that of the buffer (field-amplified sample stacking, FASS) [29, 30]. Additionally, large-volume sample stacking (LVSS) has been demonstrated to improve detection limits of charged analytes by more than 1000-fold and to be easily automated and controlled by software [31–37]. Sweeping is another on-line sample

concentration method for either charged or neutral analytes, the sample concentration effect relies on how the pseudostationary phase enters the sample solution zone (nonmicelle buffer) and sweeps the analytes [38–43]. Prof. Terabe has reviewed the online concentration of neutral analytes for MEKC [44–48].

Furthermore, microchip electrophoresis (MCE) as one of the principal analytical techniques for micrototal analysis system (μ-TAS) is also a research area of increasing importance [49–51]. Although a variety of conventional detection schemes has been accomplished in these microfluidic devices, including optical methods which employ laser-induced fluorescence (LIF), absorbance [52, 53], chemiluminescence [54–56], electrochemiluminescence [57, 58], electrochemical methods [59, 60], mass spectrometric measurements [61, 62] etc.. LIF detection is the most widely utilized detection mode in microchip devices [63–65], due to its high sensitivity and compatibility with the typical chip dimensions (i.e., the ease of focusing a laser beam onto the mm-sized channels). However, despite the success that LIF has enjoyed with microfabricated instrumentation, it is difficult to realize the ultimate integration scheme due to the larger volume of conventional lasers. Furthermore, these conventional lasers are generally expensive, relatively unstable (flicker noise), and have short lifetimes (~3000 h) [66], so they are not the ideal light sources for miniaturized systems.

Electrochemical (EC) detection due to characteristics such as inherent miniaturization, sensitivity, low cost, portability and compatibility with microfabrication technology has been successfully employed in capillary electrophoresis (CE) microchips. Among possible electrochemical techniques, amperometric detection, since first reported in 1998 by Wolley et al. [67], has been the most widely employed for CE microchips. In this detection mode, an appropriate design of the detection cell is required for ensuring electrical isolation from the high separation voltage. Three strategies have been reported for coupling EC detection to CE: in-channel, off-channel and end-channel [68]. Although, CE-microchips were initially

fabricated using glass substrates, in the last years, polymer materials such as poly (methylmethacrylate) (PMMA) [69,70], poly(dimethylsiloxane) (PDMS) [71], polycarbonate (PC) [72], polyester [73] and poly(ethylenterephthalate) (PET) [74] have been also employed owing to their mechanical and chemical properties, low cost, ease of fabrication and higher flexibility. Recently, we developed an exceedingly simple technique for microfluidic device fabrication using a dry film photoresist in conjunction with photolithographic and hot roll lamination techniques [75]. Dry film photoresists offer many advantages over the use of liquid photoresists, including good conformability, excellent adhesion to other substrates, good flatness, and absence of liquids, uniform photoresist distribution, low exposure energy, low cost, and short processing times [76].

1.2. Objective of the work

The goal of this work was to develop a simple and highly sensitive method for the detection of abused drugs and their major metabolite using GC/MS, on-line preconcentration (stacking or sweeping) capillary electrophoresis, and microchip electrophoresis, respectively. Accordingly, sweeping in conjunction with MEKC or microchip electrophoresis in conjunction with electrochemical detection is an accurate, sensitive and rapid approach that should be considered for use in rapid drug screening; it is a sufficiently reliable and complementary method to GC/MS for use in clinical and forensic analyses.

1.2.1. Comparison of analysis of abused drug by the use of GC/MS in conjunction with liquid–liquid and solid phase extraction methods

Both the liquid-liquid extraction (LLE) and solid phase extraction (SPE) methods are sensitive enough to monitor ketamine and its major metabolites in urine and hair samples, but, although the LLE extraction procedure is easier and faster to perform than the SPE method, SPE is preferable to LLE when considering the efficiency of the extraction, concentration, and

detection. The results would be discussed in chapter 2.

1.2.2. Research of on-line preconcentration and determination of abused drugs by micellar electrokinetic chromatography: Complementary method to gas chromatography/mass spectrometry

In this work, we have investigated a rapid, simple, and highly efficient on-line preconcentration method using in micellar electrokinetic chromatography (MEKC) for the analysis of abused drugs. In chapter 3, we describe a simple and highly sensitive method for the detection of ketamine and its major metabolite, norketamine, in urine using the techniques of on-line preconcentration and sample sweeping, and combined with MEKC. In chapter 4, we also report on an approach involving the use of a sweeping technique combined with MEKC for the simultaneous determination of flunitrazepam and its major metabolites, 7-aminoflunitrazepam and *N*-desmethylflunitrazepam. Furthermore, in chapter 5, we also successfully established and validated a screening procedure for the analysis of cocaine, heroin, and opiates in powders and urine samples using cation-selective exhaustive injection/sweeping micellar electrokinetic chromatography (CSEI-sweep-MEKC).

1.2.3. Research of separation and identification of abused drugs using MEKC /dry-film-based microchip capillary electrophoresis with electrochemical detection

In chapter 6, we report the first separation of amphetamine, methamphetamine, and ephedrine using micellar electrokinetic chromatography (MEKC) and dry-film-based microchip capillary electrophoresis (DFB-MCE) in conjunction with electrochemical detection. We determined the optimal separation conditions for this method to decrease the amount of sample consumed and the separation time; we then used the optimized conditions to successfully determine the presence of these compounds in urine sample after solid phase

extraction. Herein, we compare the results obtained when using the DFB-MCE and GC/MS methods for the analyses of these compounds. The capillary electrophoresis-based methods are extremely complementary to GC/MS-based forensic analyses.

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Chapter 2

Simultaneous determination and quantitation of ketamine and its major metabolites by the use of GC/MS in conjunction with liquid–liquid and solid phase extraction methods

2.1. Introduction

Ketamine [(R,S)-2-(O-chlorophenyl)-2-(methylamino)cyclohexanone] hydrochloride, also known colloquially as "special K" or "K", is a general anesthetic for human and veterinary use. It is a legitimate manufactured anesthetic that is used primarily by vets to immobilize cats and monkeys. Although it is also used in human surgery, it has been in the most part replaced by safer and more-effective drugs. Developed in the late 1960s as an analogue of phencyclidine (PCP) [1], ketamine produces effects similar to PCP in conjunction with the visual effects of lysergic acid diethylamide (LSD) [2]. Users tout ketamine's overt hallucinatory effects as being preferable to those of PCP or LSD because they last a short time—an hour or less. The drug, however, can affect the senses, judgment, and coordination for up to 18–24 h [3]. Moreover, the effects on an individual vary widely according to body size and the presence of alcohol or other drugs.

Ketamine can be detected in blood, plasma and urine [4]. When ketamine is snorted, smoked, or mixed into drinks, its detectable metabolites include ketamine, norketamine, dehydronorketamine, deaminonorketamine, and other derivatives (see Table 2.1). It is believed that norketamine contributes the greatest pharmacological effect of ketamine, and it has been demonstrated to exhibit depressant effects similar to those of ketamine. Figure 2.1 displays that the mechanism of ketamine metabolism. Ketamine is metabolized to at least two compounds of pharmacological interest; first, ketamine undergoes N-demethylation mediated by CYP-450 enzyme to form norketamine in the liver, and then its cyclohexanone ring undergoes oxidative metabolism to form the second metabolite dehydronorketamine [5].

When urine or hair samples that are suspected to contain ketamine are collected usually ketamine itself cannot be detected in these samples when using gas chromatography/mass spectroscopy. It is possible that ketamine is rapidly transformed in humans into its major metabolites, norketamine, and dehydronorketamine. Because of the rapid growing in the abuse of ketamine, a simple, fast, and consistent method for its determination is necessary. Some analytical techniques for detecting ketamine have been presented, including high-performance liquid chromatography (HPLC) [6-8] and gas chromatography/mss spectrometry (GC/MS) [9]. These techniques have almost always been combined with liquid–liquid extraction (LLE), solid phase extraction (SPE), or solid phase microextraction (SPME) techniques to obtain the target substances. Although GC/MS can provide good data that can be used as scientific proof that may be accepted in the court, it often results in running times that are too long (often > 15min) when a 30-m GC column is used. In particular, if hundreds of samples require analysis, this method is not practical. In addition, the pretreatment of the bio-samples usually results in further wasted time. In this paper, we report our approach that uses GC/MS combined with simple LLE and SPE procedures for the rapid analysis (< 10 min) and comparison of ketamine and its metabolites in urine and hair samples. The methods have proven useful in forensic cases for the simultaneous determination and quantitation of the components of ketamine and its metabolites in suspect samples. Furthermore, we also compare the analytical differences between ketamine and its metabolites obtained from urine and hair samples from criminal suspects.

2.2. Materials and methods

2.2.1. Apparatus

All analyses were performed using a Hewlett–Packard (HP; Palo Alto, CA) gas chromatography/mass spectrometry (GC/MS) system that consisted of an HP 6890 series GC, an HP 5973 quadrupole mass selective detector (MSD), and an HP 7683 auto-injector; data were collected using an HP Chem-Station computer system. Helium was the carrier gas and was used at a flow-rate of 1 mL/min. The injector temperature was 250 ˚C. A Zebron ZB-5 MS fused-silica capillary column (30 m × 0.25 mm i.d.; 0.25-μm film thickness of 5% phenylmethylsilicone) provided the analytical separation. The retention times for ketamine, norketamine, dehydronorketamine, deaminonorketamine, and ketamine-D_4 (I.S.) were 9.87, 9.60, 9.81, 9.32, and 9.84 min, respectively. The oven temperature program was as follows: beginning at 120 °C (held for 1 min), it was ramped to 200 °C at 15 °C /min and then held for 2 min. Next, it was ramped to 250 °C at 18 °C /min and then finally held at that temperature 5.0 min. The total analysis time was 16.12 min. The MSD was operated in the electron ionization and SIM modes. The spectrometer was operated under the following conditions: SIM mode; ionization energy, 70 eV; the ion source temperature was maintained at 280 °C; 40–300 amu at 1.84 scans per second.

2.2.2. Chemicals

Ketamine•HCl (Catalog No. K-002; 1 mg/mL methanol), norketamine•HCl (Catalog No. N-036; 1mg/mL methanol), and the internal standard ketamine-D_4 • HCl (Catalog No. K-003; 1 μg/mL methanol) were obtained from Radian International. Methanol, dichloromethane, n-hexane, isopropanol, acetic acid, ammonium hydroxide, acetone and phosphoric acid were obtained in analytical grade (Aldrich). All of the suspected urine and hair samples were donated by the Command of the Army Force of Military Police, Forensic Science Center,

Taiwan.

2.2.3. Procedures for sample preparation and extraction from urine and hair samples

2.2.3.1. Liquid–liquid extraction

A simple, fast extraction method was used. A urine sample (2 mL) was mixed with a preformed mixture of ketamine-D_4, saturated K_2CO_3, and the extraction solvent (n-hexane/dichloromethane, 3:1, v/v); we used saturated K_2CO_3 to make the analyte alkaline. The solution was then shaken for 15min before the upper organic layer was collected into a clean tube and then evaporated (< 40 °C) to dryness under nitrogen. The residue was dissolved in 50 μL of methanol and 2 μL of this sample was injected into a GC/MS. Details of procedures are presented in Table 2.2a (left-hand column).

Hair samples were collected from the occipital crown regions of the heads of several subjects. Hair usually has many contaminants on its surface, which may influence the analytical results. Thus, to clean the samples we cut the hairs into segments (1–2 cm), placed them into a microtube, and then decontaminated them by vortexing in an ultrasonic bath with n-hexane (1 mL, 1min) and acetone (1 mL, 1min). Finally, the rinsing extracts were evaporated and the hairs were cut into 1–2-mm sections; a portion was weighed (10.0 mg), methanol was added (0.5 mL), and the sample was placed in ultrasonic bath (40 °C) for 1 h before liquid–liquid extraction was performed followed by the same method as that used for the urine samples. We summarize these procedures in Table 2.2a (right-hand column).

2.2.3.2. Solid-phase extraction

The cartridges (part No. 1211-3052; column type, LRC) were obtained from Varian (CA, USA). The cartridges were conditioned with methanol (3 mL), DI H_2O (3 mL), and 0.1 M phosphate buffer (pH 6.0, 1 mL). Urine (2 mL; Table 2.2b, left-hand column) and rinsed hairs

(Table 2.2b, right-hand column) were mixed with ketamine-D_4 solution (100 μL) and 0.1 M phosphate buffer (pH 6.0, 1 mL). We washed the column with DI H_2O (3 mL), 0.1 M acetic acid (1 mL) and methanol (3 mL) and then dried it under vacuum for 10 min. The analytes were eluted with dichloromethane/isopropanol/ammonium hydroxide (78:20:2, v/v/v). The clean organic phase was then evaporated to dryness. The residue was also dissolved in 50 μL of methanol and 2 μL of this sample was injected into the GC/MS. Details of these procedures are presented in Table 2.2b.

2.3. Results and discussion

2.3.1. Mass spectra of ketamine and its major metabolites

Figure 2.2 displays typical electron ionization mass spectra, obtained from a library research database, of ketamine, its major metabolites, and ketamine-D_4 (I.S.) from urine. We can easily distinguish the selective ion that we wish to analyze. The primary ions at *m*/*z* 180, 166, 153, 173, and 184, obtained in the selective ion monitoring (SIM) mode, were confirmed to be those of ketamine (K), norketamine (NK), 5,6-dehydronorketamine (DHNK), deaminonorketamine (DANK), and ketamine-D_4 (K-D_4), respectively. According to these results, Figure 2.3 displays the postulated fragmentations of ketamine and its major metabolites.

2.3.2. Linearity of the concentration

Standard solutions of K, NK and K-D_4 (I.S.) were prepared at concentrations between 100 and 3000 ng/mL (100, 200, 1000, 2000, and 3000) in methanol to obtain a calibration graph. This plot indicates that a linear relationship exists in the 100-3000 ng/ml range for each of K, NK, and K-D_4; the linearity was excellent. We obtained the following equations when using the LLE method: $y = 3.18 \times 10^5x - 7.15 \times 10^3$ ($r^2 = 0.9930$) for K; $y = 2.94 \times 10^5x - 7.41 \times 10^3$ ($r^2 = 0.9964$) for NK; $y = 4.12 \times 10^5x - 8.11 \times 10^3$ ($r^2 = 0.9931$) for K-D_4; y is the peak area and x is the concentration (mg/mL) of analyte. Using the SPE method, we obtained the following equations: $y = 2.81 \times 10^5x - 2.77 \times 10^3$ ($r^2 = 0.9992$) for K; $y = 2.15 \times 10^5x - 3.31 \times 10^3$ ($r^2 = 0.9991$) for NK; $y = 3.52 \times 10^5x - 3.66 \times 10^3$ ($r^2 = 0.9993$) for K-D_4. Table 2.3 summarizes these data.

2.3.3. Limit of detection (LOD) and limit of quantitation (LOQ)

The limit of detection (LOD) is calculated by taking the concentration of the peak of

interest divided by three times the signal-to-noise ratio (LOD；S/N = 3). The limit of quantitation (LOQ) is calculated as same way as LOD, but divided by 10 times the signal-to-noise ratio (LOQ；S/N = 10). For the analyses using the LLE procedure, we obtained values for the LOD and LOQ of 18.1 and 58.8 ppb, respectively, for K and 21.9 and 74.6 ppb, respectively, for NK. When we used the SPE procedure, the values of LOD and LOQ that we obtained are 5.4 and 17.9 ppb, respectively, for K and 7.1 and 23.3 ppb, respectively, for NK. These results are also summarized in Table 2.3.

2.3.4. Repeatability tests

The intra-day and inter-day precisions are listed in Table 2.3. The retention times are characterized by RSDs of 1–2% at a concentration of 1 ppm for each extraction procedure. At a concentration of 1 ppm, the peak areas are characterized by RSDs of 3–5% under the conditions of LLE and 1–2% by SPE. According to these results, both of these processes are fairly good extraction methods, but the SPE procedure is slightly superior to the LLE one.

2.3.5. Analysis and comparison of ketamine and its major metabolites

2.3.5.1. Urine samples

As Figure 2.4 indicates, from GC/MS analysis, we detected K and its major metabolites in the urine sample of suspect No. 001 after both liquid–liquid (Fig. 2.4A) and solid phase (Fig. 2.4B) extraction. Using the SIM mode in LLE, we found K and its metabolites NK and DHNK. The concentrations decrease in the order NK > DHNK > K. When we chose the SPE method, we detected K and its metabolites NK, DHNK, and DANK. The concentrations decrease in the order NK > DHNK > K > DANK. In the urine samples of 20 suspects (listed in Table 2.4) that contained positive K, we found that NK and DHNK were usually the major metabolites present. When we used the SPE procedure, however, we often found another

metabolite, DANK, which we seldom detected after LLE, and we used the ions at *m/z* 173, 129, and 208 in the SIM mode. Because the half-life ($T_{1/2}$) of K is 3–4 h and over a 72-h period a single dose of K is eliminated primarily in the urine [5], it is necessary to collect the suspected urine samples as soon as possible.

Table 2.4 displays an obvious result: even if we cannot determine the presence of K, the suspect cannot necessarily be proven innocent because we usually observe its metabolites, NK or DHNK, in the urine samples. Thus, it has been suggested that NK and DHNK are the most important metabolites for this analytical procedure.

2.3.5.2. Hair samples

Figure 2.5 depicts a comparison of the methods for extracting the hair samples. We collected hair from suspect No. 001, whose urine we had also tested. As in the analysis of this suspect's urine, K was the major metabolite in the hair samples. Although we detected two other metabolites (NK and DANK) when using the SPE procedure, their concentrations were lower than that of K. The concentrations decrease in the order K >> NK ≈ DHNK. Another interesting phenomenon is that that we could not detect DANK in the hair samples of any of the 20 suspects when using either LLE or SPE. Table 2.4 lists all of these results. Although the extraction procedures are more multifarious than that used for the urine samples, the analyses of the hair extracts were very clear and provided superior analytical results. Furthermore, we could also obtain much useful information in these analyses, for example, the drug type and the time when it was used.

2.3.6. Recovery of extraction

Figure 2.6 presents a comparison of the recoveries of K and NK when using the LLE and SPE techniques. In Figure 2.6A, K and NK were spiked at 1 ppm in blank urine samples and K-D_4 (I.S., 1ppm) was added to one set of aliquots after LLE. We determined the extraction

efficiency of LLE for K and NK to be ca. 72% and ca. 67%, respectively. The average recoveries for K and NK were 74 ± 4% and 71 ± 6%, respectively. Using this same procedure, we determined (Fig. 2.6B) the extraction efficiencies for K and NK when using SPE to be ca. 88% and ca. 81%, respectively. The average recoveries for K and NK were 90 ± 3% and 84 ± 5%, respectively. As concluded earlier, SPE is more efficient and provides cleaner extracts than does LLE.

2.4. Conclusion

In this paper, we report that gas chromatography/mass spectrometry combined with LLE and SPE techniques can be used to determine ketamine and its two major metabolites, norketamine and 5,6-dehydronorketamine, easily, rapidly, and successfully without the need for a derivatization step. Using this approach, we have found that the major metabolites that can be monitored in urines samples collected from suspects were ketamine, norketamine, and 5,6-dehydronorketamine. In contrast, if hair samples are collected then we could often determine only the major amount of ketamine. When using the extraction procedure, we could obtain better values for the LOD, LOQ, and RSD of both urine and hair samples. Both the LLE and SPE methods are sensitive enough to monitor ketamine and its major metabolites in urine and hair samples, but, although the LLE extraction procedure is easier and faster to perform than the SPE method, SPE is preferable to LLE when considering the efficiency of the extraction, concentration, and detection.

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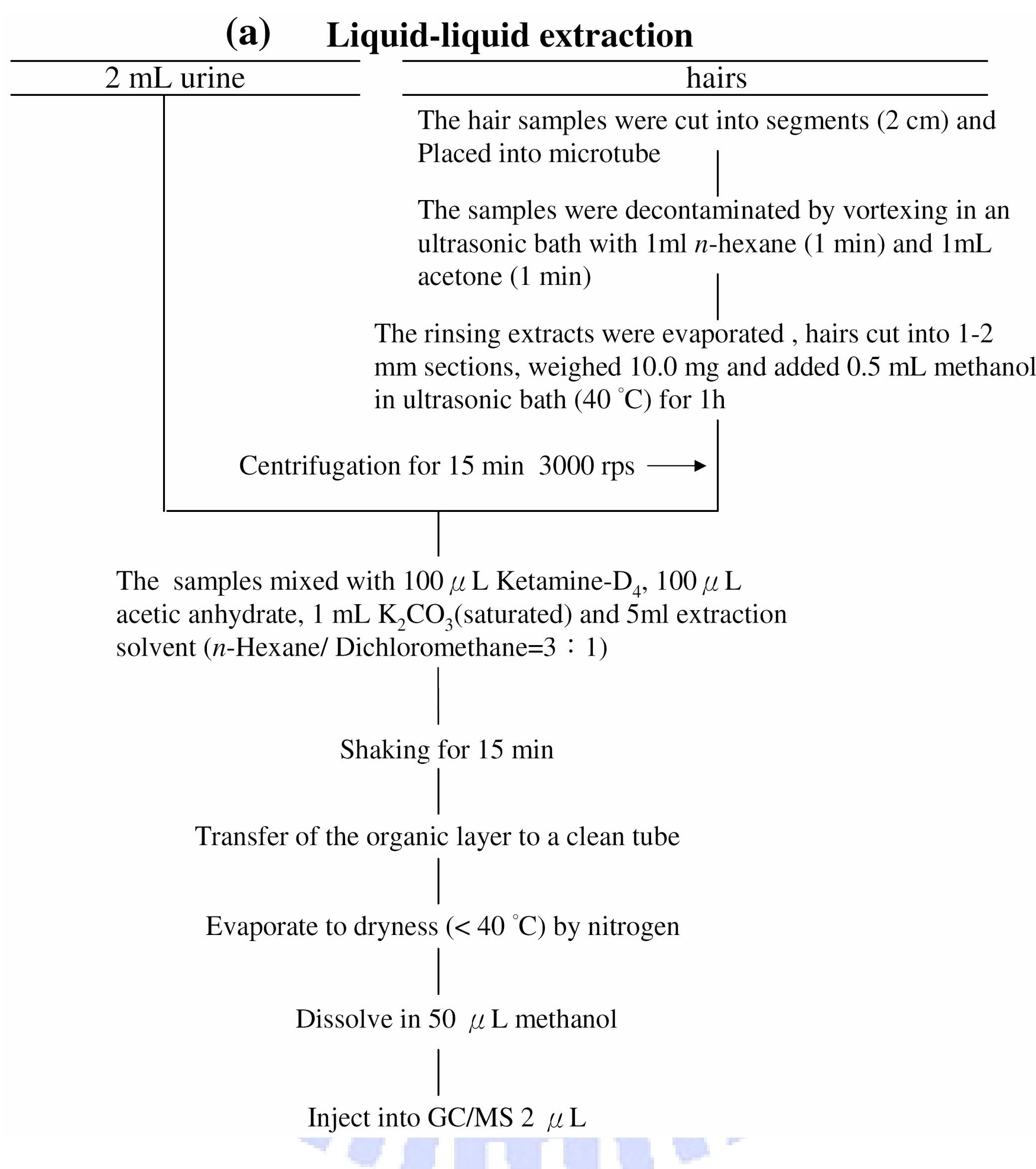
Table 2.1 The structures of ketamine and its metabolites.

Analyte	Structure	m/z
Ketamine (K)		237.7
Norketamine (NK)		223.7
5, 6-Dehydronorketamine (DHNK)		221.7
Deaminonorketamine (DANK)		208.7
Ketamine-D_4* ($K-D_4$)		241.7

* Internal standard (I.S.)

Table 2.2 Procedures for the preparation of analytical samples from urine and hair using (a) liquid–liquid extraction and (b) solid phase extraction.



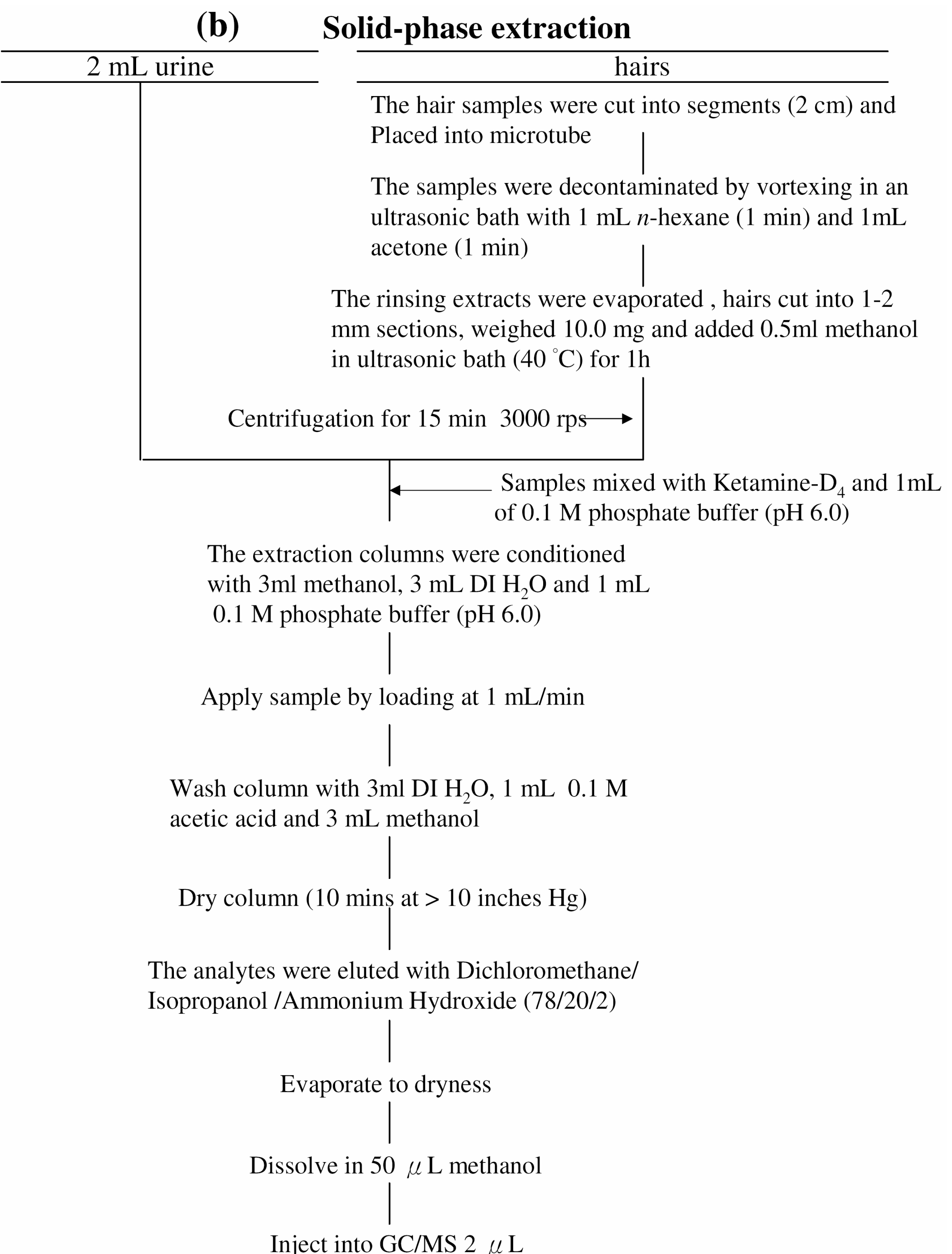

(b) Solid-phase extraction
2 mL urine
hairs
The hair samples were cut into segments (2 cm) and Placed into microtube
The samples were decontaminated by vortexing in an ultrasonic bath with 1 mL n-hexane (1 min) and 1mL acetone (1 min)
The rinsing extracts were evaporated , hairs cut into 1-2 mm sections, weighed 10.0 mg and added 0.5ml methanol in ultrasonic bath (40 °C) for 1h
Centrifugation for 15 min 3000 rps
Samples mixed with Ketamine-D4 and 1mL of 0.1 M phosphate buffer (pH 6.0)
The extraction columns were conditioned with 3ml methanol, 3 mL DI H2O and 1 mL 0.1 M phosphate buffer (pH 6.0)
Apply sample by loading at 1 mL/min
Wash column with 3ml DI H2O, 1 mL 0.1 M acetic acid and 3 mL methanol
Dry column (10 mins at > 10 inches Hg)
The analytes were eluted with Dichloromethane/ Isopropanol /Ammonium Hydroxide (78/20/2)
Evaporate to dryness
Dissolve in 50 μL methanol
Inject into GC/MS 2 μL

Table 2.3 Values of the coefficient of determination (r^2), limits of detection (LOD), limits of quantitation (LOQ), and RSD for ketamine, norketamine, and ketamine-D_4 after liquid–liquid extraction (LLE) and solid phase extraction (SPE).

	Ketamine	Norketamine	Ketamine-D_4[a]
Diagnostic ions	180*,209,152	166*,195,131	184*,213,156
Part A . LLE			
Equation of line of best fit	$y = 3.18 \times 10^5 x - 7.15 \times 10^3$	$y = 2.94 \times 10^5 x - 7.41 \times 10^3$	$y = 4.12 \times 10^5 x - 8.11 \times 10^3$
Coefficient of determination	$r^2 = 0.9930$	$r^2 = 0.9964$	$r^2 = 0.9931$
LOD ($S/N = 3$)	18.1 ppb	21.9 ppb	17.2 ppb
LOQ ($S/N = 10$)	58.8 ppb	74.6 ppb	55.5 ppb
RSD % (1 μg/mL)			
Ⅰ.Retention time (n=5)			
Intra-day	1.11	1.02	1.31
Inter-day	1.89	1.97	2.01
Ⅱ.Peak area (n=5)			
Intra-day	3.12	3.01	4.11
Inter-day	4.98	5.32	5.13
Part B. SPE			
Equation of line of best fit	$y = 2.81 \times 10^5 x - 2.77 \times 10^3$	$y = 2.15 \times 10^5 x - 3.31 \times 10^3$	$y = 3.52 \times 10^5 x - 3.66 \times 10^3$
Coefficient of variation	$r^2 = 0.9992$	$r^2 = 0.9991$	$r^2 = 0.9993$
LOD ($S/N = 3$)	5.4 ppb	7.1 ppb	4.5 ppb
LOQ ($S/N = 10$)	17.9 ppb	23.3 ppb	14.5 ppb
RSD % (1 μg/mL)			
Ⅰ.Retention time (n=5)			
Intra-day	1.01	1.03	1.0
Inter-day	1.64	1.83	1.91
Ⅱ.Peak area (n=5)			
Intra-day	2.11	1.99	2.01
Inter-day	2.56	2.33	2.76

[a] Internal Standard, * Primary ion

Table 2.4 Concentrations of ketamine and its major metabolites in the urine and hair samples of a series of suspects obtained using liquid-liquid extraction and solid- phase extraction.

	Urine								Hair							
	LLE(ppm)				SPE(ppm)				LLE(ppm)				SPE(ppm)			
Case	K	NK	DHNK	DANK	K	NK	DHNK	DANK	K	NK	DHNK	DANK	K	NK	DHNK	DANK
001	12.31	37.52	12.72	ND	11.31	32.45	13.36	0.21	3.62	ND	ND	ND	3.97	0.04	0.06	ND
002	1.34	3.11	0.88	ND	1.53	4.25	1.11	0.03	0.54	ND	ND	ND	0.86	0.09	0.07	ND
003	0.93	1.37	1.14	ND	1.20	1.17	0.92	ND	1.62	0.15	ND	ND	1.41	0.21	ND	ND
004	0.45	0.27	0.85	ND	0.58	0.43	0.71	ND	0.87	ND	ND	ND	0.91	0.01	ND	ND
005	1.03	5.77	2.13	ND	0.99	5.67	2.34	ND	0.66	ND	ND	ND	0.69	ND	ND	ND
006	0.77	0.23	0.56	ND	0.74	0.25	0.59	ND	0.89	ND	ND	ND	0.93	0.12	ND	ND
007	0.32	0.02	0.65	ND	0.28	0.04	0.71	ND	1.01	ND	ND	ND	1.06	0.32	ND	ND
008	0.89	0.12	1.22	ND	0.86	0.14	1.26	ND	ND	ND	ND	ND	0.02	ND	ND	ND
009	0.02	ND	0.78	ND	0.03	ND	0.81	ND	ND	ND	ND	ND	0.01	ND	ND	ND
010	0.12	0.08	0.22	ND	0.14	0.10	0.26	ND	ND	ND	ND	ND	0.66	0.04	ND	ND
011	0.37	0.04	0.77	ND	0.35	0.03	0.80	ND	ND	ND	ND	ND	ND	ND	ND	ND
012	ND	ND	0.04	ND	0.01	ND	0.05	ND	ND	ND	ND	ND	ND	ND	ND	ND
013	ND	0.34	0.66	ND	ND	0.43	0.78	ND	ND	ND	ND	ND	ND	ND	ND	ND
014	ND	0.78	0.34	ND	ND	0.73	0.38	ND	ND	ND	ND	ND	ND	ND	ND	ND
015	ND	ND	0.22	ND	ND	ND	0.24	ND	ND	ND	ND	ND	ND	ND	ND	ND
016	ND	ND	0.14	ND	ND	ND	0.16	ND	ND	ND	ND	ND	ND	ND	ND	ND
017	ND	ND	ND	ND	ND	ND	0.01	ND	ND	ND	ND	ND	ND	ND	ND	ND
018	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
019	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
020	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

ND: Not detected

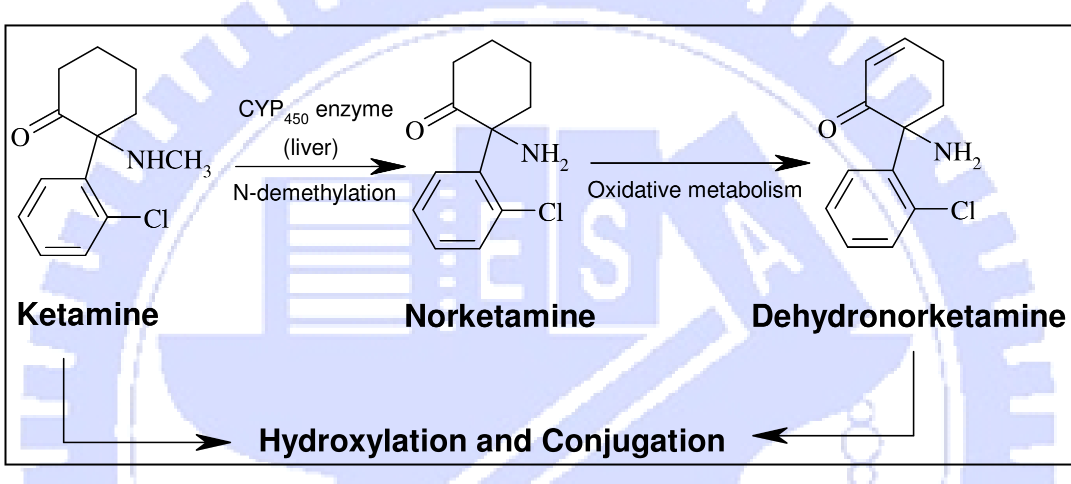


Figure 2.1 The major mechanisms of ketamine metabolism in humans.

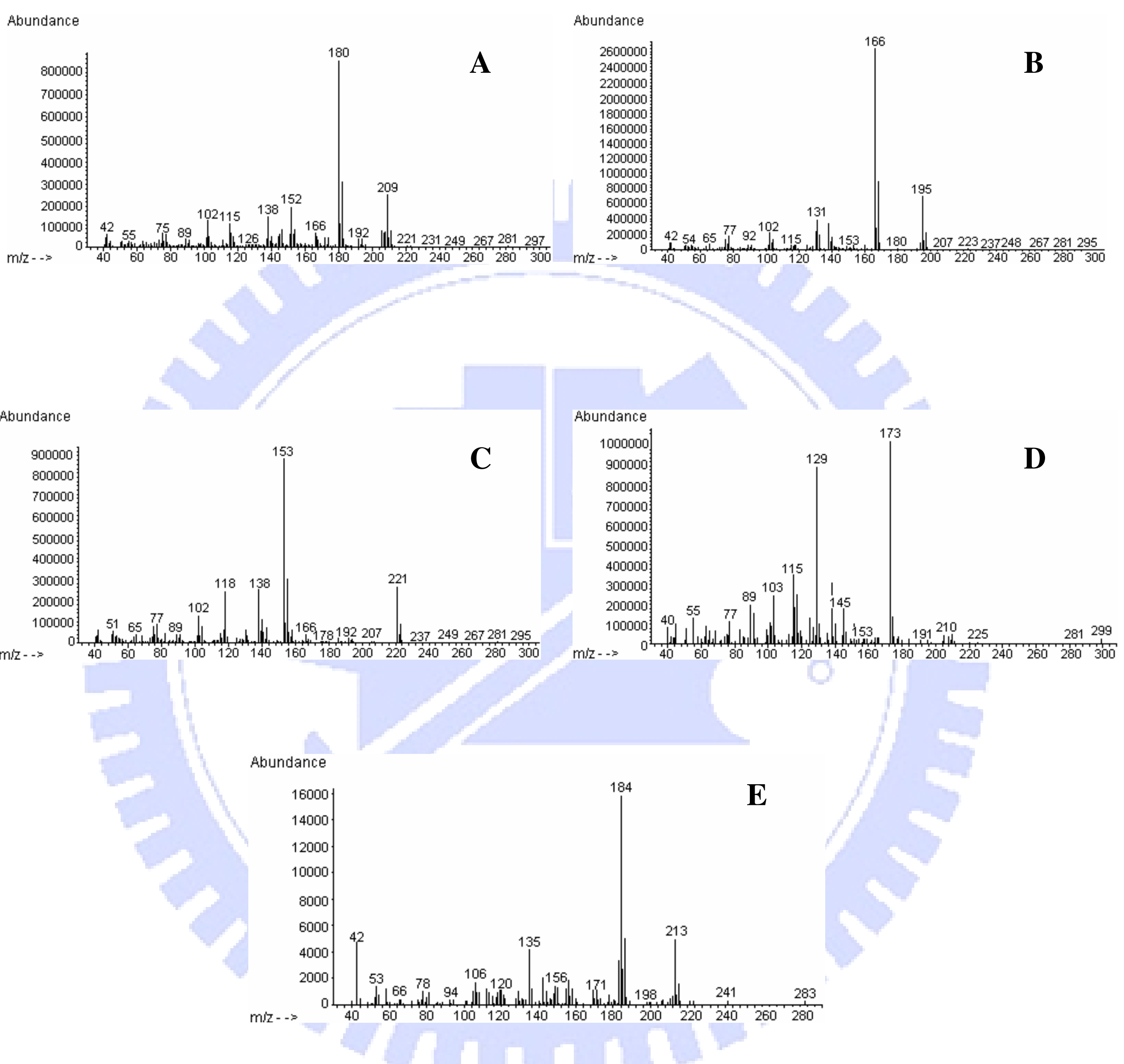


Figure 2.2 Electron-ionization mass spectra of (A) K, (B) NK, (C) DHNK, (D) DANK, and (E) K-D_4 (IS).

(A)

Ketamine

m/z=209

m/z=180

m/z=152

(B)

Norketamine

m/z=195

m/z=166

m/z=131

(C)

5,6-Dehydronorketamine

m/z=221

m/z=153

m/z=138

(D)

Deaminonoketamine

m/z=173

m/z=129

m/z=115

Figure 2.3 Postulated fragmentations of (A) ketamine, (B) norketamine, (C) 5,6-dehydronorketamine, and (D) deaminonorketamine.

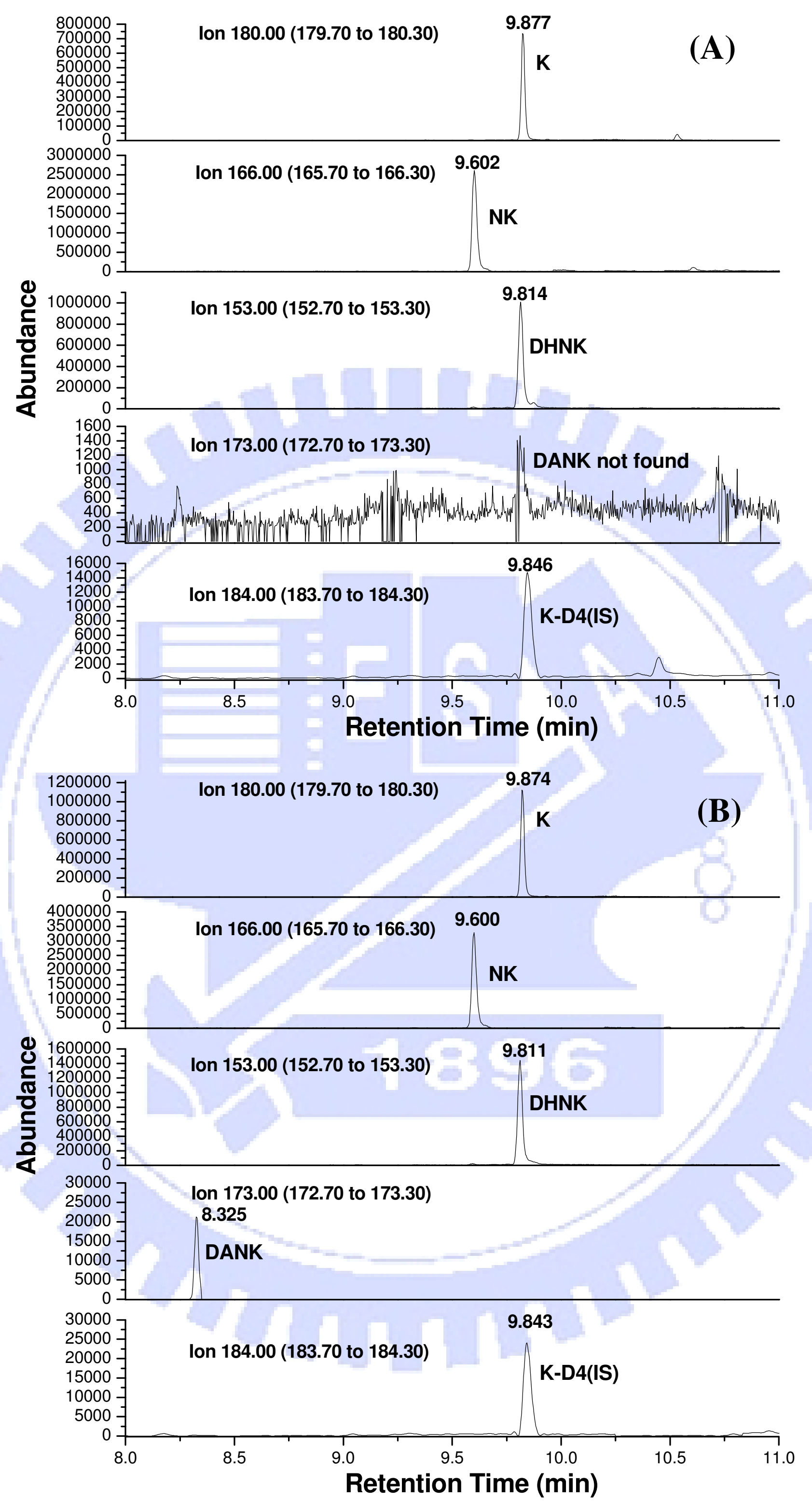


Figure 2.4 A comparison of the methods for extracting the urine samples from suspect no. 001: (A) liquid–liquid extraction; (B) solid phase extraction.

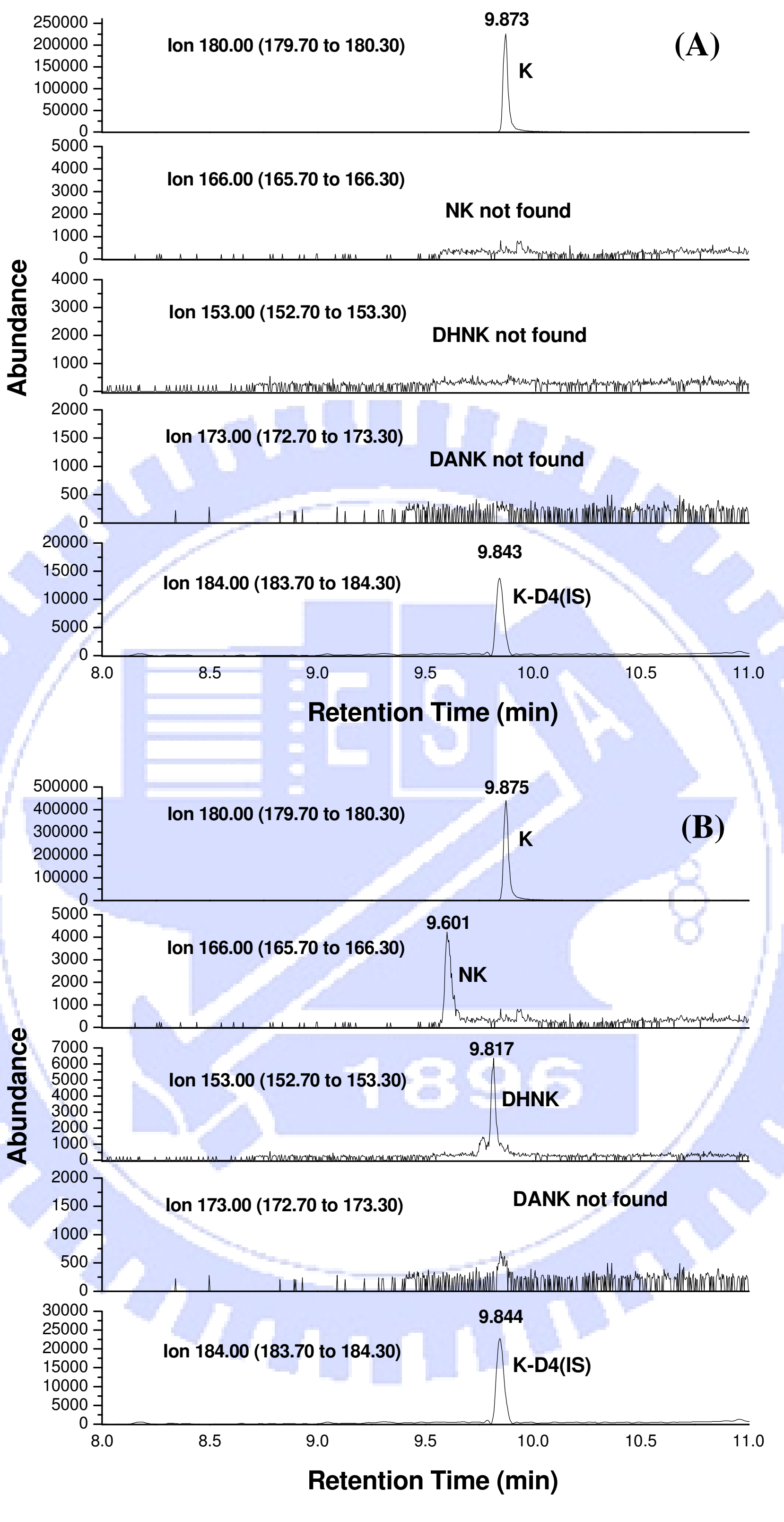


Figure 2.5 A comparison of the methods for extracting of hair samples from suspect no. 001: (A) liquid–liquid extraction; (B) solid phase extraction.

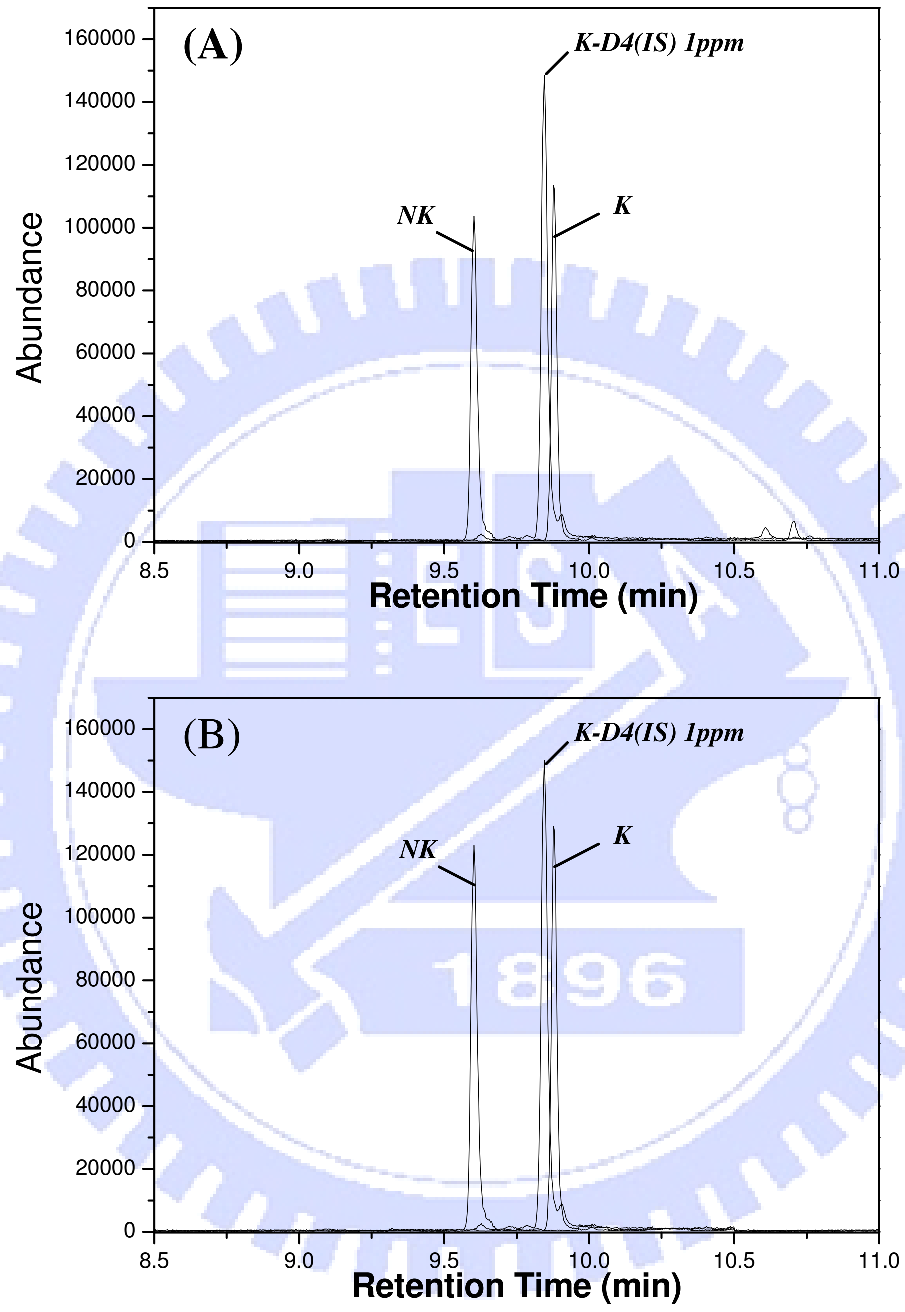


Figure 2.6 A comparison of the recoveries when using (A) liquid–liquid extraction and (B) solid phase extraction. Ketamine and norketamine were spiked at 1 ppm; ketamine-D_4 (internal standard, 1 ppm) was added to one set of aliquots after extraction.

Chapter 3

On-line preconcentration and determination of ketamine and norketamine by micellar electrokinetic chromatography: Complementary method to gas chromatography/mass spectrometry

3.1. Introduction

Ketamine is familiar to emergency physicians as a dissociative anesthetic that has been abused as a hallucinogen for almost 30 years. Ketamine produces effects similar to phencyclidine (PCP) in conjunction with the visual effects of lysergic acid diethylamide (LSD) [1]. Ketamine is available as either a powder or a liquid; in its powdered form, it can be inhaled nasally, smoked, or mixed into drinks; in its liquid form, it can be injected or applied to, for example, cigarettes. Ketamine is metabolized to at least two compounds of pharmacological interest. First, ketamine undergoes N-demethylation mediated to form norketamine in the liver. Then, norketamine's cyclohexanone ring undergoes oxidative metabolism to form dehydronorketamine. Current techniques for analyzing ketamine include the use of high-performance liquid chromatography (HPLC) [2–5] and gas chromatography in conjunction with mass spectroscopy (GC/MS) [6]. These approaches almost always employ liquid–liquid extraction (LLE), solid-phase extraction (SPE), or solid-phase microextraction (SPME) techniques to obtain the target substances.

Capillary electrophoresis (CE) is a separation method – based on a physical process quite different from that of chromatography – that has been the focus of much attention for developing new analytical methodologies [7–9]. CE is a powerful technique that is simple, provides rapid results, has high efficiency, resolution, and sensitivity, and involves low sample consumption; additionally, many CE instruments are available commercially. CE is a rapidly growing separation technique that is being applied in bioscience, pharmaceuticals, environmental, food science, and forensic research [10]. Micellar electrokinetic

chromatography (MEKC), which is one of the basic modes of CE, has become a popular technique for improving CE separation efficiency for both neutral and charges analytes [11]. Unfortunately, the benefits provided by the high number of theoretical plates obtained with CE can be overshadowed by the low sensitivity of UV detection systems. Because of the small dimensions of a CE capillary – the typical inside diameter (I.D.) and length are 25–100 μm I.D. and 40–80 cm, respectively – only very small sample volumes may be loaded onto the column. Additionally, for most common optical detection techniques, CE suffers from a drastically reduced pathlength relative to, for example, LC. Overcoming the poor sensitivity of CE with on-line sample preconcentration has been the focus of many investigations [12–14]. For example, Quirino and Terabe [15–18] found that neutral compounds could be analyzed effectively when utilizing the technique of MEKC combined with stacking. In 1998, they reported a sweeping method that can effect infiltration of analytes into the pseudostationary phase of the sample zone by applying an electric potential [19]. This technique is a new one for the on-line sample concentration of neutral or charged analytes in MEKC [20, 21]. The sample solution does not need to be prepared in a low-conductivity matrix, but the conductivity equal to or higher than the running micellar solution is favored.

In this paper, we describe a simple and highly sensitive method for the detection of ketamine and its major metabolite, norketamine, in urine using the techniques of on-line preconcentration and sample sweeping, and combined with MEKC. We have optimized several electrophoresis parameters to effect successful separations, such as the concentration of sodium dodecyl sulfate (SDS), the injection time, the applied voltage, and the temperature. We provide a three-dimensional representation to present a clear visualization of the improvements in the number of theoretical plates with respect to the different separation conditions. We determined the optimal separation conditions for this method and decreased the amount of sample consumed and the separation time. Finally, we also compare the results of this analytical approach with those obtained when using MEKC, sweeping MEKC, and

GC/MS.

3.2. Experimental

3.2.1. Chemicals

Ketamine hydrochloride (K・HCl, 1 mg/mL methanol), norketamine hydrochloride (NK・HCl, 1 mg/mL methanol), and the internal standard, [$^2H_4$] ketamine hydrochloride (ketamine-d$_4$, K-D$_4$・HCl, 1 μg/mL methanol), were obtained from Radian International. Fig. 3.1 displays their structures. SDS was purchased from Sigma (St. Louis, MO, USA). Disodium hydrogen phosphate (Na_2HPO_4) and sodium hydroxide (NaOH) were purchased from Fluka (Buchs, Switzerland). Citric acid was obtained from Merck (Darmstadt, Germany). Methanol, dichloromethane, n-hexane, isopropanol, acetic acid, ammonium hydroxide, acetone, and phosphoric acid were obtained in analytical grade (Aldrich). Water was purified by using a Milli-Q water system (Millipore, Bedford, MA, USA) and filtered through a 0.22 μm filter. All of the urine samples were donated by the Command of the Army Force of Military Police, Forensic Science Center, Taiwan.

3.2.2. Apparatus

A Beckman P/ACE 5500 capillary electrophoresis system (Beckman Instruments, Fullerton, CA, USA)was used to effect the separations. A diode-array detector was employed for detection. Separations were performed in a 47 cm (40 cm to detector) × 50 μm I.D. fused-silica capillary tube (Polymicro Technologies, Phoenix, AZ, USA). The capillary tube was assembled in the cartridge format. A personal computer using System Gold software controlled the P/ACE instrument and allowed data analysis. The separation capillary was preconditioned prior to use with 1 M NaOH for 30 min, 0.1 M NaOH for 30 min, and then deionized water for 30 min. The sample was injected hydrodynamically and then a negative voltage was applied with the micellar background electrolyte (BGE) at both ends of the capillary to effect separation. Between runs, the capillary was flushed sequentially with 0.1 M

NaOH, water, and BGE for 10 min each. The optimal buffer (pH 2.6) consisted of 25 mM citric acid/disodium hydrogen phosphate.

3.2.3. Sweeping and separation procedures

The column we used was a bare fused-silica capillary that we conditioned initially using a low-pH micellar electrolyte. The electroosmotic flow was suppressed by the low pH (2.6). Samples were pressure-injected at 0.5 psi. The detection wavelength was set at 200 nm. The neutral sample moved slowly because the velocity of the electroosmotic flow was very slow. The inlet and outlet of the capillary were placed in vials containing the BGE, and a negative voltage (15–30 kV) was applied. After the anionic micelles entered the sample zone, sweeping and separation were achieved through MEKC [21]. Stock sample solutions were prepared in methanol at a concentration of 100–1000 ppm. Different sample concentrations were obtained by diluting concentrated samples while keeping the sample matrix as 25 mM citric acid/disodium hydrogen phosphate and a low percentage of organic solvent (around 5–10%, v/v).

3.2.4. GC/MS apparatus and method

A Hewlett-Packard (HP; Palo Alto, CA, USA) system was used for gas chromatography/mass spectrometry (GC/MS). It consisted of an HP 6890 series GC, an HP 5973 quadrupole mass-selective detector, and an HP 7683 auto-injector; data were collected using an HP Chem Station computer system. Helium was the carrier gas and was used at a flow-rate of 1 mL/min. The injector temperature was 250 °C. A Zebron ZB-5 MS fused-silica capillary column (30 m × 0.25 mm I.D.; 0.25 μm film thickness of 5% phenylmethylsilicone) provided the analytical separation. The retention times for ketamine, norketamine, and ketamine-d_4 (I.S.) were 9.87, 9.60, and 9.84 min, respectively. The oven temperature was programmed as follows: beginning at 120 °C (held for 1 min), the temperature was ramped to

200 °C at 15 °C /min and then held for 2 min. Next, it was ramped to 250 °C at 18 °C /min and then finally held at that temperature for 5.0 min. The total analysis time was 16.12 min. The MS system was operated in electron ionization and selected ion monitoring (SIM) modes. The spectrometer was operated under the following conditions: SIM mode; ionization energy, 70 eV; the ion temperature was maintained at 280 °C; 40–300 u at 1.84 scans/s.

3.2.5. Solid-phase extraction procedure

The cartridges (column type, LRC) were obtained from Varian (CA, USA). The cartridges were conditioned with methanol (3 mL), water (3 mL) and 0.1 M phosphate buffer (pH 6.0; 1 mL). The urine sample (2 mL) was mixed with ketamine-d_4 (100 L) and 0.1 M phosphate buffer (pH 6.0; 1 mL). The column was washed with deionized water (3 mL), 0.1 M acetic acid (1 mL), and methanol (3 mL), and then it were dried under vacuum for 10 min. The analytes were eluted with dichloromethane/isopropanol/ammonium hydroxide (78:20:2, v/v/v). The clean organic phase was then evaporated to dryness. The residue was dissolved in methanol (50 μL) and a sample (2 μL) was injected into the GC/MS system. Fig. 3.2 provides detailed procedures.

3.3. Results and discussion

3.3.1. Optimizing the conditions for separation by sweeping MEKC

SDS is the most commonly additive used for MEKC during its separation. Fig. 3.3 displays typical MEKC chromatograms of ketamine (K), norketamine (NK), and ketamine-d_4 ($K\text{-}D_4$) that were separated in the presence of different concentrations of SDS. In Fig. 3.3, in addition to SDS, the buffer also consisted of 25 mM citric acid/disodium hydrogen phosphate (pH 2.6). As indicated in chromatogram of Fig. 3.3a, when 25 mM SDS was used, the separation of the analytes within 5 min was poor. When 50 mM SDS was used, however, the separation (Fig. 3.3b) began to improve as a result of increased interactions between the analytes and SDS micelles. The separation of the analyte was optimized (Fig. 3.3c) at an SDS concentration of 75 mM. In the acidic buffer solution (pH 2.6), the electrophoretic mobility of the neutral analytes toward the outlet (anode) is provided by the negative charged SDS micelles. The migration sequence of analytes to the outlet is based on their interaction with SDS. Thus, NK with the highest interaction with SDS migrated first. Under these conditions, we observed migration times in the following order: NK (peak 2) <K (peak 1) < $K\text{-}D_4$ (peak 3). When the concentration of the SDS was 100 mM (Fig. 3.3d), peaks K and $K\text{-}D_4$ became broad and overlapped.

Fig. 3.4 illustrates the effects of different injection times on the analyte's resolution during sweeping MEKC separation. We performed hydrodynamic injection at a pressure at 0.5 psi, injected the sample solution into the capillary for 90, 120, 150, 180, or 210 s, and then applied a −25 kV potential to effect sweeping MEKC separation. The concentration enhancement of the analytes increased as the injection time increased. Injecting the sample for 150 s provided an excellent separation efficiency (Fig. 3.4c), but longer injection times led to incomplete peak separation; peaks 1 (K) and 2 (NK) gradually overlapped as the injection time increased, which would not allow qualitative analyses in a forensic environment.

The influence that the applied voltage had on the sweeping MEKC separation was examined in the range of potential from −15 to −30 kV (data not shown). Clearly, an applied voltage of −25 kV provided the optimal separation. Joule heating occurs upon increasing the applied voltages and results in the occurrence of diffusion phenomena, which leads to poor separation at −30 kV. Finally, we examined the effect that temperature had on the separation condition by varying the capillary temperature from 18 to 30 ˚C (data not shown). We found that the resolution reduced at 30 ˚C, so we chose 25 ˚C as an optimum separation temperature.

3.3.2. Three-dimensional representation of the effects

The number of theoretical plates changed as a function of the conditions of the many different experiments, i.e., the injection time, SDS concentration, applied voltage, and temperature; Fig. 3.5 provides a clear visualization of these data for K and NK in three-dimensional representation. Fig. 3.5a indicates the plate numbers for K and NK, respectively, in the range from 1.0×10^5 to 3.6×10^5.We have fitted continuous analytical functions to the experimental values to guide the eye; they indicate that the optimized plate numbers for K and NK of 3.48×10^5 and 2.81×10^5, respectively, occur for injection times in the neighborhood of 150 s at an SDS concentration of 75 mM. Fig. 3.5b illustrates the plate numbers for K and NK, respectively, as a function of injection time and applied voltage. By comparing the sub-figures in Fig. 3.5, we find that the SDS concentration is the most important condition, more so than the applied voltage or temperature (data not shown), for affecting the plate number of the separation. In comparison, the temperature effect is minimal. We believe that such a three-dimensional representation is useful for determining a range of the optimized conditions for CE separation.

3.3.3. Comparing MEKC and sweeping MEKC

Fig. 3.6 depicts the analysis of K and NK by MEKC and sweeping MEKC methods. The concentrations of the analytes K, NK, and K-D_4 were 50, 30, and 20 ppm, respectively in Fig. 3.6a. However, the sample concentration was diluted 100-fold used in Fig. 3.6b. Under these conditions, K, NK, and K-D_4 had ca. ~760-, ~540-, and ~800-fold enhancements in their detection sensitivities, respectively, relative to those obtained in Fig. 3.6a. Table 3.1 presents values for the range of linearity, coefficient of determination (r^2), limit of detection (LOD), RSD, and the number of theoretical plates for K, NK, and K-D_4 using the MEKC and sweeping MEKC methods; in addition, we compare these values with those obtained when using the GC/MS method. The results indicate that the sweeping MEKC method provides better results than do the other methods for the separation of these analytes.

3.3.4. Separating and determining of ketamine and norketamine in suspect urine samples

Finally, we have used the sweeping MEKC method combined with SPE, was compared it with the GC/MS method, to analyze real urine samples obtained from suspected K users. First, we attempted to analyze the urine sample without extraction or sweeping, but we could not obtain a signal for K or NK (Fig. 3.7a). Next, we applied the same conditions as those used to obtain Fig. 3.7a, but with an injection time of 150 s; the resulting separation remained poor, but peaks for the target of analytes gradually appeared (Fig. 3.7b). Then, when we utilized SPE in conjunction with sweeping, we were able to clearly distinguish peaks for K, NK, and K-D_4 from the urine sample within 5 min (Fig. 3.7c). The concentrations of K and NK are 61.2 and 55.4 ppb, respectively. We also compared these results with those obtained by GC/MS for the same sample (Fig. 3.7d). Although the separation remained similarly as that in Fig. 3.7c, the analysis time was almost twice that required for using the sweeping MEKC technique.

3.4. Conclusion

In this study, we have demonstrated successfully the use of on-line sample preconcentration for determining the presence of K and NK by sweeping MEKC, which is an easy, rapid, and efficient technique. We have presented our results in a three-dimensional plot to provide a clear depiction of the conditions that effect the optimal separation. Under the optimized separation parameters, the analysis times for K, NK, and K-D_4 were less than 5 min, which is much faster than similar results obtained by GC/MS. The optimized parameters for the sweeping MEKC method were: running buffer, 25 mM citrate/phosphate (pH 2.6); applied voltage, −25 kV; temperature, 25 °C; SDS concentration, 75 mM. The limits of detection were 2.8, 3.4, and 3.3 ng/mL for K, NK, and K-D_4, respectively, and the enrichment factor for each compound fell within the range of 540–800. Accordingly, sweeping in conjunction with MEKC represents a good method that is complementary to GC/MS for use in clinical and forensic analyses.

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Table 3.1 Values of the range of linearity, coefficient of determination (r^2), limit of detection (LOD), RSD, and the number of theoretical plates for ketamine, norketamine, and ketamine-D_4 during separation by normal MEKC, MEKC/ sweeping and GC/MS, respectively.

	Ketamine	**Norketamine**	**Ketamine- D_4**
Normal MEKC			
Range of linearity	5–500 μg/mL	5–500 μg/mL	5–500 μg/mL
Coefficient of determination	$r^2 = 0.9921$	$r^2 = 0.9963$	$r^2 = 0.9938$
LOD ($S/N = 3$)	1.1 μg/mL	1.2 μg/mL	1.9 μg/mL
RSD (%; $n = 5$)			
Ⅰ. Migration time	3.12	4.74	3.87
Ⅱ. Peak area	4.22	3.85	4.66
Number of theoretical plates (N/m)	2.58×10^5	2.45×10^5	2.41×10^5
MEKC-sweeping			
Range of linearity	5–500 ng/mL	5–500 ng/mL	5–500 ng/mL
Coefficient of variation	$r^2 = 0.9957$	$r^2 = 0.9984$	$r^2 = 0.9961$
LOD ($S/N = 3$)	2.8 ng/mL	3.4 ng/mL	3.3 ng/mL
RSD (%; $n = 5$)			
Ⅰ. Migration time	2.11	2.03	1.89
Ⅱ. Peak area	1.76	1.92	2.04
Number of theoretical plates (N/m)	3.48×10^5	2.81×10^5	3.18×10^5
GC-MS			
Range of linearity	10–1000 ng/mL	10–1000 ng/mL	10–1000 ng/mL
Coefficient of variation	$r^2 = 0.9992$	$r^2 = 0.9991$	$r^2 = 0.9993$
LOD ($S/N = 3$)	5.4 ng/mL	7.1 ng/mL	4.5 ng/mL
RSD (%; $n = 5$)			
Ⅰ. Retention time	1.01	1.03	1.0
Ⅱ. Peak area	2.11	1.99	2.01

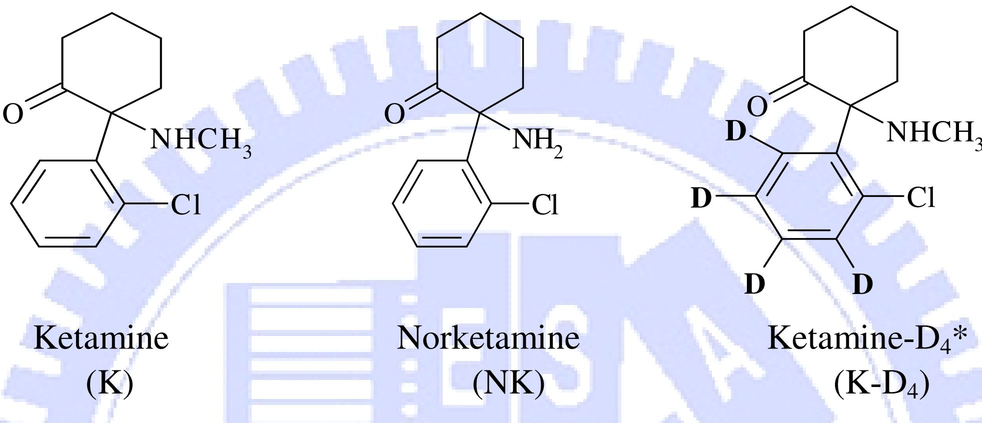

Figure 3.1. The structures of ketamine, norketamine and ketamine-D_4 (I.S.)

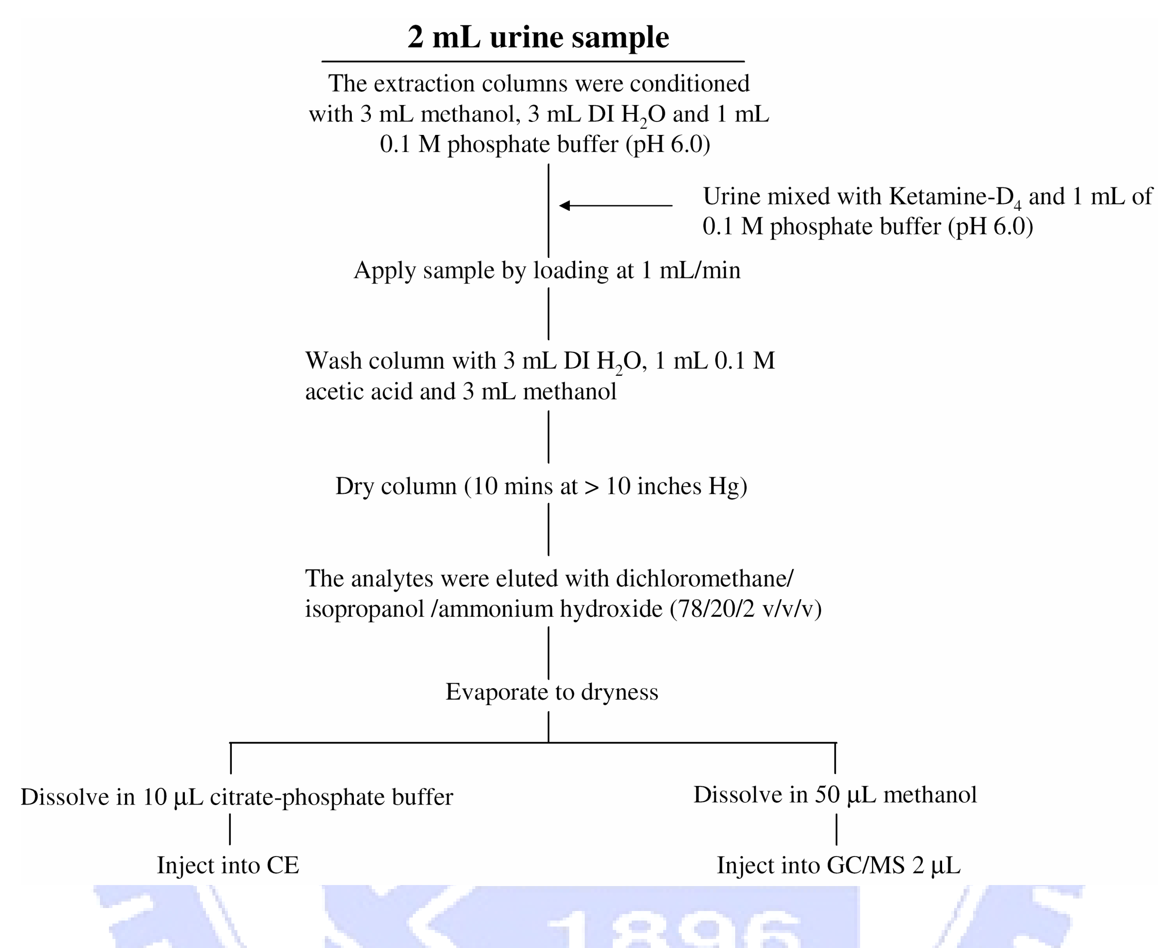

Figure 3.2 The procedures used for sample preparation for urine by solid-phase extraction of urine.

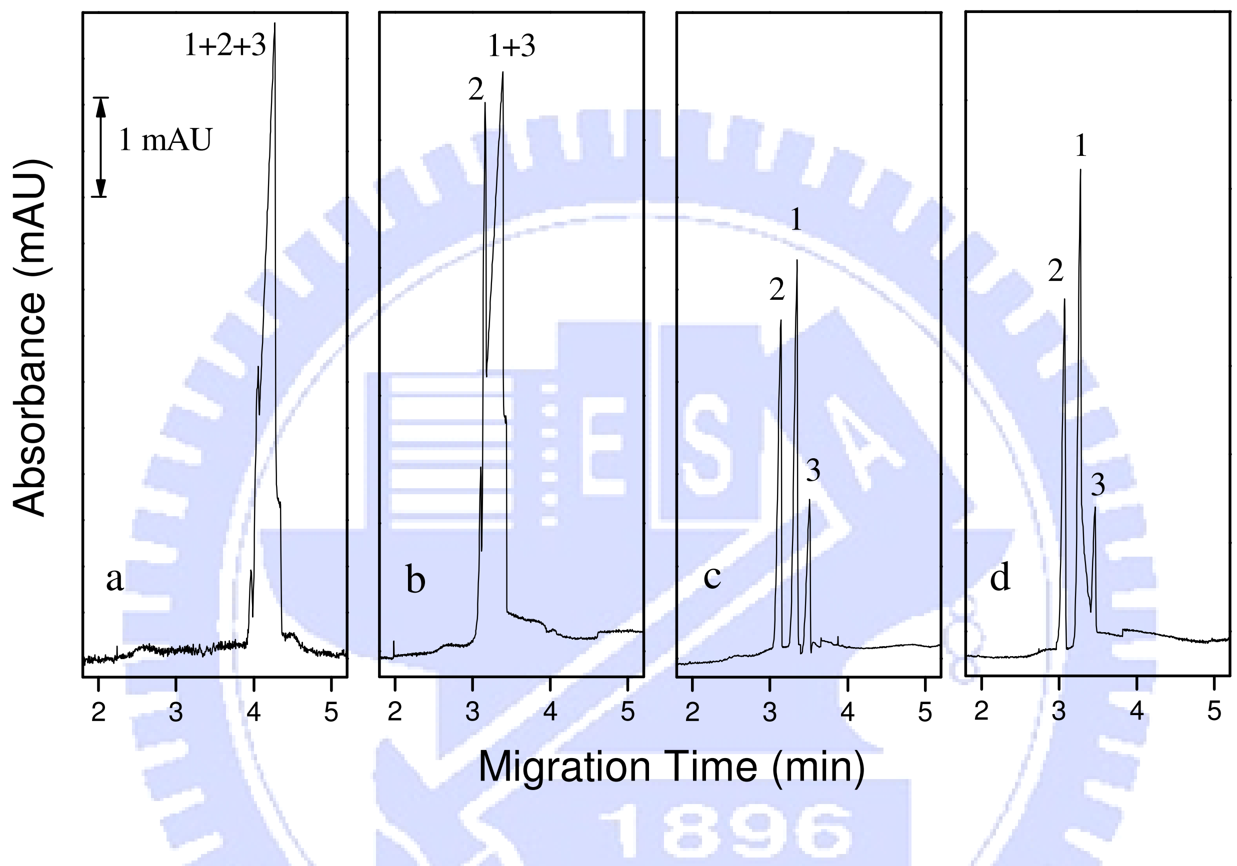

Figure 3.3 Effects that different SDS concentrations have on MEKC separations: (a) 25 mM; (b) 50 mM; (c) 75 mM; and (d) 100 mM. Conditions: capillary, 47 cm long (40 cm to detector), 50 μm I.D.; 25 mM citrate/phosphate buffer (pH 2.6); applied voltage, −25 kV; detection wavelength, 200 nm; temperature, 25°C; injection time, 4 s (0.5 psi); sample concentrations: 50, 30, and 20 ppm for K (peak 1), NK (peak 2), and K-D_4 (peak 3), respectively.

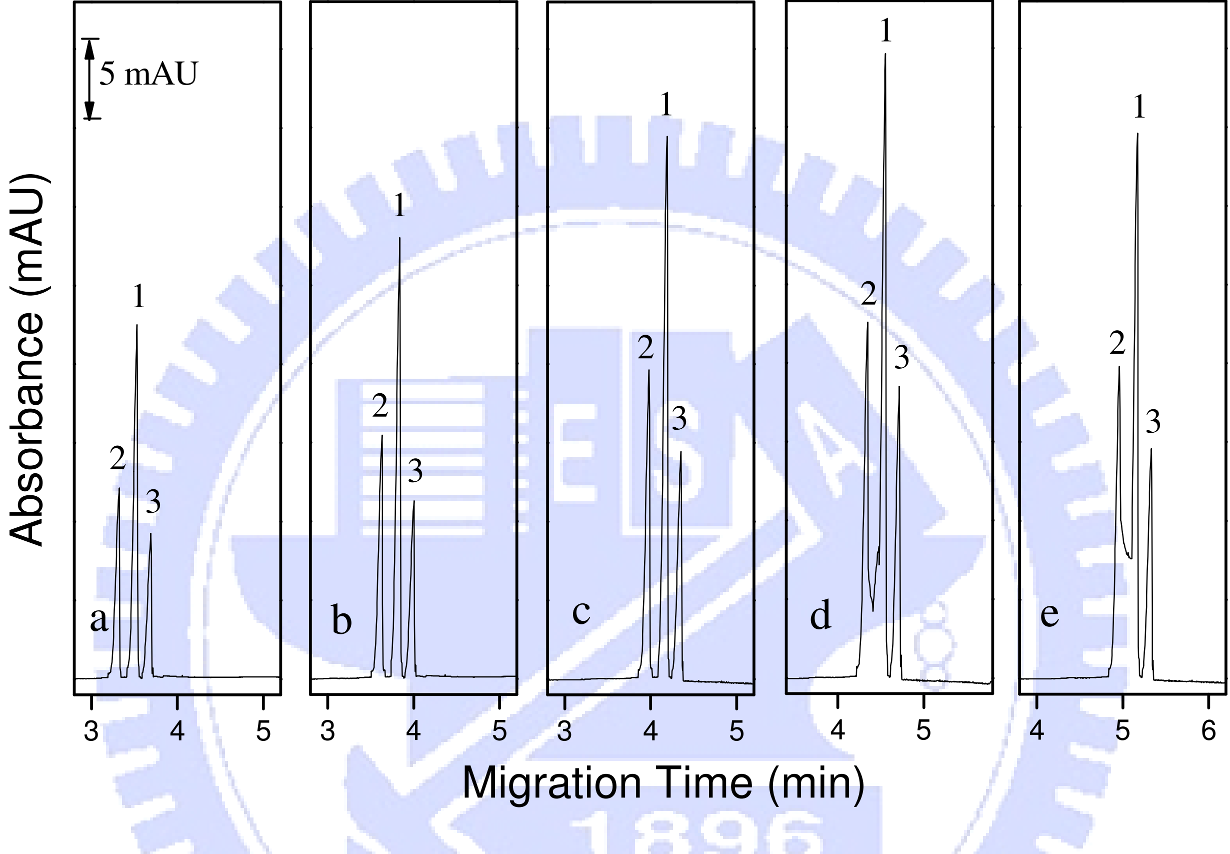

Figure 3.4 Effects that different injection times have on sweeping MEKC separations. (a) 90 s, (b) 120 s, (c) 150 s, (d) 180 s, and (e) 210 s. Conditions: SDS concentration, 75 mM; sample concentrations: 500, 300, and 200 ppb for K (peak 1), NK (peak 2), and K-D_4 (peak 3), respectively. Other conditions are the same as those in Fig. 3.3

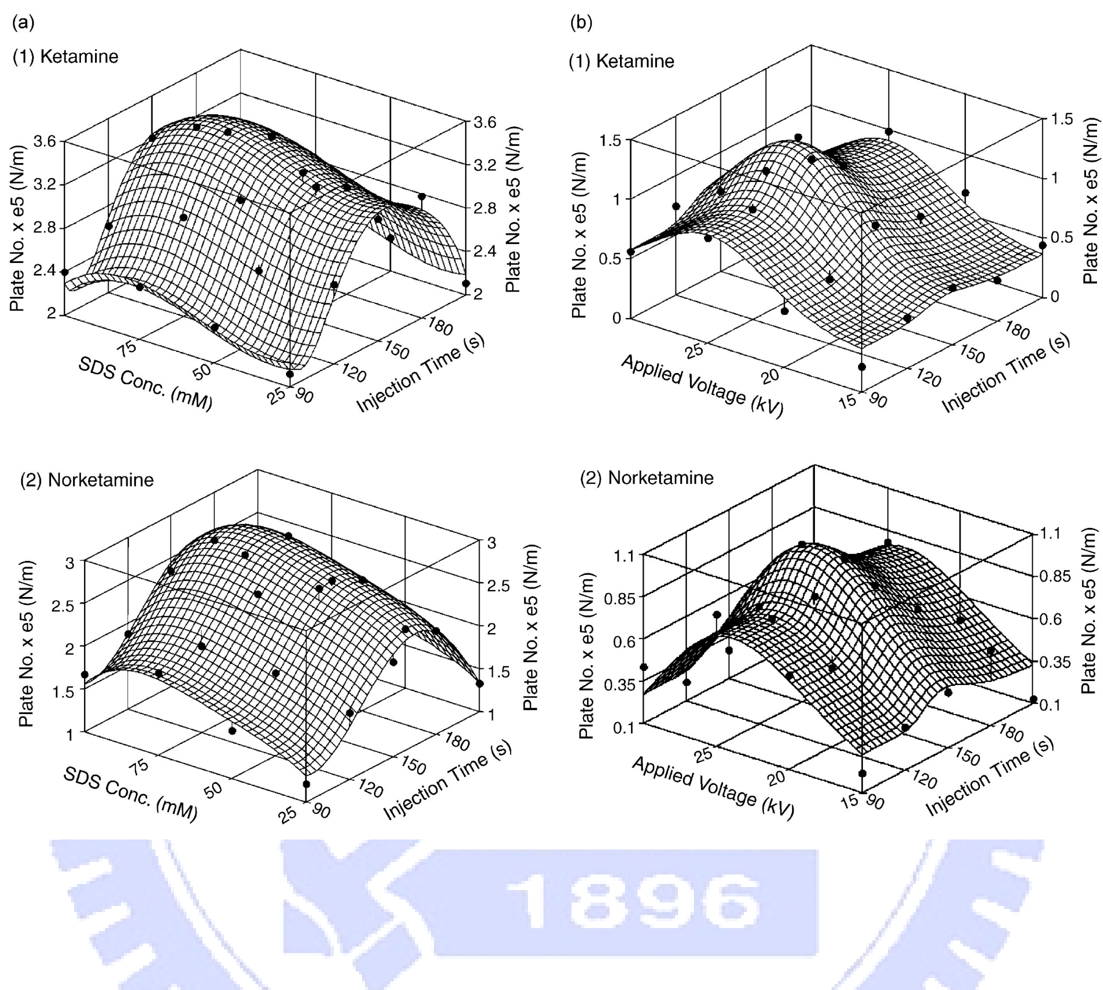

Figure 3.5 Three-dimensional representation of the effects that (a) SDS concentration and injection time, (b) applied voltage and injection time have on the number of theoretical plates for (1) ketamine and (2) norketamine.

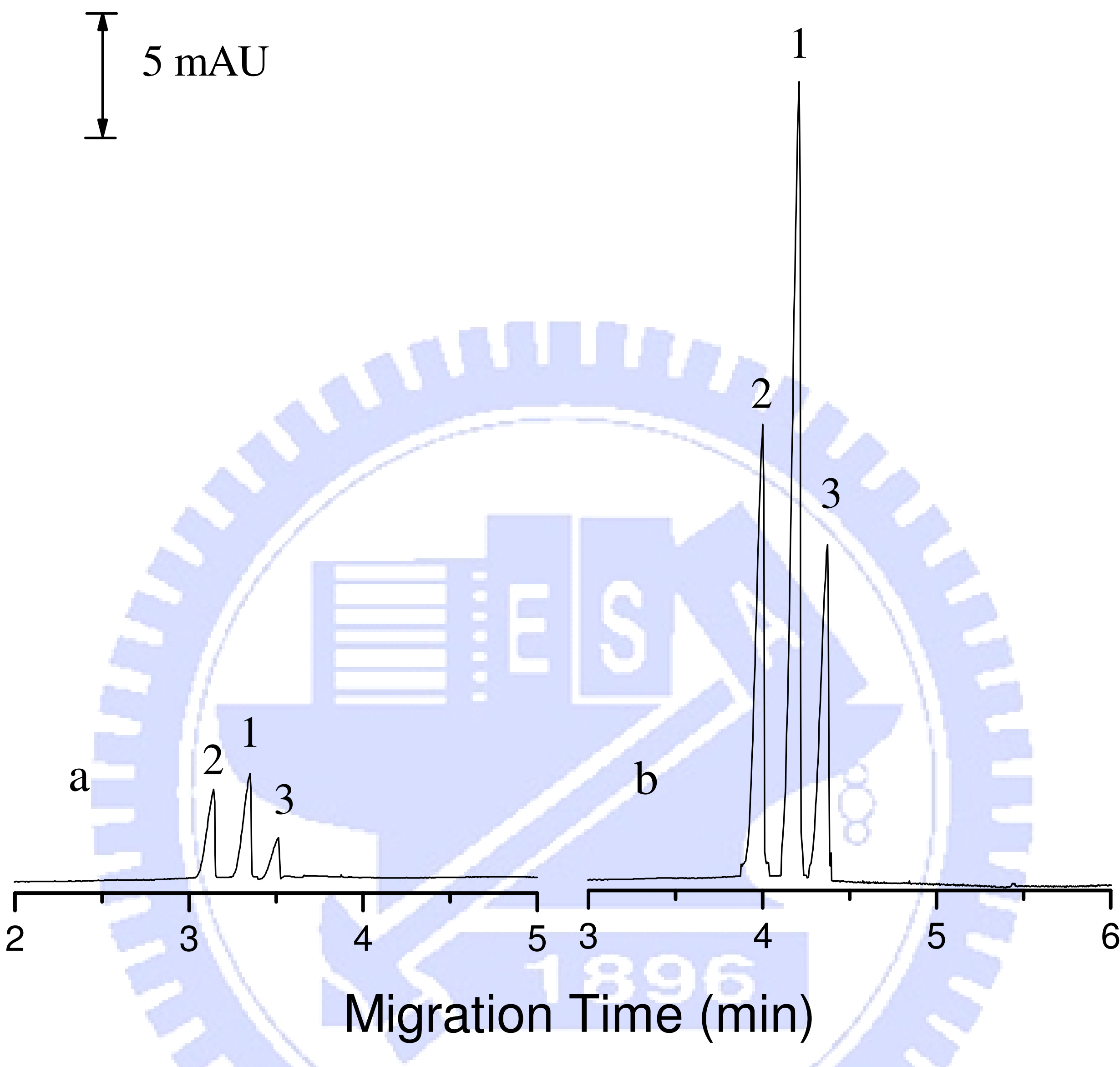

Figure 3.6 Analysis of ketamine and norketamine by (a) MEKC and (b) sweeping MEKC methods. Sample concentrations: (a) 50, 30, and 20 ppm for K (peak 1), NK (peak 2), and K-D_4 (peak 3), respectively and (b) 500, 300, and 200 ppb for K (peak 1), NK (peak 2), and K-D_4 (peak 3), respectively. Other conditions are the same as those in Figs. 3.3 and 3.4.

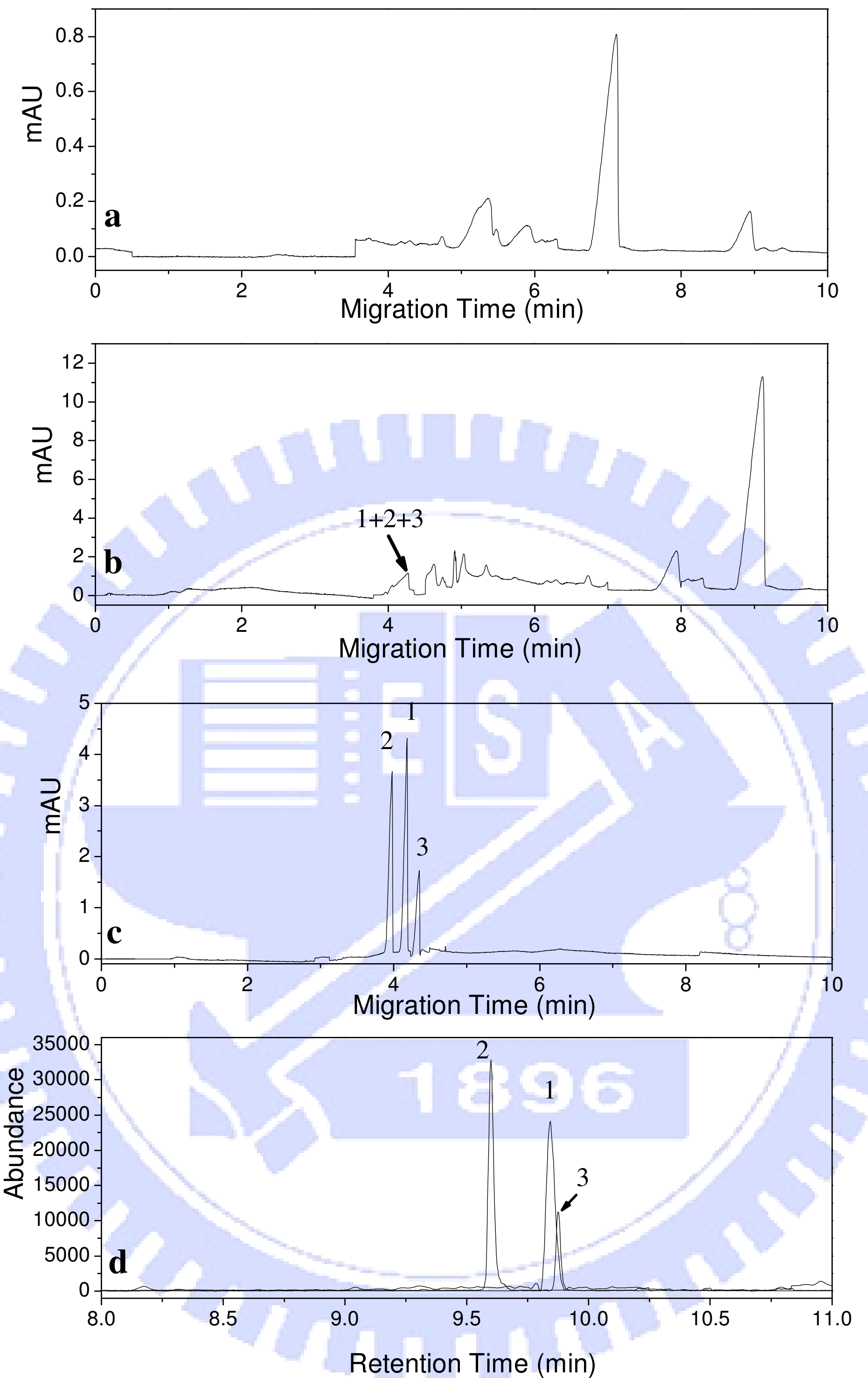

Figure 3.7 Electropherograms and GC/MS traces for the analysis of a urine sample of a suspected ketamine user via (a) MEKC; (b) sweeping MEKC; (c) solid-phase extraction and sweeping MEKC; and (d) selective ion current profile measured using GC/MS methods. Conditions are the same as those in Figs. 3.3 and 3.4.

Chapter 4

Sweeping technique combined with micellar electrokinetic chromatography for the simultaneous determination of flunitrazepam and its major metabolites

4.1. Introduction

Flunitrazepam (Rohypnol), a nitro-containing benzodiazepine, is used as a hypnotic and anesthetic induction agent. It is administered orally or by intravenous injection at doses of 2 mg. It has physiological effects similar to those of other benzodiazepines and has a potency that is ca. 10 times that of benzodiazepine. The illicit use of flunitrazepam usually involves a combination of other drugs, although it may be used alone. It has been used illegally in Asia since the early 1980s. In Taiwan, it appears to be used most frequently in conjunction with alcohol, with which it seems to have a synergistic effect, producing disinhibition and amnesia. This has given flunitrazepam, especially tasteless and odorless solutions, the reputation of being a "date-rape" drug.

Flunitrazepam can be detected in blood, plasma, and urine [1, 2]. Because of its low dosage, biotransformation through *N*-demethylation, and the high volume of distribution, flunitrazepam and its metabolites occur at low blood levels after therapeutic administration [3]. Fig. 4.1 shows the pathway for flunitrazepam metabolism. Two major metabolites of flunitrazepam – 7-aminoflunitrazepam and *N*-desmethylflunitrazepam – can be detected when flunitrazepam is injected or mixed into drinks [4].

Because of the rapid growth in the extent of abuse of flunitrazepam, a simple and consistent method is needed for its determination. Some analytical techniques for detecting flunitrazepam have been reported, including the use of immunoassays [5], high-performance liquid chromatography (HPLC) [6, 7], and gas chromatography/mass spectrometry (GC/MS) [8,9]. From the perspective of qualitative analysis, GC/MS provides additional spectral

information as well as excellent sensitivity. Although GC/MS is capable of providing reliable data that can usually be used as scientific proof in a court of law, the method has disadvantages in that it involves time-consuming derivatization prior to the GC/MS analysis.

In recent years, capillary electrophoresis (CE) has expanded its scope and range in both instrumentation and applications [10]. CE has proven to be a powerful analytical tool for separating charged species in diverse samples because of its many advantageous features, which include high column efficiency, rapid analysis times, and small sample volumes. However, the benefits derived by the high separation efficiency of CE can be overshadowed by its low UV detection sensitivity. Thus, using on-line sample preconcentration to overcome the poor sensitivity of CE has been the focus of a number of investigations [11, 12]. For example, Quirino and Terabe [13] reported that neutral compounds could be concentrated effectively, when the technique of micellar electrokinetic chromatography (MEKC) combined with stacking was utilized. They later reported a sweeping method that can pick and accumulate neutral or charged analytes into a narrow zone by the pseudostationary phase in MEKC [14–16].

In this paper, we report on an approach involving the use of a sweeping technique combined with MEKC for the simultaneous determination of flunitrazepam and its major metabolites, 7-aminoflunitrazepam and *N*-desmethylflunitrazepam. The effects of the buffer pH, buffer concentration, cationic surfactant, organic modifier, and injection length on the analysis are described. We optimized the sweeping MEKC conditions to enhance the detection sensitivity with satisfactory resolution. We also employed the optimized sweeping MEKC method in an examination of a spiked urine sample.

4.2. Materials and methods

4.2.1. Apparatus

CE analysis was performed on a Beckman P/ACE MDQ CE system equipped with a photodiode-array detector (Fullerton, CA, USA). A personal computer, controlled by Beckman Coulter MDQ 32 Karat software was used for data collection. A 60 cm (50 cm to the detector) × 50 μm I.D. fused-silica capillary tube (Polymicro Technologies, Phoenix, AZ, USA) was used. The capillary column was assembled in a cartridge format. The temperature of the capillary tube during electrophoresis was maintained at 25 °C. The electrophoresis separation was performed at an applied voltage of −25 kV. Sample was pressure-injected at 0.5 psi with an extended time. The UV absorption detector was set at 240 nm for sweeping MEKC.

4.2.2. Chemicals

Flunitrazepam, 7-aminoflunitrazepam, and *N*-desmethylflunitrazepam were purchased from Radian International (Austin, TX, USA). Sodium tetraborate was obtained from Sigma (St. Louis, MO, USA). Cetyltrimethylammonium bromide (CTAB) was purchased from Merck (Hohenbrunn, Germany). All other chemicals were analytical grade. Water was purified using a Milli-Q water system (Millipore, Bedford, MA, USA) and filtered through a 0.22-μm filter.

4.2.3. Procedure

New capillaries were conditioned prior to separation by washing with methanol, water, 1 M NaOH, and water for 10 min each. The capillary was flushed between runs with 0.1 M NaOH, methanol, and water for 3 min each. For the sweeping MEKC procedure, the stock solutions were diluted with a buffer solution that did not contain CTAB surfactants. The

borate background solution (BGS) contained an appropriate amount of CTAB and methanol. The BGS was first passed through the capillary for 3 min and the sample solution was then pressure-injected into the capillary. Finally, voltages were applied at negative polarity. Other experimental conditions are described in the Section 4.3.

The SPE cartridges (Oasis MCX, 3 mL/60 mg) were conditioned with 2mL methanol and 2 mL H_2O.A 3-mL urine sample was spiked with 30-μL of a standard solution (10 μg/mL), then mixed with 60 μL pH 5.0 phosphate buffer (1 M) and passed through the cartridge. The cartridge was washed with 2 mL HCl (0.1 M) and 2mL methanol, and was then dried under a vacuum for 10 min. The analytes were eluted with 3 mL dichloromethane/2-propanol /ammonium hydroxide solution (78:20:2, v/v/v). This organic solution was then evaporated to dryness. The residue was dissolved in 300 μL buffer and was used directly for MEKC.

4.3. Results and discussion

4.3.1. Effects of separation conditions for flunitrazepam and its major metabolites

Flunitrazepam and its metabolites (Fig. 4.1) are hydrophobic substances, with a neutral charge in slightly or strongly basic environments, which could interact with micelles. Thus, a sweeping technique using CTAB was employed to achieve online sample concentration [15, 16]. After the voltage was applied with a negative polarity from inlet (cathode), the EOF, under the influence of the cationic CTAB surfactant, moved toward the outlet (anode). Because the velocity of the EOF was higher than that of the CTAB micelle, the analytes stacked at the boundary by the CTAB micelle and moved toward the anode.

When performing the analysis using MEKC, the pH and concentration of the buffer solution were adjusted so as to obtain adequate separation. The migration times of the analytes increased with increasing pH value from pH 9.5 to pH 10.5 with a similar sensitivity enhancement. The peaks also broadened and their heights decreased for electrolyte concentrations lower than 25 mM. The migration time of the analytes was delayed with increasing electrolyte concentration. Taking all of these phenomena into consideration, we conclude that the pH 9.5 buffer with 25 mM electrolyte is the most suitable for the separation.

In the basic buffer solution, flunitrazepam and its metabolites acted as neutral analytes, and migrated with the electroosmotic flow. When they interacted with the positively charged CTAB micelles, however, the decrease in their apparent electrophoretic velocities caused these analytes to become focused. When the CTAB concentration was increased from 10 to 50 mM, separation and peak height improved, suggesting that the sweep effect became more efficient. Nevertheless, when the CTAB concentrations exceeded 50 mM, the separations became poor. These results suggest that the use of 50 mM CTAB provides the best condition for the separation.

Increasing the percentage of methanol in the buffer had a dramatic influence on the

analyte migration time and the peak focusing effect. The results showed that adding an organic solvent to the buffer modified the polarity of the BGS, which further changed the EOF. It also improved the resolution by modifying the partition of the analytes between the solution phase and the micelle phase. The experimental results indicate that adding 30 % methanol to the buffer solution provided the best condition for the separation.

In general, prolonging the sample injection length in sweeping MEKC is advantageous, in terms of achieving better sensitivity for a separation. A long sample zone, however, increases the sweeping time and may have a negative influence on the efficiency of the sweeping procedure. Using the optimal conditions discussed above, we found that an injection length of 151 mm is suitable for the complete separation of all the peaks.

4.3.2. Comparing normal MEKC and sweeping MEKC

Fig. 4.2 depicts the results of normal MEKC and the sweeping MEKC separation of flunitrazepam, 7-aminoflunitrazepam, and *N*-desmethylflunitrazepam under optimized conditions. Fig. 4.2a was obtained when the sample solution was the same as the running buffer, but did not contain micelles. The concentration of each analyte was 100 μg/mL. Fig. 4.2b was obtained in a manner similar to that of Fig. 4.2a, but the injection length was 151 mm and the sample concentration was diluted 100-fold. The sensitivity enhancement in terms of peak heights (SE_{height}) for the three analytes was calculated. Flunitrazepam, 7-aminoflunitrazepam, and *N*-desmethylflunitrazepam had ca. 110-, 140-, and 200-fold enhancements in their detection sensitivities, respectively.

Table 4.1 presents the calibration lines, coefficient of determination (r^2), limits of detection (LODs), migration times, and RSDs for the three analytes using MEKC and sweeping MEKC techniques. For analyses conducted using the normal MEKC procedure, the LODs were in the low μg/mL range. When the sweeping MEKC procedure was used, the LODs were less than 13.4 ng/mL. Table 4.1 also presents the reproducibility of the migration

times and peak area. The RSD for the migration time was less than 0.62 % for either separation procedure. The RSD of the peak area was also less than 4.10 %. According to these results, both processes are acceptable separation methods, but the sweeping MEKC procedure is superior to MEKC in detection sensitivity.

Fig. 4.3 illustrates the use of the sweeping MEKC method in analyzing a urine sample spiked with flunitrazepam and its metabolites. The separation of these analytes in urine was adequate. The total separation time was less than 12 min. Thus, sweeping MEKC can be used as a rapid screening method for the analysis of flunitrazepam and its major metabolites.

4.4. Conclusion

In conclusion, we report that a sweeping technique combined with MEKC permits the simultaneous determination of flunitrazepam and its major metabolites through a process that is easily performed, and does not require a derivatization step. The optimized parameters for the sweeping MEKC method were: running buffer, 25 mM borate buffer (pH 9.5); CTAB, 50 mM; organic modifier, 30 % MeOH (v/v); injection length, 151 mm. The LODs ranged from 5.6 to 13.4 ng/mL. Accordingly, sweeping in conjunction with MEKC represents an alternative approach with enhanced sensitivity for analyzing flunitrazepam and its major metabolites.

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Table 4.1 Calibration lines, coefficient of determination (r^2), limits of detection (LODs), migration times, and values of RSD for flunitrazepam, 7-amino-flunitrazepam, and *N*-desmethylflunitrazepam using the MEKC and sweeping MEKC techniques

	flunitrazepam	**7-amino-flunitrazepam**	***N*-desmethyl-flunitrazepam**
<u>Normal MEKC</u>			
Calibration line[a]	$y = 101\ x + 224$	$y = 130\ x - 121$	$y = 71.5\ x - 228$
Coefficient of determination	$r^2 = 0.997$	$r^2 = 0.999$	$r^2 = 0.997$
LOD ($S/N = 3$, μg/mL)	1.87	0.52	1.74
Migration time (min)	10.54	7.17	13.10
RSD (%; $n = 5$)			
Ⅰ. Migration time	0.55	0.25	0.62
Ⅱ. Peak area	2.85	2.04	1.48
<u>Sweeping MEKC</u>			
Calibration line[b]	$y = 11.5\ x - 682$	$y = 28.2\ x - 853$	$y = 24.7\ x - 3.1 \times 10^3$
Coefficient of determination	$r^2 = 0.996$	$r^2 = 0.999$	$r^2 = 0.998$
LOD ($S/N = 3$, ng/mL)	13.4	5.6	12.0
Migration time (min)	8.48	6.21	10.24
RSD (%; $n = 5$)			
Ⅰ. Migration time	0.39	0.28	0.51
Ⅱ. Peak area	1.14	2.01	4.10
SE_{height}[c]	110	140	200

[a] Calibration line (10–200 μg/mL): peak area (arbitrary units) = slope × concentration (μg/mL) + y-intercept.

[b] Calibration line (50–1000 ng/mL): peak area (arbitrary units) = slope × concentration (ng/mL) + y-intercept.

[c] SE_{height} = (peak height obtained with sweeping MEKC/peak height obtained with MEKC)·dilution factor.

7-aminoflunitrazepam

7-acetylflunitrazepam

flunitrazepam

N-demethylflunitrazepam

3-hydroxylflunitrazepam

Glucuronide conjugation

Figure 4.1. The major metabolic pathway for the detoxification of flunitrazepam in humans.

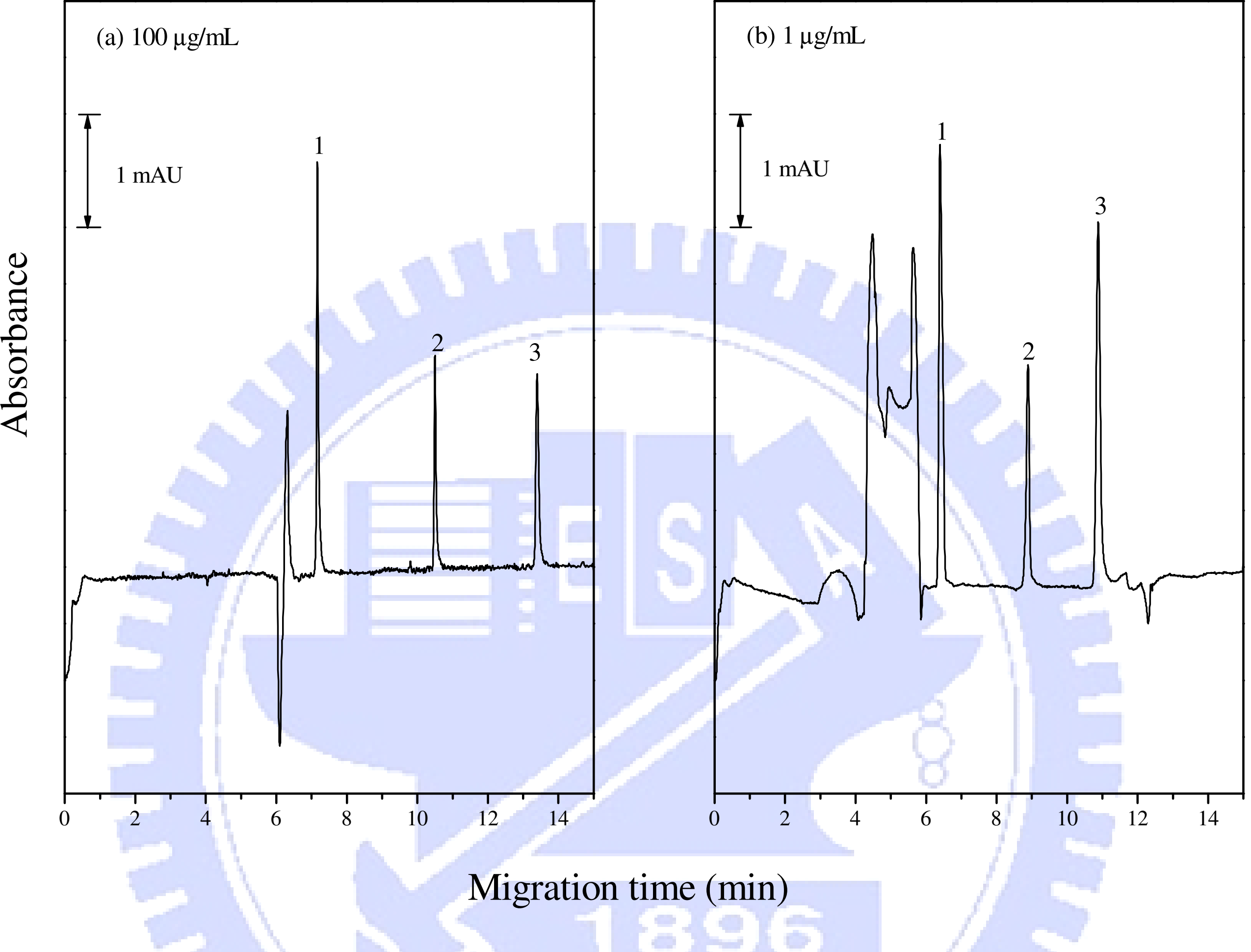

Figure 4.2. Normal MEKC and sweeping MEKC analysis of flunitrazepam and its major metabolites. (a) MEKC analysis. Analyte concentration: 100 µg/mL; injection length: 1.51 mm. (b) Sweeping MEKC analysis. Analyte concentration: 1 µg/mL; injection length: 151 mm. Conditions: capillary, 60 cm long (50 cm to detector), 50 µm I.D.; buffer solution: 25 mM borate buffer (pH 9.5), 50 mM CTAB, 30 % CH_3OH (v/v), conductivity 7.28 mS/cm; sample matrix: 25 mM borate buffer (pH 9.5); separation voltage: −25 kV; UV detection at 240 nm. Peak identification: peak 1, 7-aminoflunitrazepam; peak 2, flunitrazepam; peak 3, *N*-desmethylflunitrazepam.

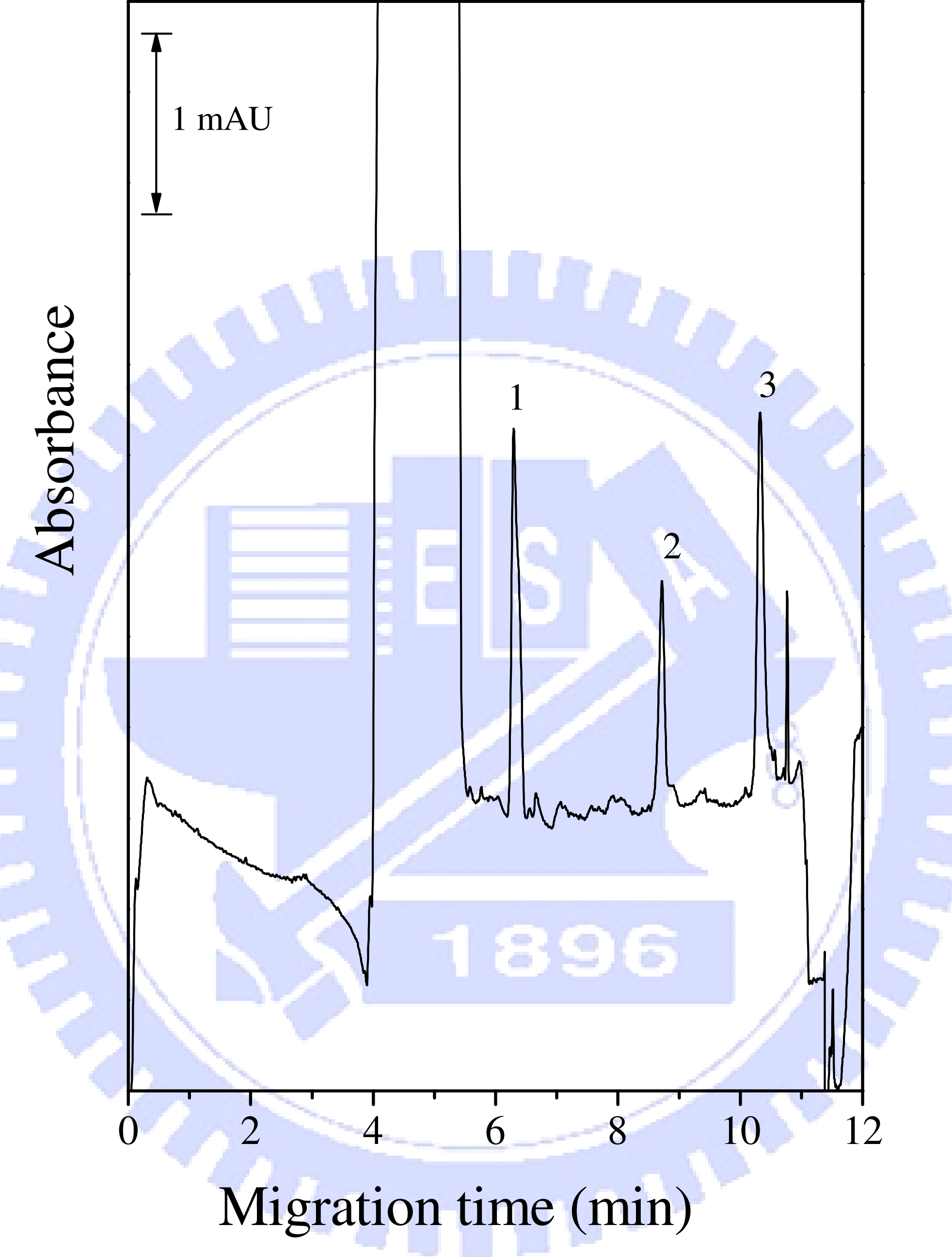

Figure 4.3. Sweeping MEKC electropherogram of a spiked urine sample. Analyte concentration: 0.3 μg spiked in a 3-mL urine sample before SPE extraction. The other conditions are the same as in Fig. 4.2.

Chapter 5

Analysis of a wide variety of illicit drugs using cation-selective exhaustive injection/sweep-micellar electrokinetic chromatography

5.1. Introduction

Cocaine, heroin, and opiates are among the most widely abused illicit drugs in America and Asia. Their abuse has increased dramatically during the past three decades. Cocaine is one of the most potent of the naturally occurring stimulants of the central nervous system. Heroin is processed from morphine, a naturally occurring substance extracted from the seedpods of the Asian poppy plants. Heroin usually appears as a white or brown powder, and affects the central nervous system in the same manner as opiates. Heroin and opiates are commonly abused substances that clinical and forensic laboratories are often asked to identify within urine samples [1–6]. Morphine can be eliminated from the human body without metabolization, and it is also the main metabolite of codeine and heroin. In order to identify the correct origin form heroine or codeine, the 6-acetylmorphine is considered the best marker for heroin use because there is no known natural source; it is not a codeine metabolite. Several publications describe the development of methods for the detection and quantification of cocaine, heroin, and opiate drugs in powders and biological matrices, such as blood and urine [7–10]. Because of the rapid growth in the abuse of these substances, simple, economical, rapid, and consistent methods for their determination are necessary for both forensic research and clinical analysis. As a result, many procedures and methods have been developed for the analysis of cocaine, heroin, 6-acetylmorphine, morphine, and codeine (Fig. 5.1), including those using immunoassays [11,12], high-performance liquid chromatography (HPLC) [1,13–16], gas chromatography [17,18], high-performance liquid chromatography/mass spectrometry (LC/MS) [19], and gas chromatography/mass spectrometry (GC/MS) [20 – 24]. Of these techniques, GC/MS is the most popular and powerful one for the analyzing these

abused drugs. Unfortunately, this method has its drawbacks: a derivatization step and an additional amount of sample handling are required, and, thus, it may not be practical if hundreds of samples require analysis.

Capillary electrophoresis (CE) is a separation technique that is characterized by extremely high efficiencies and short analysis times. The use of capillary-scale columns provides several advantages over conventional-scale separation methods [25, 26]. The minimal separation volumes can result in mass detection limits as low as the femtomole level with appropriate detection [27, 28]. Because of volumetric restrictions, however, the corresponding concentration detection limits are not as impressive, but they can be improved when using laser-induced fluorescence (LIF) detection or increasing the sample capacity of the system. Unfortunately, the LIF method requires rather expensive and somewhat complex hardware [29, 30]. To overcome this limitation, while still enhancing the detectability, several on-line sample preconcentration techniques have been developed and applied to CE systems, including micellar electrokinetic chromatography (MEKC). Stacking [31] and sweeping [32] techniques both can be effective at significantly improving the detection sensitivity in MEKC, especially under acidic conditions.

In this study, we established and validated a screening procedure for the analysis of cocaine, heroin, and opiates in powders and urine samples using cation-selective exhaustive injection/sweeping micellar electrokinetic chromatography (CSEI-sweep-MEKC) [33–35]. This method does not require any derivatization procedure and permits the detection of very small amounts of cocaine, heroin, and opiates in small powder and urine samples. It provides a more sensitive method of detection than merely using sweeping alone. We determined the optimal separation conditions for this method such that we decreased both the amount of sample consumed and the separation time. We applied the optimized method to the successful determination of these compounds in powders (after liquid–liquid extraction) and in spiked urine samples (after solid-phase extraction).

5.2. Experimental

5.2.1. Chemicals

Cocaine (CC, 1.0 mg/mL in acetonitrile), heroin (HR, 1.0 mg/mL in acetonitrile), 6-acetylmorphine (AM, 1.0 mg/mL in acetonitrile), morphine (MP, 1.0 mg/mL in methanol), and codeine (CD, 1.0 mg/mL in methanol) were obtained from Radian International. Disodium hydrogen phosphate and sodium hydroxide were obtained from Fluka. Sodium dodecyl sulfate (SDS) was obtained from Sigma (St.Louis, MO, USA). Citric acid, methanol, and acetonitrile were purchased from Merck–Schuchardt. All chemicals were obtained in analytical grade. The urine samples were donated by the Command of the Army Force of Military Police, Forensic Science Center, Taiwan.

5.2.2. Apparatus

CE analysis was performed on a P/ACE MDQ CE system equipped with a photodiode-array detector (Beckman Instruments, Fullerton, CA, USA); the personal computer was run using Beckman – Coulter MDQ 32 Karat software. A 60 cm (50 cm to the detector) 50 μm I.D. fused-silica capillary tube (Polymicro Technologies, Phoenix, AZ, USA) was used. The capillary column was assembled in a cartridge format. The temperature of the capillary tube during electrophoresis was maintained at 25 °C. The electrophoresis separation was performed with an applied voltage of –20 kV. Samples were injected electrokinetically at a potential of 10 kV and were focused through sample stacking for a long period of time. The resulting stacked zones were then focused for a second time by sweeping using the pseudostationary phase. Focused zones were separated using MEKC. The UV absorption detector was set at 240 nm during CSEI-sweeping-MEKC.

5.2.3. Capillary electrophoresis procedures

The new capillaries were conditioned prior to separation by washing them sequentially with methanol (10 min), purified water (2 min), 1 M NaOH (10 min), and purified water (10 min). The capillary was flushed between runs sequentially with 0.1 M NaOH, methanol, and water (3 min each). For the CSEI-sweep-MEKC procedure, the capillary was initially conditioned with a non-micellar buffer [50 mM citric acid/disodium hydrogen phosphate (pH 4.0)], followed by injection of a high-conductivity buffer [HCB: 100 mM citric acid/disodium hydrogen phosphate (pH 4.0) containing 10 % acetonitrile (v/v)], and finally injection of a short plug of water. Using electrokinetic injection (at a positive polarity), cationic analytes were prepared in a low-conductivity matrix or water. The cationic analytes entered the capillary through the water plug at high velocities and then the velocities decreased gradually such that the analytes focused or stacked at the boundary between the water and the HCB. Both ends of the capillary were inserted into the background solution (BGS) containing anionic micelles (SDS) and then the voltage was switched to negative polarity. The micelles from the cathodic vial entered the capillary to sweep the stacked introduced analytes to narrow their bands. Eventually, the separation was performed using MEKC [34].

5.2.4. Stock standard solution

A standard solution (5-μg/mL) was prepared by mixing a stock solution (1.0 mg/mL) in DI water. Sample solutions of various concentrations were prepared by diluting the standard solutions with buffer (1 mM, pH 2.2 citric acid/disodium hydrogen phosphate buffer).

5.2.5. Liquid–liquid extraction of tablets

The powder (ca. 1.5 mg) was dissolved in methanol (1.0 mL) under sonication for 30 min. The mixture was centrifuged for 10 min at 3000 rpm at room temperature; the upper layer was collected and diluted 1000-fold with 1 mM citric acid/disodium hydrogen

phosphate buffer (pH 2.2). The sample solutions were then filtered through a Polypore polypropylene membrane filter (0.2 μm; Alltech, Laarne, Belgium) before their direct use in the CE system. Fig. 5.2A provides detailed procedures.

5.2.6. Solid-phase extraction of urine samples

Oasis® HLB solid phase extraction cartridges (3 cc/60 mg) were obtained from Waters (Milford, MA, USA). The cartridges were conditioned with methanol (2 mL) and 10 mM phosphate buffer (2 mL, pH 8.0). The urine sample (2 mL) spiked with mixed standard solution (5 μg/mL, 200 μL) was loaded into the column. The column was washed with 10 % methanol (2 mL) and then dried under vacuum for 10 min. The analytes were eluted using methanol (3 mL). The eluants were evaporated to dryness at 40 °C under nitrogen. The residue was reconstituted in water (conductivity: ca. 55.6 μS/cm; 2 mL). Fig. 5.2B provides detailed procedures.

5.3 Results and discussion

5.3.1 Optimizing separation conditions

Figs. 5.3A–D display the electropherograms of the analytes under different separation conditions. In frame A, to investigate the effect of the pH of the buffer solution, we fixed the concentrations such that the CE buffer was 100 mM citric acid/disodium hydrogen phosphate (i.e., HCB) containing acetonitrile 15% (v/v) and the background solutions were 50 mM citric acid/disodium hydrogen phosphate containing 150 mM SDS. The values of pH of the buffer solutions in electropherograms a–d were 2.2, 3.0, 4.0, and 5.0, respectively. The sample concentration for each analyte was 500 ng/mL. The optimum pH was 4.0 (i.e., in electropherogram c). We also investigated the effects that different concentrations of SDS (Fig. 5.3B) had on the separation. When the concentration of SDS increased (cf. electropherograms e–g: 50, 100, and 150 mM, respectively), the separations of the analytes improved because of the increased degree of interaction between the analytes and the SDS micelles. The separation of the analytes was optimized (Fig. 5.3B-g) at an SDS concentration of 150 mM. Adding a small portion of organic solvent can modify the polarity difference between the solution phase and the micellar phase; consequently, it will also change the EOF. Figure 5.3C depicts the results we obtained after gradually increasing the percentage of acetonitrile/water [electropherograms h–k: 0, 10, 15, and 20% (v/v), respectively], under otherwise identical experimental conditions, in an attempt to improve the resolution of the analytes. Unfortunately, the migration time increased and the peak focusing effect had a tremendous influence. Our results suggest that the presence of 10% acetonitrile/water (v/v) in the buffer solution provides the best conditions for separation (i.e., Fig. 5.3C-i). Finally, we tested the injection time (frame D; electropherograms l–n: 300, 600, and 900 s, respectively). We found that an injection time of 600 s allowed complete separation of all of the peaks (i.e., Fig. 5.3D-m). Accordingly, we achieved the optimal separation of the analytes when using

high-conductivity buffer [100 mM citric acid/disodium hydrogen phosphate buffer (pH 4.0)] containing 10% acetonitrile (v/v) and 150 mM SDS in conjunction with electrokinetic injection for 600 s.

5.3.2. Comparing the separations using normal MEKC, sweeping-MEKC, and CSEI-sweep-MEKC

Figure 5.4 displays a comparison of the results obtained from separations using normal MEKC, sweeping-MEKC, and CSEI-sweeping-MEKC (electropherograms a – c: 200 μg/mL, 1.0 μg/mL, and 50 ng/mL). The concentration effect in terms of peak height (SE_{height}) for preservatives is defined [36] as

$$SE_{height} = \frac{H_{stack}}{H} \times \frac{C}{C_{stack}}$$

where Hstack is the peak height of the analytes by sweeping, H is the peak height of the standard analytes, Cstack is the concentration of the analytes by sweeping, and C is the concentration of the standard analytes. The normal MEKC hydrodynamic injection time was 3 s at 0.5 psi (Fig. 5.4A), the sweeping-MEKC hydrodynamic injection time was 300 s at 0.5 psi, and the CSEI-sweeping-MEKC electrokinetic injection time was 600 s at 10 kV. Clearly, CSEI-sweeping-MEKC displays a notable increasing concentration in response over those of the normal MEKC and sweeping-MEKC modes; its analysis times for CC, HR, AM, MP, and CD were all less than 10 min. Table 5.1 lists the values of SE, which were 2200–3200-fold improvements for each of these analytes.

Table 5.1 presents the regression equations and the values of r^2, LOD, LOQ, migration times, RSD, and SE for the separations of cocaine, heroin, and opiates when using the CSEI-sweeping-MEKC technique. Especially, the values of SE were 2,200–3,200-fold improvements for each of these analytes (CSEI-sweep-MEKC compared with normal MEKC). Our results indicate that CSEI-sweeping-MEKC provides better results for the separation of

these samples than do the normal MEKC and sweeping-MEKC modes. For analyses conducted using the CSEI-sweeping-MEKC procedure, we obtained LOD values for cocaine, heroin, 6-acetylmorphine, morphine, and codeine of 0.13, 0.40, 0.43, 0.31, and 0.37 ng/mL, respectively. As a result of these favorable findings, we were encouraged to use this CSEI-sweeping-MEKC technique for the simultaneous determination and quantitation of cocaine, heroin, and opiates in powder and urine samples.

5.3.3. Simultaneous determination and quantitation of cocaine, heroin, and opiates in powder samples

We used GC/MS (data not shown) and CE (Fig. 5.5) to analyze different powders that had been seized from illicit markets in Taiwan. In the CE analyses of the suspect powders I, II, and III, performed in conjunction with simple liquid–liquid extraction, we detected morphine (Fig. 5.5B), heroin (Fig. 5.5C), and cocaine (Fig. 5.5D), respectively. Although we can readily identify each analyte from its migration time relative to those of the standards (Fig. 5.5A), we confirmed our results by spiking cocaine, heroin, and morphine standard solutions (200 ng/mL each) into the extracts of the three suspect powders. As is evident (data not shown), the heights of the three peaks increased quite obviously and their migration times were identical to those of the standard solutions. Furthermore, we determined directly that the purities of morphine, heroin, and cocaine in the suspect powders were 5.2, 3.3, and 15.4%, respectively (Table 5.2). Thus, CSEI-sweeping-MEKC can be used successfully to simultaneously determine and quantify cocaine, heroin, and opiates in powder samples, with the results of these analyses being the same as those obtained using GC/MS.

5.3.4. Simultaneous determination and quantitation of cocaine, heroin, and opiates in urine samples

Figure 5.6 illustrates the results of using both CE and CSEI-sweeping-MEKC in

conjunction with SPE for the analyses of urine samples, which had been spiked with standard solutions, obtained from volunteers. Whereas the CE analyses conducted using urine samples that had not been extracted were very poor (Fig. 5.6A), those performed using CSEI-sweeping-MEKC combined with simple SPE resulted (Fig. 5.6B) in clearly distinguishable peaks for each analyte. Although we could also successfully determine the presence of CC, HR, AM, MP, and CD by using GC/MS in conjunction with SPE and derivatization (data not shown), the extraction and analysis times were more than three times longer than those required for the corresponding CSEI-sweeping-MEKC analyses. We believe that the CSEI-sweeping-MEKC method is a successful, versatile, and superior approach to the analysis of these analytes.

5.4. Conclusion

We have demonstrated that the CSEI-sweeping-MEKC technique can be used successfully for the simultaneous determination and quantitation of cocaine, heroin, and opiates in powder and urine samples. The CSEI-sweeping-MEKC method is a useful means of preconcentrating analytes from biological matrices prior to their analyses. The performance of this method depends upon the selectivity required and would require optimization during validation of a particular analytical system. The optimized conditions we obtained involve using 100 mM citric acid/disodium hydrogen phosphate buffer (pH 4.0) containing 10% acetonitrile (v/v) and 150 mM SDS in conjunction with electrokinetic injection for 600 s. Accordingly, on-line preconcentration performed in conjunction with MEKC is an excellent method that is readily adaptable for use in forensic toxicology.

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Table 5.1. Regression equations and values of r^2, LOQ, LOD, migration times, RSD, and stacking efficiency (SE) in term of peak height for cocaine (CC), heroin (HR), 6-acetylmorphine (AM), morphine (MP), and codeine (CD) for their separations using the CSEI-sweeping-MEKC technique.

	CC	HR	AM	MP	CD
Line of best fit[a]	$y = 363.9x + 704.1$	$y = 85.4x + 4102.3$	$y = 169.7x + 6927.4$	$y = 289.8x + 120.1$	$y = 206.6x + 1124.7$
Coefficient of determination (r^2)	0.9969	0.9961	0.9946	0.9937	0.9927
LOD (ng/mL; $S/N = 3$)	0.13	0.40	0.43	0.31	0.37
LOQ (ng/mL; $S/N = 10$)	0.45	1.36	1.43	1.05	1.25
Migration time (min)	6.95	7.07	7.79	9.33	8.13
RSD% (1 µg/mL; n =5)					
I. migration time	0.32	0.30	0.34	0.48	0.37
II. peak area	2.31	3.26	2.55	3.02	4.78
SE_{height}[b]	2400	2200	3100	3200	3000
SE_{height}[c]	46	50	46	47	43

[a]: Line of best fit (50 ng/mL-1000 ng/mL): peak area (arbitrary units) = slope × concentration (ng/mL) + y-intercept

[b]: Peak height (CSEI-sweep-MEKC)/peak height (normal MEKC) × dilution factor.

[c]: Peak height (CSEI-sweep-MEKC)/peak height (sweep-MEKC) × dilution factor.

Table 5.2 Identified compounds within three suspect powders and their purities.

Suspect powder	Identified compound	Purity (%)[a]
I	Morphine	5.2
II	Heroin	3.3
III	Cocaine	15.4

[a]: Purity (%) = [peak area of suspect powder (20 μg/mL)/peak area of standard (20 μg/mL)] × 100 %

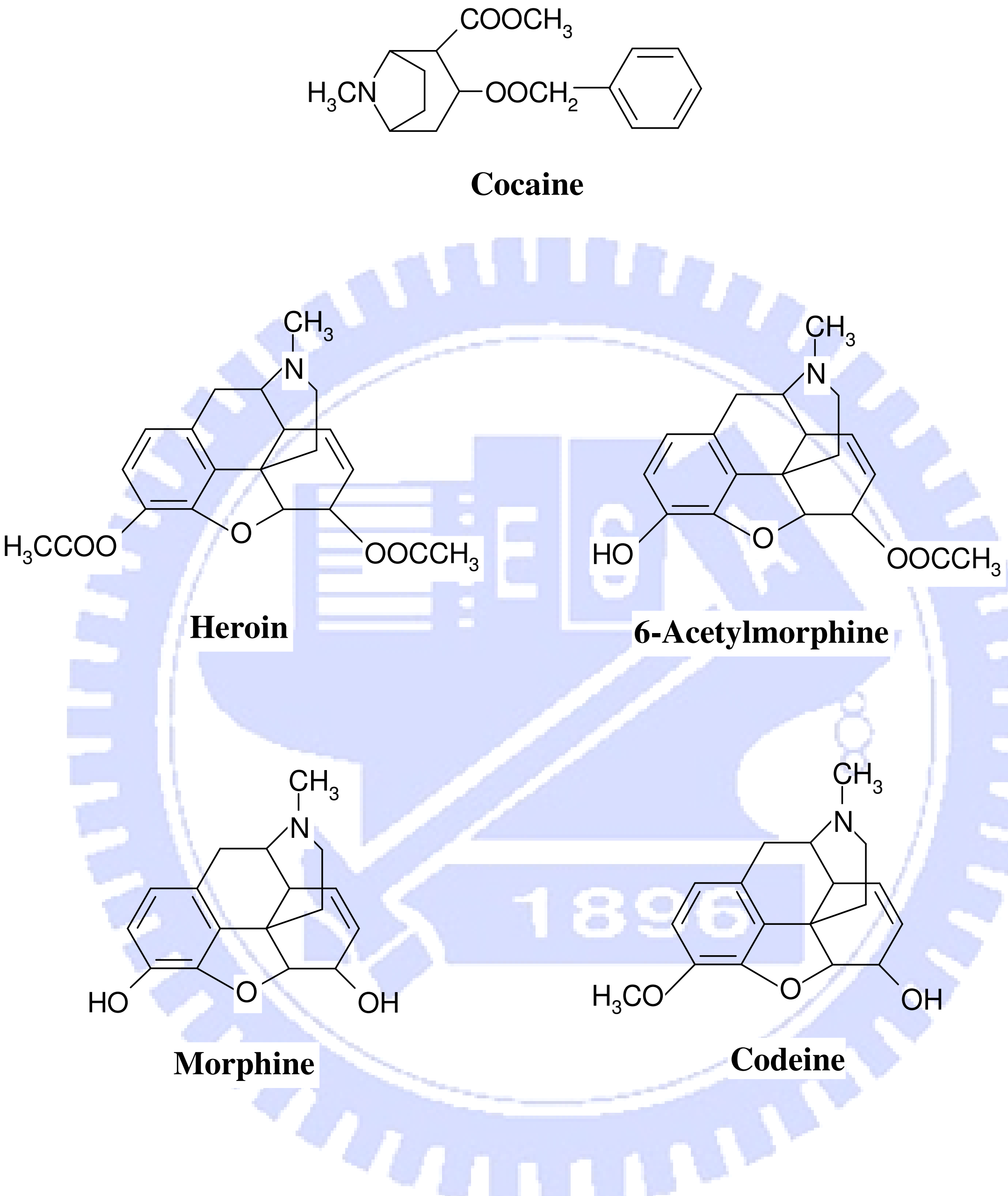

Figure 5.1. Structures of cocaine, heroin, and the major human metabolites of opiates.

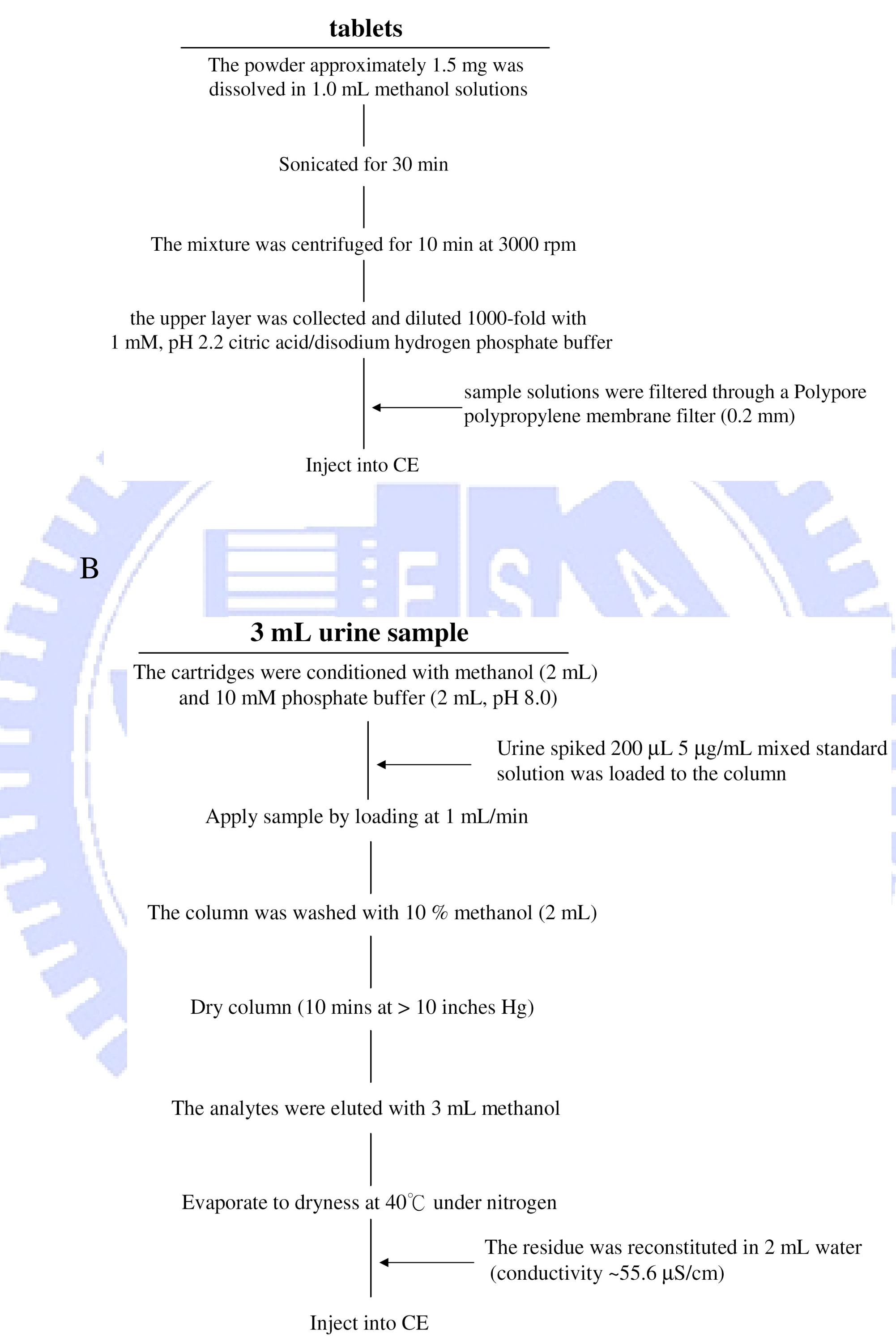

Figure 5.2. Procedures for the preparation of analytical samples from (A) tablets (through liquid–liquid extraction) and (B) urine (through solid phase extraction).

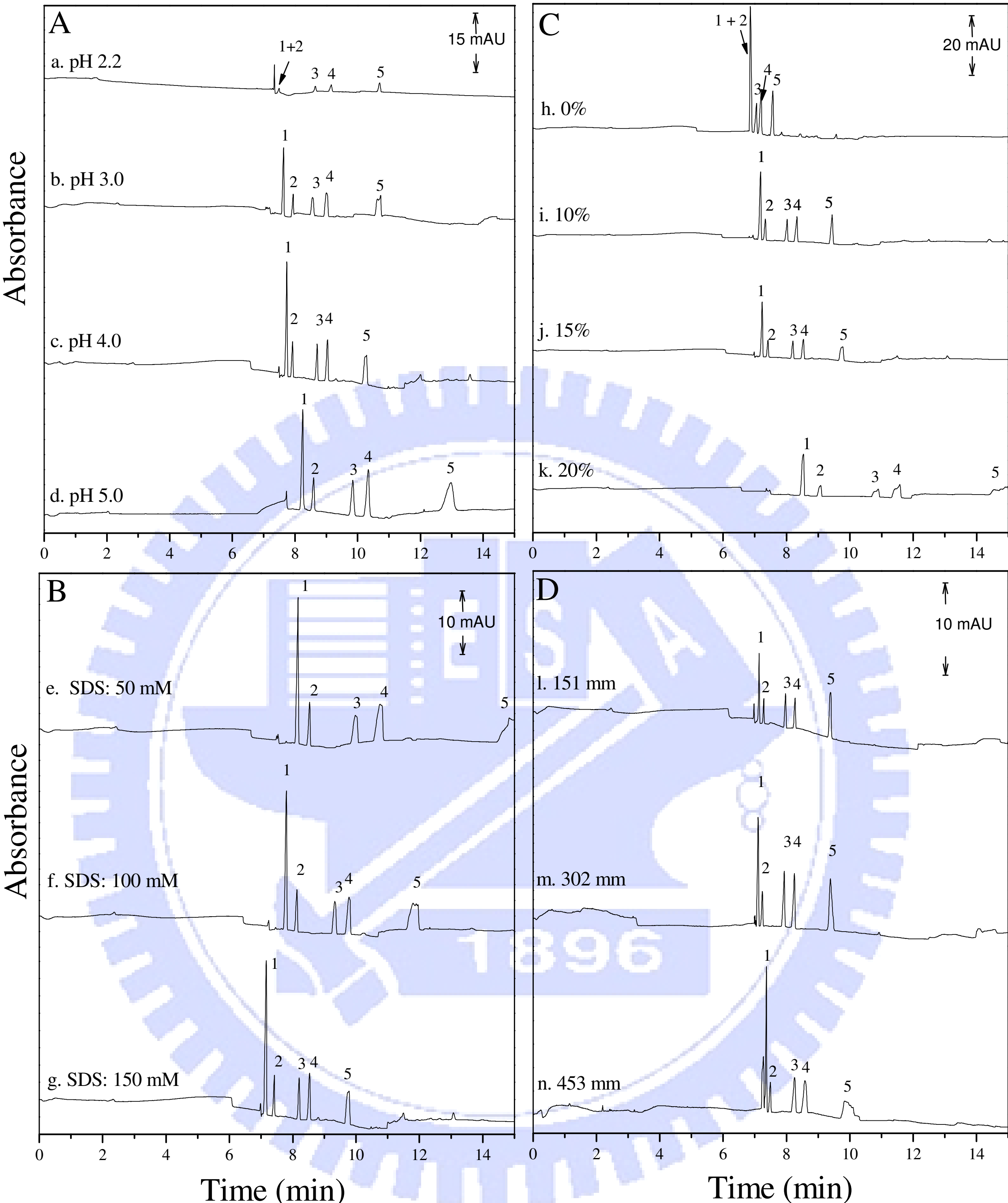

Figure 5.3. Effects of different parameters on CE separation. Conditions: capillary, 60 cm (50 cm to detector), 50 μm I.D.; applied voltage, –20 kV; high-conductivity buffer (HCB), 100 mM citric acid/disodium hydrogen phosphate; sample solution, 1 mM citric acid/disodium hydrogen phosphate (pH

2.2); sample concentrations: 500 ng/mL each. (A) 150 mM SDS; 15% acetonitrile; electrokinetic injection length, 151 mm; pH of HCB: (a) 2.2, (b) 3.0, (c) 4.0, and (d) 5.0. (B) HCB: pH 4.0; 15 % acetonitrile; electrokinetic injection length: 151 mm; SDS concentrations: (a) 50, (b) 100, and (c) 150 mM. (C) HCB: pH 4.0; 150 mM SDS; electrokinetic injection length, 151 mm; acetonitrile content (v/v): (h) 0, (i) 10, (j) 15, and (k) 20%. (D) HCB: pH 4.0; 150 mM SDS; 10% acetonitrile; electrokinetic injection lengths: (l) 151, (m) 302, and (n) 453 mm.

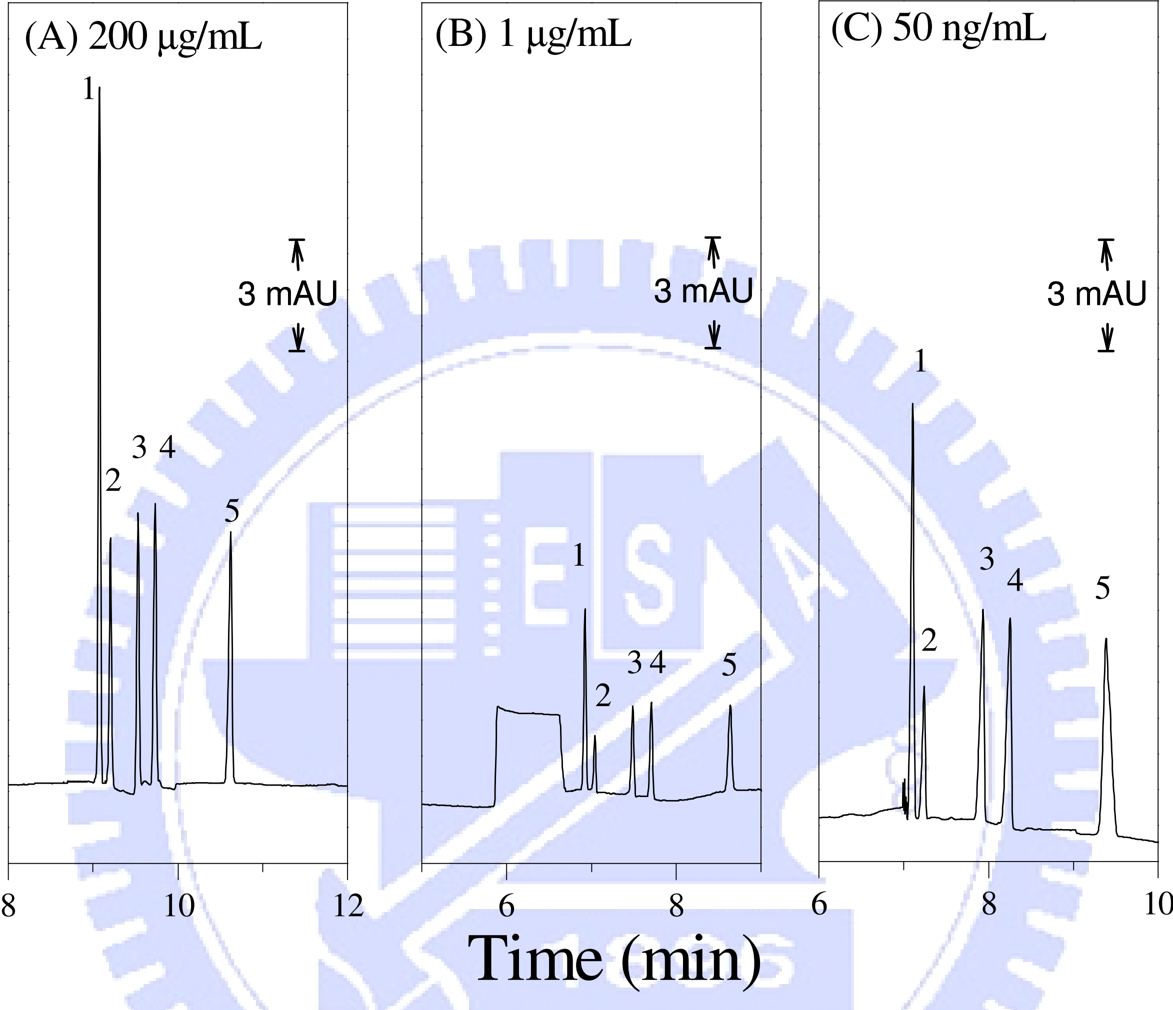

Figure 5.4. Comparison of the CSEI-sweeping-MEKC, sweeping-MEKC, and normal MEKC methods. (A) Normal MEKC. Sample concentrations: 200 μg/mL; hydrodynamic injection: 3.0 s. (B) Sweeping-MEKC. Sample concentrations: 1 μg/mL; hydrodynamic injection: 151 mm. (C) CSEI-sweeping-MEKC. Sample concentrations: 50 ng/mL; electrokinetic injection: 10 kV, 302 mm.

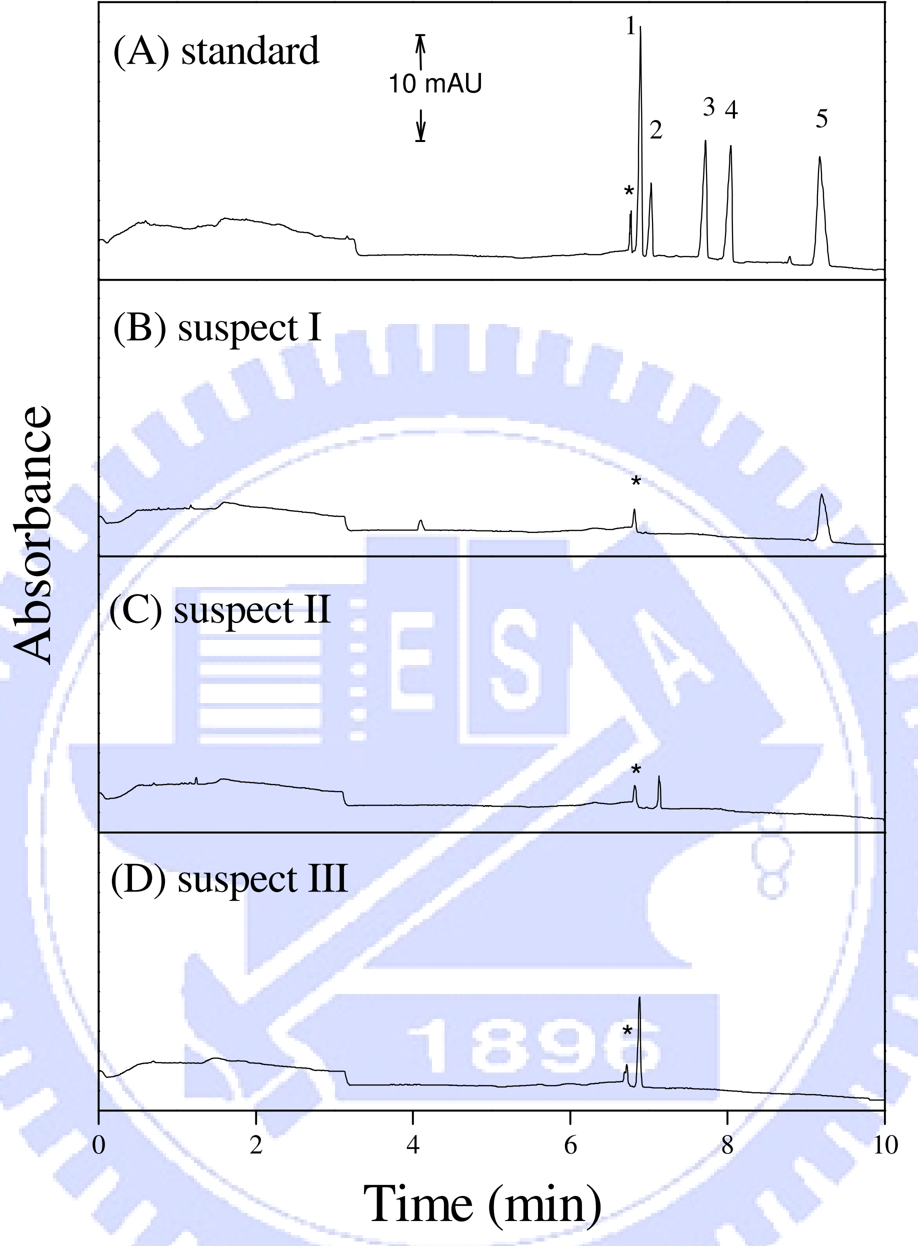

Figure 5.5. CSEI-sweeping-MEKC electropherograms of (A) the standard sample, (B) suspect sample I, (C) suspect sample II, and (D) suspect sample III. Analytes: peak 1, cocaine (300 ng/mL); peak 2, heroin (100 ng/mL); peak 3, 6-acetylmorphine (100 ng/mL); peak 4, codeine (100 ng/mL); peak 5, morphine (100 ng/mL); *, system peak.

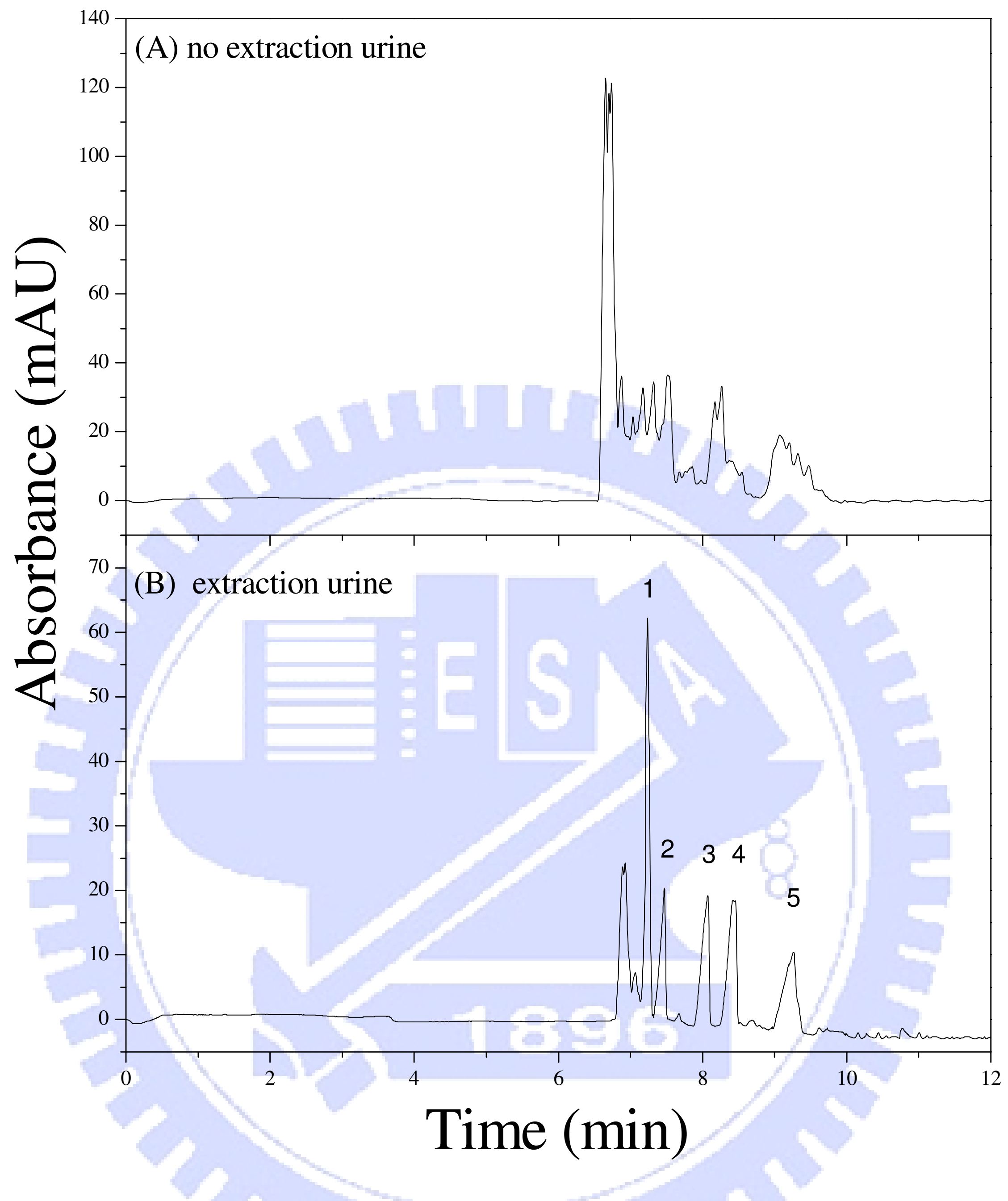

Figure 5.6. CSEI-sweeping-MEKC electropherogram of (A) unextracted urine (1/50 urine; conductivity: ca. 55.6 μS/cm), (B) solid-phase-extracted urine. Spiked standards: cocaine (peak 1, 800 ng/mL); heroin (peak 2, 250 ng/mL); 6-acetylmorphine (peak 3, 150 ng/mL); codeine (peak 4, 150 ng/mL); morphine (peak 5, 150 ng/mL).

Chapter 6

On-Chip Micellar Electrokinetic Chromatographic Separation of Amphetamine, Methamphetamine, and Ephedrine with Electrochemical Detection

6.1. Introduction

The detection of amphetamine, methamphetamine, and ephedrine is of great importance in toxicological, clinical, and forensic analysis because in many countries they are the most widely abused illicit drugs. The abuse of these substances, which exhibit potent stimulating effects on the central nervous system (CNS), has increased dramatically during the past three decades. They are usually sold as white or brown powders, but clinical and forensic laboratories are often asked to identify their presence in biological matrices such as urine, sweat, plasma, and whole blood [1–3]. Figure 6.1 displays the major mechanisms and structures of amphetamine, methamphetamine, and ephedrine, and their major metabolites in humans.

Because of the increasing abuse of such drugs, rapid, simple, cost-effective, and consistent methods for their determination are desirable. Several methods have been developed for this purpose, including the use of immunoassays [4], high-performance liquid chromatography (HPLC) [5], gas chromatography/mass spectrometry (GC/MS) [6–7], and liquid chromatography/mass spectrometry (LC/MS) [8]. Each of these methods has its advantages and disadvantages regarding its sensitivity, precision, and simplicity. Although GC/MS is the most popular and powerful technique for the analysis of these abused drugs, the sample requires derivatization prior to injection into the GC system, as well as additional handling. This method becomes impractical when hundreds of samples require analysis. Therefore, the discovery of a rapid and efficient method—especially one that is complementary to GC/MS—for forensic and clinical analyses remains highly desirable.

One such complementary technique that has been used previously for rapid separation of illicit drugs is capillary electrophoresis (CE) [9–13]. Microchip electrophoresis (MCE), which is a miniaturized version of CE [14], is a sensitive, efficient, and fast separation method that has become a very popular, powerful tool in the analysis of biomolecules [15–18]. The most commonly used detection technique in MCE is fluorescence detection, performed in conjunction with a monochromatic laser, because of its high sensitivity and ease of implementation [19]. Unfortunately, fluorescence detection is very expensive and it requires that most of the analytes be derivatized prior to analysis, which is time-consuming [20]. For these reasons, we choose to detect the analytes electrochemically. Electrochemical detection is attractive for microchip systems because of its high sensitivity, tunable selectivity, independence of path length, and amenability to miniaturization.

Recently, we developed an exceedingly simple technique for microfluidic device fabrication using a dry film photoresist in conjunction with photolithographic and hot roll lamination techniques [21]. Dry film photoresists offer many advantages over the use of liquid photoresists, including good conformability, excellent adhesion to other substrates, good flatness, absence of liquids, uniform photoresist distribution, low exposure energy, low cost, and short processing times [22].

In this paper, we report the first separation of amphetamine, methamphetamine, and ephedrine using micellar electrokinetic chromatography (MEKC) and dry-film-based microchip capillary electrophoresis (DFB-MCE) in conjunction with electrochemical detection. We determined the optimal separation conditions for this method to decrease the amount of sample consumed and the separation time; we then used the optimized conditions to successfully determine the presence of these compounds in urine sample after solid phase extraction. Herein, we compare the results obtained when using the DFB-MCE and GC/MS methods for the analyses of these compounds.

6.2. Materials and methods

6.2.1. Chemicals

(±)-Amphetamine (catalog no. A-007; 1.0 mg/mL methanol; AP), (±)-methamphetamine (catalog no. M-009; 1.0 mg/mL methanol; MA), and (1*R*, 2*S*)-(–)-ephedrine (catalog no. E-023; 1.0 mg/mL methanol; EP) were obtained from Radian International. The negative dry film photoresist (AF5050) was purchased form ChangChun Inc. Co. (Hsin-chu, Taiwan). Disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), and sodium hydroxide (NaOH) were obtained from Fluka. Sodium dodecyl sulfate (SDS) was obtained from Sigma (St. Louis, MO, USA). Pentafluoropropionic anhydride (PFPA) was purchased from Supelco. All chemicals were obtained in analytical grade. All aqueous solutions were prepared using water purified through a Milli-Q water system (Millipore, Bedford, MA, USA) and filtered through a 0.22-µm filter. The urine samples were donated by the Command of the Army Force of Military Police, Forensic Science Center, Taiwan.

6.2.2. Dry-film-based microchip fabrication

PMMA microchips were fabricated using a photolithography replica molding method (Fig. 6.2A). The PMMA chip size was 30 mm long, 85 mm wide and 1 mm thick. The PMMA chip was cleaned with water and ethanol, and then dried under a high pressure of dry air. The PMMA substrate was hot-pressed on a 50-µm-thick layer of negative dry film photoresist, covered with the photomask, exposed to UV light in a clean room for 6 s, and then developed for 5 min using a developing solution. The channel pattern and electrode placement are depicted in Figure 6.2B; the configuration of the holder design is indicated in Figure 6.2C. Pinched injection through a double-T injector was employed in these experiments. The separation channel length from the injection cross to the buffer waste (BW) was 62.0 mm. The injection channel length from reservoir sample 1 (S1) or sample 2 (S2) to the reservoir

sample waste 1 (SW1) or sample waste 2 (SW2) was 15.0 mm. All channels were 100 μm wide and 50 μm deep. Access holes (depth: 1.0 mm; diameter: 2 mm) were located at the ends of each channel. The electrodes (Pt and carbon fiber) were placed on a PDMS cushion that was molded with two fillisters to fix the electrodes as indicated in Figure 6.2C. Prior to positioning them on the PDMS cushion, the electrodes were washed with ethanol. The electrodes were located beneath the separation channel, thus, allowing the buffer to flow past the detector. A carbon fiber was the working electrode; a Pt wire was the ground electrode and also served as the decoupler. The distance between the two electrodes was 2.0 mm. A basic apparatus setup for electrochemical detection (Fig. 6.2D) was used for DFB-MCE to separate the analytes.

6.2.3. Instrumentation

The high-voltage power supply used for electrophoresis separation had an adjustable voltage range from 0 to +5 kV (model MP-5000-250P; Major Science, Taiwan). The photolithographic procedures were performed using a hot roller (model TCC-6000; Tah-Hsin, Taipei, Taiwan) for pressing, a UV aligner (model Union EMA-400; Tokyo, Japan) for exposing, and an auto-development machine for developing. The detection system was an electrochemical analyzer (model 8021b; CHI, USA) coupled to the working, auxiliary, and reference electrodes through sockets. The working electrode, 99.99% carbon fiber (100 μm diameter), was obtained as a present from Yeou Chuen Wire Co., Ltd. (Tao-Yuan, Taiwan).

6.2.4. Electrophoresis procedures

The new microchips were conditioned prior to separation by washing them with purified water for 10 min and then 1 mM phosphate buffer solution (pH 7.4; containing SDS anionic surfactant) for 5 min. For electrophoretic separation, the stock solutions were diluted with a relative buffer solution that did not contain SDS anionic surfactant and had conductivity

similar to that of the micellar background solution (BGS). First, the sample was loaded into the injection cross under electroosmotic pumping from S1 or S2 to SW1 or SW2 by application of a potential between the two reservoirs at 200 V/cm for 10 s. Later, another potential was applied between the buffer (B) and BW to avoid sample diffusion at the intersection. The potential applied between B and BW was also 200 V/cm.

6.2.5. GC/MS apparatus and method

A Hewlett–Packard (HP; Palo Alto, CA) system was used for gas chromatography/mass spectrometry (GC/MS). It consisted of an HP 6890 series GC, an HP 5973 quadrupole mass selective detector (MSD), and an HP 7683 auto-injector; data were collected using an HP Chem Station computer system. The injector temperature was 250 °C. A capillary column (30 m × 0.32 μm I.D.) with an HP-5 MS (5% diphenyl and 95% dimethylpolysiloxane) bonded stationary phase film (0.25-μm thick; Agilent Technologies, USA) was used. The oven temperature was programmed as follows: beginning at 70 °C (held for 1 min), the temperature was ramped to 200 °C at 15 °C/min and then held for 2 min. Next, it was ramped to 260 °C at 20 °C/min and then finally held at that temperature for 10.3 min. The total analysis time was 25.0 min. Helium, the carrier gas, was introduced at a flow rate of 1 mL/min. The MSD was operated in electron ionization and selected ion monitoring (SIM) modes. The spectrometer was operated under the following conditions: SIM mode; ionization energy, 70 eV; ion temperature maintained at 280 °C; 40–300 amu at 1.84 scans/s.

6.2.6. Solid phase extraction procedure for urine samples

The cartridges (part No. 1211-3052; column type, LRC; Varian, CA, USA) were conditioned sequentially with methanol (3 mL), H_2O (3 mL), and 0.1 M phosphate buffer (pH 6.0; 1 mL). The urine sample (2 mL) was mixed with 1 M phosphate buffer (pH 6.0; 1 mL). The column was washed sequentially with H_2O (3 mL), 1.0 M acetic acid (1 mL), and

methanol (3 mL) and then dried under vacuum for 10 min. The analytes were eluted with dichloromethane/isopropanol/ammonium hydroxide (78:20:2, v/v/v; 3 mL). The clean organic phase was then evaporated to dryness at a temperature below 40 °C. MCE: The residue was dissolved in phosphate buffer (pH 7.4; 10 μL) and used directly. GC/MS: PFPA (50 μL) was added to the residue as a derivatizing reagent, and then the sample was heated for 20 min at 70 °C. After cooling, the derivatized extracts were transferred to an autosampler vial and a 2-μL aliquot was injected into the GC/MS system.

6.3. Results and discussion

6.3.1. Optimization of electrochemical detection performance

We used the hydrodynamic voltammetric (HDV) method to obtain the optimum detection potential for separation of the analytes. Figure 6.3A displays the hydrodynamic voltammograms obtained by the carbon fiber electrode in the absence of SDS in the running buffer. The curves were recorded pointwise over a potential range from 0 to +1.5 V and using a separation voltage of 1.5 kV (200 V/cm). As indicated in the Figure, the detection potentials of buffer, AP, MA, and EP were not significantly different. The cyclic voltammogram results are also displayed (inset) for A, MA, and EP (10 ng/mL each). We could not separate the analytes when the detection potential was 1.0 V (Fig. 6.4A); thus, we added the anionic surfactant SDS to the running buffer in an attempt to obtain separation with better resolution. As indicated in Figure 6.3B, the peak heights of the analytes remained steady when the detection potential was above 0.9 V. Because the noise of the electrochemical baseline increased upon increasing the potential (data not shown), we chose 0.9 V as the optimal detection potential.

6.3.2. Effect of SDS concentration in the running buffer

Figure 6.4 displays the effects that different SDS concentrations in the running buffer have upon the separation of the target analytes. As indicated in the electropherogram in Figure 6.4A, when no SDS was added, the separation of the analytes within 80 s was very poor when using a detection potential of 1.0 V; peaks 1 and 2 cannot be identified, and the peaks appeared significantly broadened. When 5 mM SDS was used, however, the separation (Fig. 6.4B) improved—as a result of increased interactions between the analytes and the SDS micelles—but the resolution remained poor, when using a 0.9-V detection potential. After increasing the SDS concentration from 10 to 30 mM, we found that the separation of the

analytes was optimized (Fig. 6.4D) at an SDS concentration of 20 mM. Under these conditions, we observed migration times in the following order: AP (peak 1) < MA (peak 2) < EP (peak 3). When the concentration of the SDS was 30 mM (Fig. 6.4E), peaks 2 and 3 overlapped. Accordingly, we observed an interesting phenomenon: MA had the strongest interaction with SDS—its migration time changed slowly, relative to those of AP and EP, upon increasing the SDS concentration. Initially, peaks 1 (AM) and 2 (MA) were inseparable in the absence of (or in the presence of a low concentration of) SDS. When the SDS concentration was increased gradually, the migration times of all of the analytes increased. Finally, the peaks 2 (MA) and 3 (EP) overlapped and the intensities of all of the peaks decreased. This result may be due to the generation of an electroosmotic flow (EOF) and to differences in the degrees of interaction between the analytes and SDS, because the micelle phase has mobility against the EOF that is higher than those of the analytes.

6.3.3. EOF behavior of the analytes in the DFB-MCE microchannel

To investigate the influence that the presence of SDS in the running buffer has on the EOF, we measured the EOF in the DFB-MCE microchannel using phosphate buffer (pH 7.4) containing different SDS concentrations. We monitored the EOF using the simple and effective method of current monitoring as reported by Zare et al. [23]. Figure 6.5A illustrates the effects that the SDS concentration has on the effective mobilities and peak heights of AP, MA, and EP, respectively. The inset of Figure 6.5A displays the values of EOF in the freshly prepared DFB-MCE microchannel of phosphate running buffers (pH 7.4) as a function of the SDS concentration; it is clear that a gradual decrease in the EOF occurred, as is also evident in Figure 6.5A. In the presence of 20 mM SDS, the effective mobilities of the analytes had obviously changed; they occurred in the following order: AP < MA < EP. These results correspond to the migration behavior displayed in Figure 6.4D. When we increased the SDS concentration beyond 20 mM (e.g., 30 mM), however, the effective mobilities of MA and EP

became almost equal; this situation explains why peaks 2 (MA) and 3 (EP) overlap—and the resolution is poor—in Figure 6.4E upon increasing the SDS concentration. Figure 6.5B also suggests that 20 mM SDS provides the optimum peak height and better sensitivity.

Table 6.1 presents the ranges of linearity, the coefficients of determination (r^2), the limits of detection (LOD), the migration times, the values of RSD% of the migration times and peak heights, and the effective mobilities ($\mu_{effective}$) for AP, MA, and EP. Our results indicate that DFB-MCE performed in conjunction with electrochemical detection is a rapid, accurate, and precise method for the separation of these samples. For such analyses, we obtained values of the LOD of 85.4, 65.1, and 78.5 ng/mL and of $\mu_{effective}$ of –0.99, –1.5, and -1.77×10^{-4} cm^2 V^{-1} s^{-1} for AP, MA, and EP, respectively. Accordingly, we used this technique, DFB-MCE in conjunction with electrochemical detection, for analyses through simultaneous rapid screening and for the identification of AP, MA, and EP in urine samples.

6.3.4. Application of the analysis of real urine sample

Figure 6.6 illustrates a comparison of the DFB-MCE method (in conjunction with electrochemical detection) with the GC/MS method (with derivatization) for the analysis of real urine samples obtained from a suspected drug user. We applied the DFB-MCE method after solid phase extraction (i.e., no derivatization); the separation of AP, MA, and EP, respectively, in urine was rapid and provided clearly distinguishable peaks (Fig. 6.6A) within 80 s. Although the use of GC/MS in conjunction with SPE during the derivatization procedure also led to the successful determination of AP, MA, and EP (Fig. 6.6B), the extraction and analysis times were over five times higher than those required by the DFB-MCE method. It is obvious that DFB-MCE, when performed in conjunction with electrochemical detection, is a successful, powerful, and superior technique for using analysis of AP, MA, and EP.

6.4. Concluding remarks

In this paper, we demonstrate the first example of the use of DFB-MCE in conjunction with electrochemical detection for the simultaneous separation and identification of AP, MA, and EP in urine samples. Although solid phase extraction is also effective when used with GC/MS for separation of the analytes—after PFPA derivatization—it is not suitable for use if hundreds of samples are to be analyzed. The advantages of the DFB-MCE method are that sample pretreatment is simple, the extracts of the analytes are obtained rapidly, and the determination of the analytes in the urine samples occurs without the need for derivatization. The optimized separation parameters for the DFB-MCE method were as follows: running buffer solution, 1 mM phosphate buffer (pH 7.4); SDS concentration, 20 mM; separation voltage, +1.5 kV; injection voltage, + 300 V; injection time, 10 s; detection potential, 0.9 V. Thus, we conclude that DFB-MCE, performed in conjunction with electrochemical detection, is an accurate, sensitive and rapid approach that should be considered for use in rapid drug screening; it is a sufficiently reliable and complementary method for use in forensic analysis.

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Table 6.1. Values of the ranges of linearity, coefficients of determination (r^2), limits of detection (LOD), migration times, RSDs, and effective mobilities ($\mu_{effective}$) for amphetamine, methamphetamine, and ephedrine when using micellar electrokinetic chromatography and microchip capillary electrophoresis in conjunction with electrochemical detection.

	Amphetamine	**Methamphetamine**	**Ephedrine**
Range of linearity (ng/mL)	200–1000	200–1000	200–1000
Coefficient of determination (r^2)	0.9991	0.9993	0.9990
LOD (S/N = 3; ng/mL)	85.4	65.1	78.5
Migration time (s)	49.15	56.65	66.35
RSD (%; n = 5)			
Ⅰ. Migration time	3.01	4.03	3.83
Ⅱ. Peak height	1.89	2.44	2.11
$\mu_{effective}$ (10^{-4} cm^2 V^{-1} s^{-1}) (with 20 mM SDS)	–0.99	–1.52	–1.77

Analytes	Metabolite I	Metabolite II
Amphetamine (AP)	norephdrine phenylacetone	
Methamphetamine (MA)	Amphetamine (AP)	norephdrine
Ephdrine (EP)	norephdrine	

Figure 6.1. Major mechanisms and structures of amphetamine, methamphetamine, and ephedrine, and their major metabolites in humans.

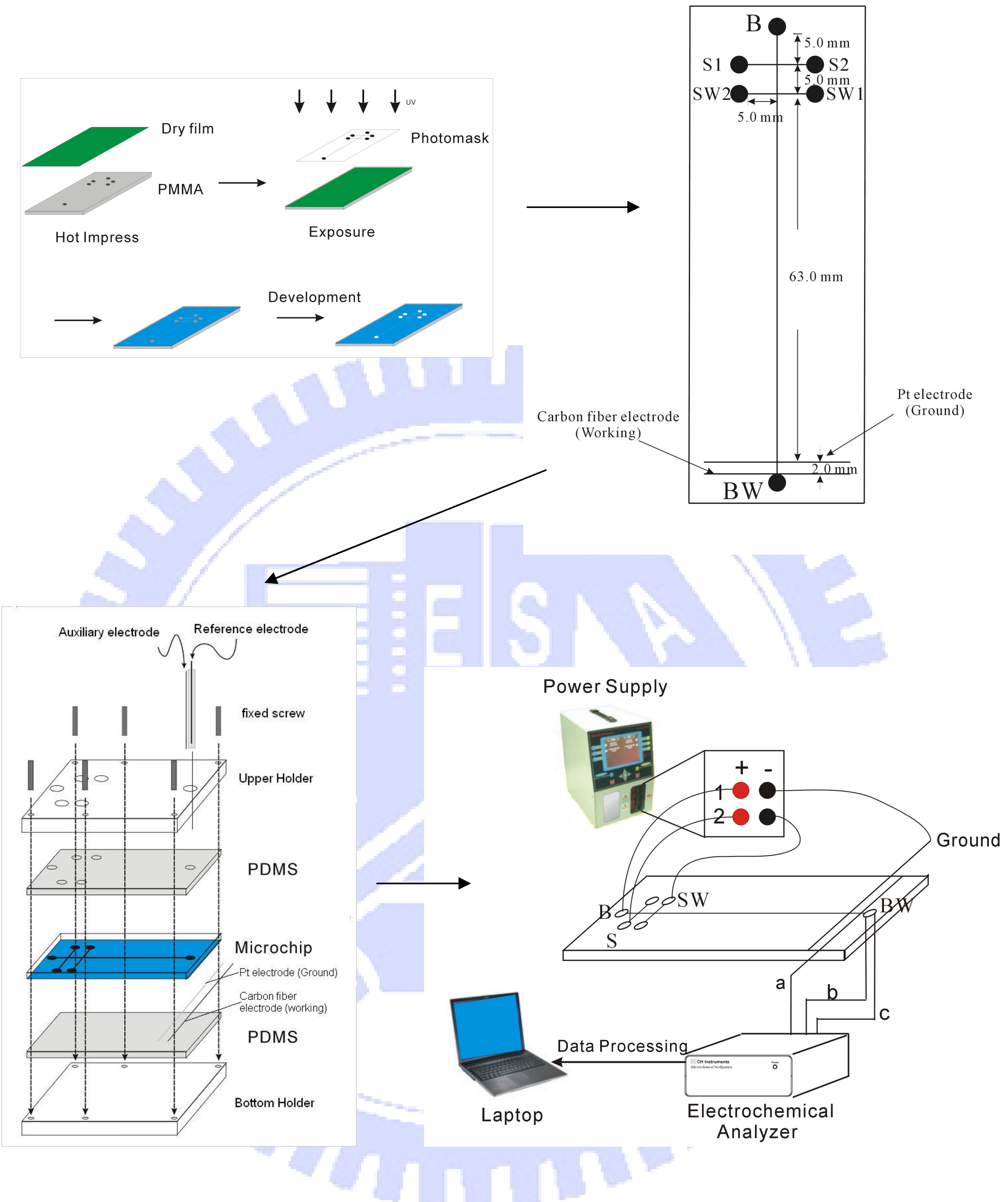

Figure 6.2. (A) Fabrication procedure for the dry-film-based microchip. (B) Microchip channel and electrode design. (C) Design and configuration of the holder of the dry-film-based microchip. (D) Basic apparatus setup for electrochemical detection in dry-film MCE.

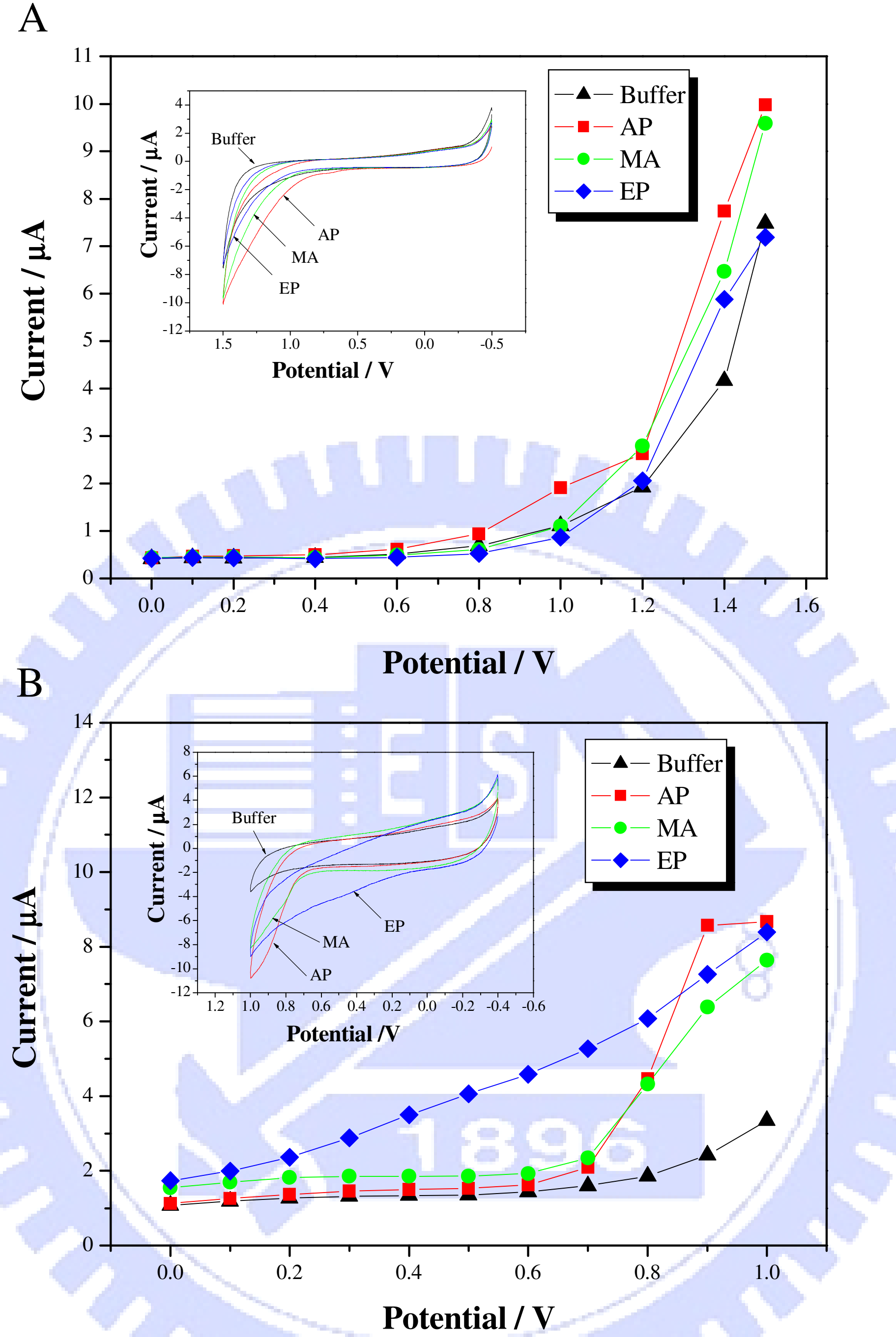

Figure 6.3. Hydrodynamic voltammograms obtained (A) with no SDS and (B) with 20 mM SDS in the running buffer. Running buffer, 1.0 mM phosphate buffer (pH 7.4); separation voltage, +1.5 kV; injection voltage, +300 V; injection time, 10 s. Inset: Cyclic voltammograms obtained for 10 ng/mL AP (red), MA (green), and EP (blue), as well as the corresponding blank sample (black), over the range of potentials from –0.6 and +1.5 V, at a scan rate of 100 mV/s, using a carbon fiber electrode in phosphate buffer (1.0 mM, pH 7.4).

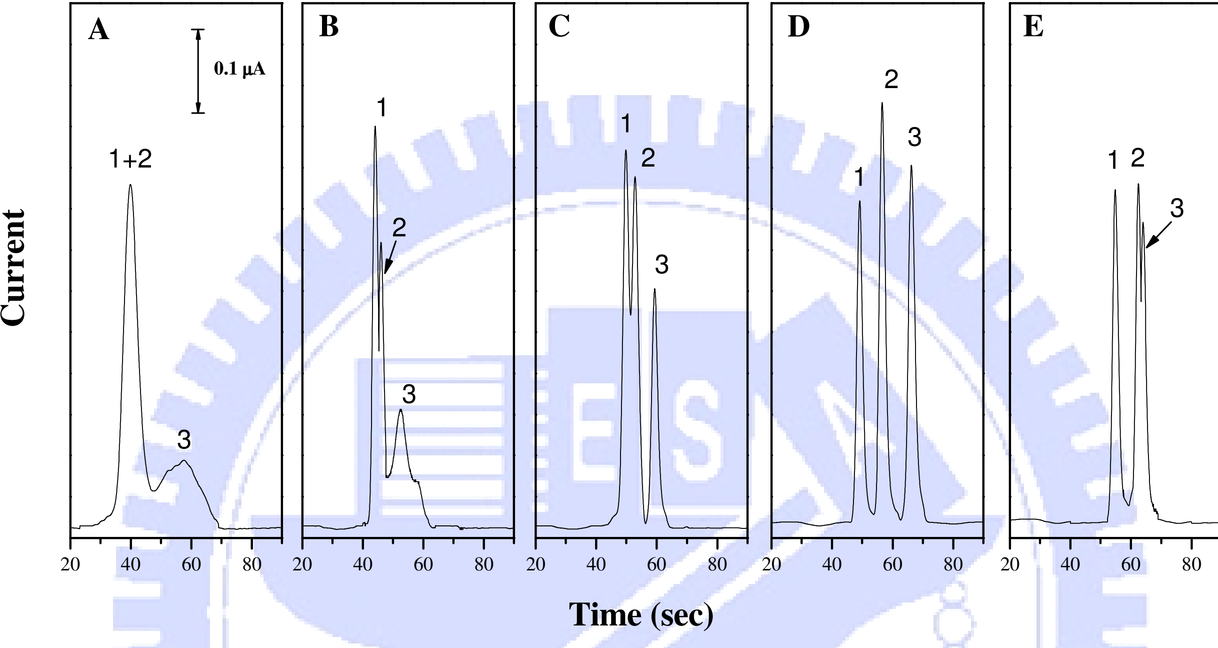

Figure 6.4. Effect of SDS concentration in the phosphate buffer (1 mM, pH 7.4) upon the separation of the target analytes: (A) 5, (B) 10, (C) 20, and (D) 30 mM. Separation buffer, 1 mM phosphate (pH 7.4); separation voltage, +1.5 kV; injection voltage, +300 V; injection time, 10 s; detection potentials at the carbon fiber electrode: (A) +1.0 V, (B–E) +0.9 V . Peak 1, AP (2.5 μg/mL); peak 2, MA (2.5 μg/mL); peak 3, EP (2.5 μg/mL).

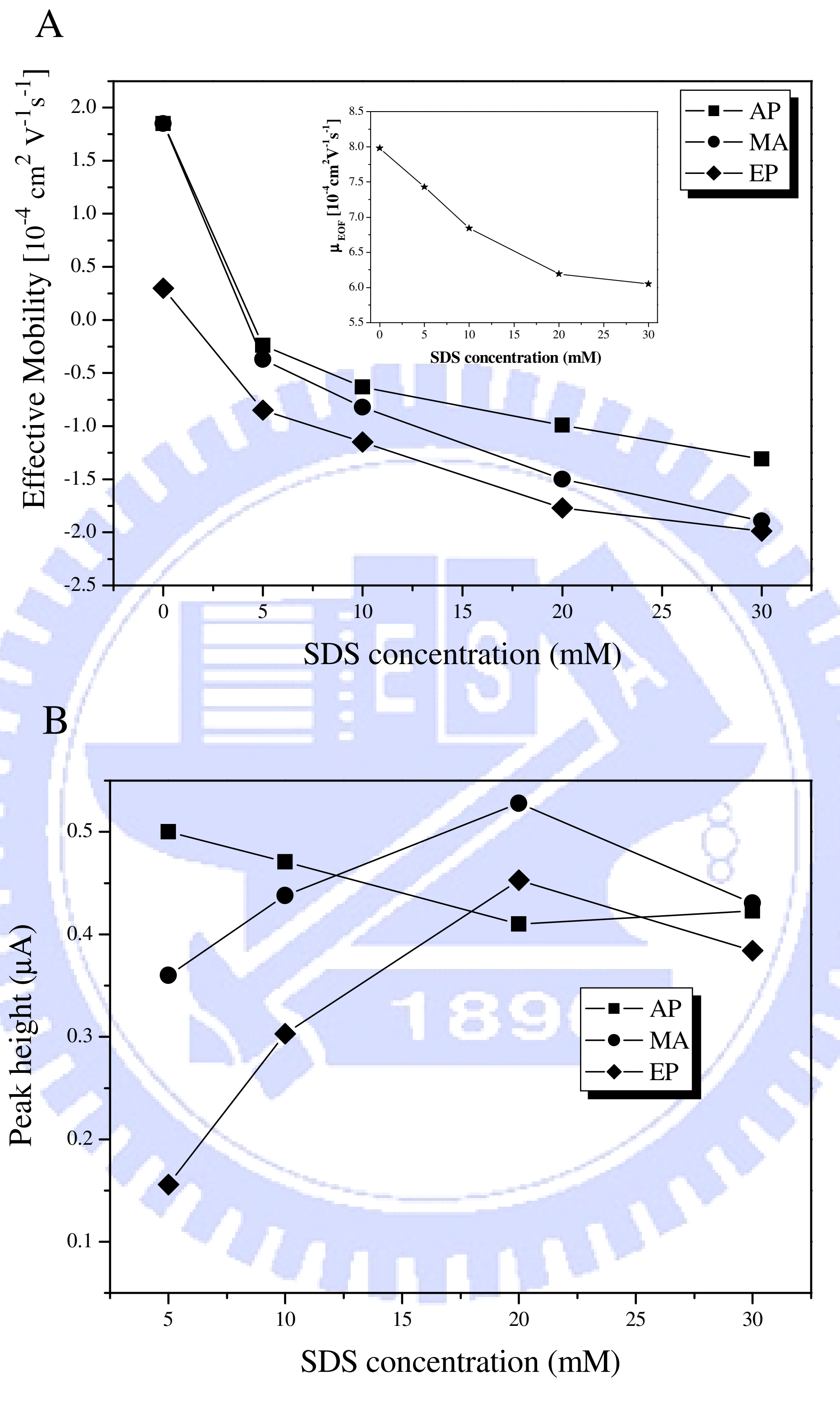

Figure 6.5. Effect of the SDS concentration on (A) the effective mobilities (inset: plot of μ_{EOF} vs. SDS concentration) and (B) the peak heights of AP, MA, and EP.

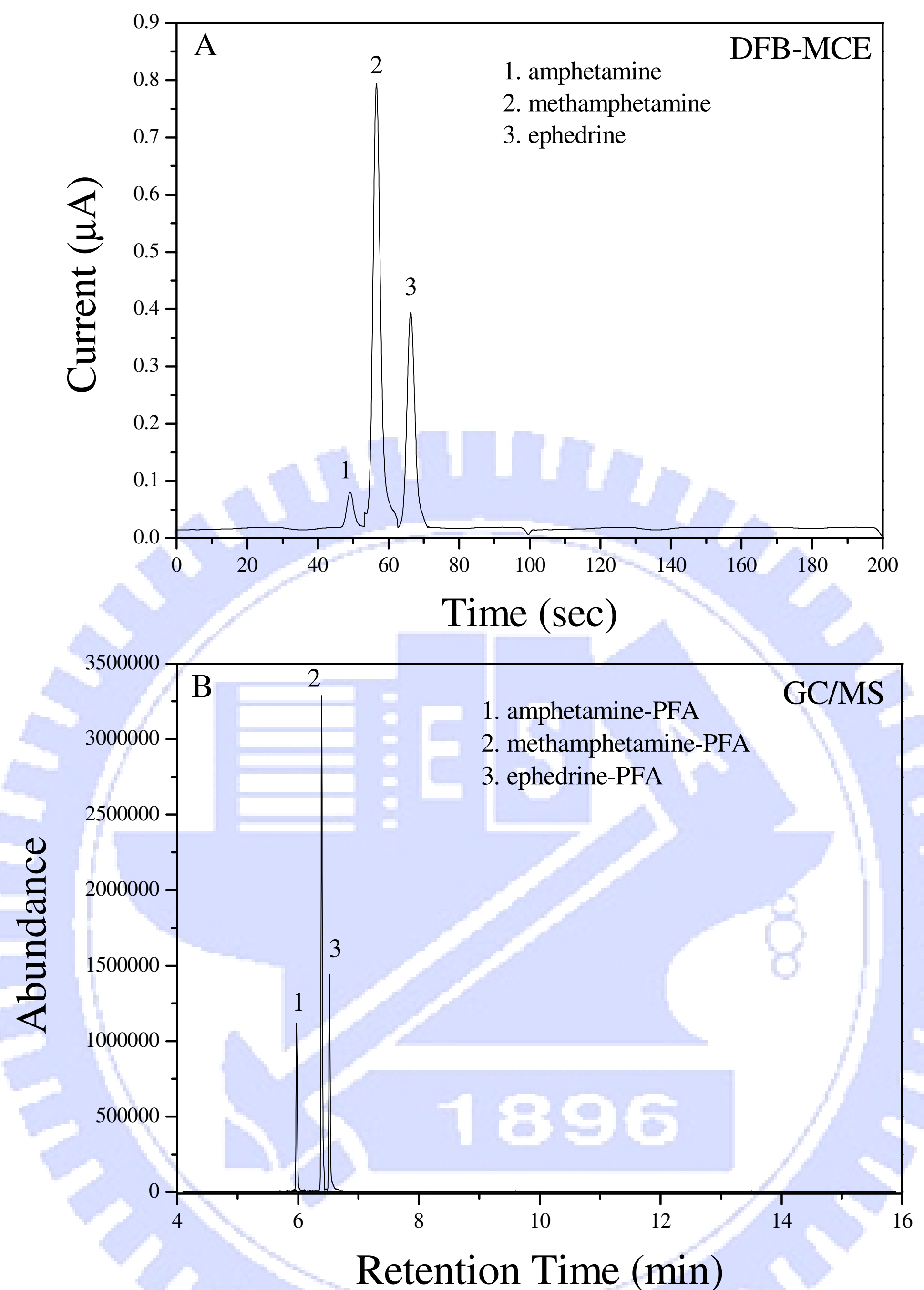

Figure 6.6. Electropherogram and GC/MS traces for the analysis—after solid-phase extraction—of a urine sample of a suspected amphetamine-like-drug user; AP (peak 1), MA (peak 2), and EP (peak 3). (A) DFB-MCE method, no derivatization; (B) GC/MS method, derivatized with PFPA. DFB-MCE conditions: SDS concentration, 20 mM; others parameters are the same as those described in Fig. 6.4.

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Journal of Chromatography A, 1111 (2006) 159–165

JOURNAL OF CHROMATOGRAPHY A

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On-line preconcentration and determination of ketamine and norketamine by micellar electrokinetic chromatography Complementary method to gas chromatography/mass spectrometry

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Available online 16 June 2005

Abstract

We have investigated a rapid, simple, and highly efficient on-line preconcentration method using in micellar electrokinetic chromatography (MEKC) for the analysis of abused drugs. Ketamine is an anesthetic that has been abused as a hallucinogen. We applied the sample sweeping technique first to ketamine and its major metabolite, norketamine, and separated the analytes with MEKC. Several of the sweeping MEKC parameters to effect successful separations, such as the concentration of sodium dodecyl sulfate (SDS), the injection time, and the applied voltage were optimized. The improvements in the number of theoretical plates under the different separation conditions are presented clearly in a three-dimensional representation. The limits of detection were 2.8, 3.4, and 3.3 ng/mL for ketamine, norketamine, and ketamine-D_4, respectively. The enrichment factor for each compound was within the range of 540–800. Experimental results are in agreement with those of analysis conducted by gas chromatography/mass spectroscopy (GC/MS). Therefore, we believe that sweeping, combined with MEKC, represents a suitable complementary method to GC/MS for use in clinical and forensic analyses of ketamine and norketamine.

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Keywords: Ketamine; Norketamine; Sweeping; Micellar electrokinetic chromatography; On-line preconcentration

1. Introduction

Ketamine is familiar to emergency physicians as a dissociative anesthetic that has been abused as a hallucinogen for almost 30 years. Ketamine produces effects similar to phencyclidine (PCP) in conjunction with the visual effects of lysergic acid diethylamide (LSD) [1]. Ketamine is available as either a powder or a liquid; in its powdered form, it can be inhaled nasally, smoked, or mixed into drinks; in its liquid form, it can be injected or applied to, for example, cigarettes. Ketamine is metabolized to at least two compounds of pharmacological interest. First, ketamine undergoes *N*-demethylation mediated to form norketamine in the liver. Then, norketamine's cyclohexanone ring undergoes oxidative metabolism to form dehydronorketamine. Current techniques for analyzing ketamine include the use of high-performance liquid chromatography (HPLC) [2–5] and gas chromatography in conjunction with mass spectroscopy (GC/MS) [6]. These approaches almost always employ liquid–liquid extraction (LLE), solid-phase extraction (SPE), or solid-phase microextraction (SPME) techniques to obtain the target substances.

Capillary electrophoresis (CE) is a separation method – based on a physical process quite different from that of chromatography – that has been the focus of much attention for developing new analytical methodologies [7–9]. CE is a powerful technique that is simple, provides rapid results, has high efficiency, resolution, and sensitivity, and involves low sample consumption; additionally, many CE instruments are available commercially. CE is a rapidly growing separation technique that is being applied in bioscience, pharmaceuticals, environmental, food science, and forensic research [10]. Micellar electrokinetic chromatography (MEKC), which is one of the basic modes of CE, has become a popular technique for improving CE separation efficiency for both neutral and

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doi:10.1016/j.chroma.2005.05.019

charges analytes [11]. Unfortunately, the benefits provided by the high number of theoretical plates obtained with CE can be overshadowed by the low sensitivity of UV detection systems. Because of the small dimensions of a CE capillary – the typical inside diameter (I.D.) and length are 25–100 μm I.D. and 40–80 cm, respectively – only very small sample volumes may be loaded onto the column. Additionally, for most common optical detection techniques, CE suffers from a drastically reduced pathlength relative to, for example, LC. Overcoming the poor sensitivity of CE with on-line sample preconcentration has been the focus of many investigations [12–14]. For example, Quirino and Terabe [15–18] found that neutral compounds could be analyzed effectively when utilizing the technique of MEKC combined with stacking. In 1998, they reported a sweeping method that can effect infiltration of analytes into the pseudostationary phase of the sample zone by applying an electric potential [19]. This technique is a new one for the on-line sample concentration of neutral or charged analytes in MEKC [20,21]. The sample solution does not need to be prepared in a low-conductivity matrix, but the conductivity equal to or higher than the running micellar solution is favored.

In this paper, we describe a simple and highly sensitive method for the detection of ketamine and its major metabolite, norketamine, in urine using the techniques of on-line preconcentration and sample sweeping, and combined with MEKC. We have optimized several electrophoresis parameters to effect successful separations, such as the concentration of sodium dodecyl sulfate (SDS), the injection time, the applied voltage, and the temperature. We provide a three-dimensional representation to present a clear visualization of the improvements in the number of theoretical plates with respect to the different separation conditions. We determined the optimal separation conditions for this method and decreased the amount of sample consumed and the separation time. Finally, we also compare the results of this analytical approach with those obtained when using MEKC, sweeping MEKC, and GC/MS.

2. Experimental

2.1. Chemicals

Ketamine hydrochloride (K·HCl, 1 mg/mL methanol), norketamine hydrochloride (NK·HCl, 1 mg/mL methanol), and the internal standard, [$^2H_4$]ketamine hydrochloride (ketamine-d$_4$, K-D$_4$·HCl, 1 μg/mL methanol), were obtained from Radian International. Fig. 1 displays their structures. SDS was purchased from Sigma (St. Louis, MO, USA). Disodium hydrogen phosphate (Na_2HPO_4) and sodium hydroxide (NaOH) were purchased from Fluka (Buchs, Switzerland). Citric acid was obtained from Merck (Darmstadt, Germany). Methanol, dichloromethane, *n*-hexane, isopropanol, acetic acid, ammonium hydroxide, acetone, and phosphoric acid were obtained in analytical grade (Aldrich). Water was purified by using a Milli-Q water system (Millipore, Bedford, MA, USA) and filtered through a 0.22 μm filter. All of the urine samples were donated by the Command of the Army Force of Military Police, Forensic Science Center, Taiwan.

Fig. 1. The structures of ketamine, norketamine and ketamine-D$_4$.

2.2. Apparatus

A Beckman P/ACE 5500 capillary electrophoresis system (Beckman Instruments, Fullerton, CA, USA) was used to effect the separations. A diode-array detector was employed for detection. Separations were performed in a 47 cm (40 cm to detector) × 50 μm I.D. fused-silica capillary tube (Polymicro Technologies, Phoenix, AZ, USA). The capillary tube was assembled in the cartridge format. A personal computer using System Gold software controlled the P/ACE instrument and allowed data analysis. The separation capillary was preconditioned prior to use with 1 M NaOH for 30 min, 0.1 M NaOH for 30 min, and then deionized water for 30 min. The sample was injected hydrodynamically and then a negative voltage was applied with the micellar background electrolyte (BGE) at both ends of the capillary to effect separation. Between runs, the capillary was flushed sequentially with 0.1 M NaOH, water, and BGE for 10 min each. The optimal buffer (pH 2.6) consisted of 25 mM citric acid/disodium hydrogenphosphate.

2.3. Sweeping and separation procedures

The column we used was a bare fused-silica capillary that we conditioned initially using a low-pH micellar electrolyte. The electroosmotic flow was suppressed by the low pH (2.6). Samples were pressure-injected at 0.5 psi. The detection wavelength was set at 200 nm. The neutral sample moved slowly because the velocity of the electroosmotic flow was very slow. The inlet and outlet of the capillary were placed in vials containing the BGE, and a negative voltage (15–30 kV) was applied. After the anionic micelles entered the sample zone, sweeping and separation were achieved through MEKC [21]. Stock sample solutions were prepared in methanol at a concentration of 100–1000 ppm. Different sample concentrations were obtained by diluting concentrated samples while keeping the sample matrix as 25 mM citric acid/disodium hydrogen phosphate and a low percentage of organic solvent (around 5–10%, v/v).

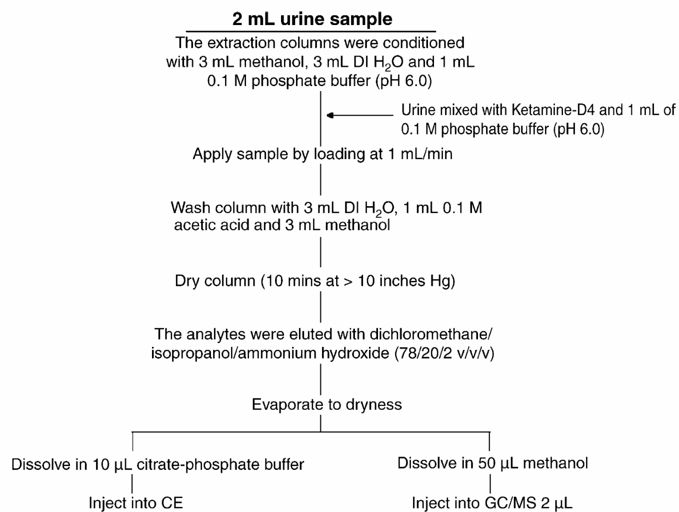


Fig. 2. The procedures used for sample preparation for urine by solid-phase extraction of urine.

2.4. GC/MS apparatus and method

A Hewlett-Packard (HP; Palo Alto, CA, USA) system was used for gas chromatography/mass spectrometry (GC/MS). It consisted of an HP 6890 series GC, an HP 5973 quadrupole mass-selective detector, and an HP 7683 auto-injector; data were collected using an HP Chem Station computer system. Helium was the carrier gas and was used at a flow-rate of 1 mL/min. The injector temperature was 250 °C. A Zebron ZB-5 MS fused-silica capillary column (30 m × 0.25 mm I.D.; 0.25 μm film thickness of 5% phenylmethylsilicone) provided the analytical separation. The retention times for ketamine, norketamine, and ketamine-d_4 (I.S.) were 9.87, 9.60, and 9.84 min, respectively. The oven temperature was programmed as follows: beginning at 120 °C (held for 1 min), the temperature was ramped to 200 °C at 15 °C/min and then held for 2 min. Next, it was ramped to 250 °C at 18 °C/min and then finally held at that temperature for 5.0 min. The total analysis time was 16.12 min. The MS system was operated in electron ionization and selected ion monitoring (SIM) modes. The spectrometer was operated under the following conditions: SIM mode; ionization energy, 70 eV; the ion temperature was maintained at 280 °C; 40–300 u at 1.84 scans/s.

2.5. Solid-phase extraction procedure

The cartridges (column type, LRC) were obtained from Varian (CA, USA). The cartridges were conditioned with methanol (3 mL), water (3 mL) and 0.1 M phosphate buffer (pH 6.0; 1 mL). The urine sample (2 mL) was mixed with ketamine-d_4 (100 μL) and 0.1 M phosphate buffer (pH 6.0; 1 mL). The column was washed with deionized water (3 mL), 0.1 M acetic acid (1 mL), and methanol (3 mL), and then it were dried under vacuum for 10 min. The analytes were eluted with dichloromethane/isopropanol/ammonium hydroxide (78:20:2, v/v/v). The clean organic phase was then evaporated to dryness. The residue was dissolved in methanol (50 μL) and a sample (2 μL) was injected into the GC/MS system. Fig. 2 provides detailed procedures.

3. Results and discussion

3.1. Optimizing the conditions for separation by sweeping MEKC

SDS is the most commonly additive used for MEKC during its separation. Fig. 3 displays typical MEKC chromatograms of ketamine (K), norketamine (NK), and ketamine-d_4 (K-D_4) that were separated in the presence of different concentrations of SDS. In Fig. 3, in addition to SDS, the buffer also consisted of 25 mM citric acid/disodium hydrogenphosphate (pH 2.6). As indicated in chromatogram of Fig. 3a, when 25 mM SDS was used, the separation of the analytes within 5 min was poor. When 50 mM SDS was used, however, the separation (Fig. 3b) began to improve as a result of increased interactions between the analytes and SDS micelles. The separation of the analyte was optimized (Fig. 3c) at an SDS concentration of 75 mM. In the acidic buffer solution (pH 2.6), the electrophoretic mobility of the neutral analytes toward the outlet (anode) is provided by the negative charged SDS micelles. The migration sequence of analytes to the outlet is based on their interaction with SDS. Thus, NK with the highest interaction with SDS migrated first. Under these conditions, we observed migration times in the following order: NK (peak 2) < K (peak 1) < K-D_4 (peak 3). When the concentration of the SDS was 100 mM (Fig. 3d), peaks K and K-D_4 became broad and overlapped.

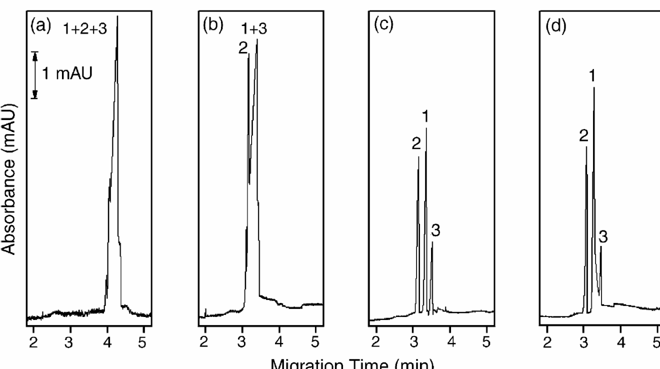


Fig. 3. Effects that different SDS concentrations have on MEKC separations: (a) 25 mM; (b) 50 mM; (c) 75 mM; and (d) 100 mM. Conditions: capillary, 47 cm long (40 cm to detector), 50 μm I.D.; 25 mM citrate/phosphate buffer (pH 2.6); applied voltage, −25 kV; detection wavelength, 200 nm; temperature, 25 °C; injection time, 4 s (0.5 psi); sample concentrations: 50, 30, and 20 ppm for K (peak 1), NK (peak 2), and K-D_4 (peak 3), respectively.

Fig. 4 illustrates the effects of different injection times on the analyte's resolution during sweeping MEKC separation. We performed hydrodynamic injection at a pressure at 0.5 psi, injected the sample solution into the capillary for 90, 120, 150, 180, or 210 s, and then applied a −25 kV potential to effect sweeping MEKC separation. The concentration enhancement of the analytes increased as the injection time increased. Injecting the sample for 150 s provided an excellent separation efficiency (Fig. 4c), but longer injection times led to incomplete peak separation; peaks 1 (K) and 2 (NK) gradually overlapped as the injection time increased, which would not allow qualitative analyses in a forensic environment.

The influence that the applied voltage had on the sweeping MEKC separation was examined in the range of potential from −15 to −30 kV (data not shown). Clearly, an applied voltage of −25 kV provided the optimal separation. Joule heating occurs upon increasing the applied voltages and results in the occurrence of diffusion phenomena, which leads to poor separation at −30 kV. Finally, we examined the effect that temperature had on the separation condition by varying the capillary temperature from 18 to 30 °C (data not shown). We found that the resolution reduced at 30 °C, so we chose 25 °C as an optimum separation temperature.

3.2. Three-dimensional representation of the effects

The number of theoretical plates changed as a function of the conditions of the many different experiments, i.e., the injection time, SDS concentration, applied voltage, and temperature; Fig. 5 provides a clear visualization of these data for K and NK in three-dimensional representation. Fig. 5a indicates the plate numbers for K and NK, respectively, in the range from 1.0×10^5 to 3.6×10^5. We have fitted continuous analytical functions to the experimental values to guide the eye; they indicate that the optimized plate numbers for K and NK of 3.48×10^5 and 2.81×10^5, respectively, occur for injection times in the neighborhood of 150 s at an SDS

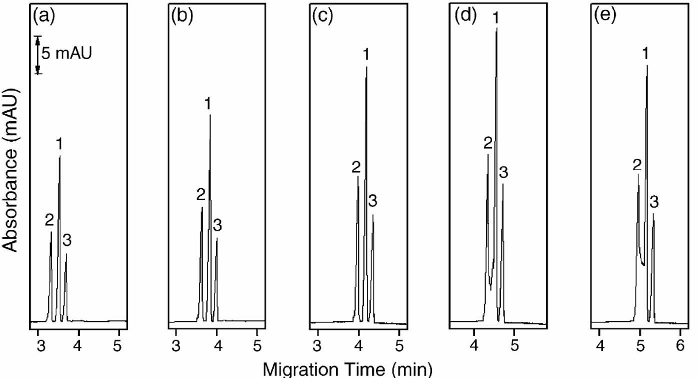


Fig. 4. Effects that different injection times have on sweeping MEKC separations. (a) 90 s, (b) 120 s, (c) 150 s, (d) 180 s, and (e) 210 s. Conditions: SDS concentration, 75 mM; sample concentrations: 500, 300, and 200 ppb for K (peak 1), NK (peak 2), and K-D_4 (peak 3), respectively. Other conditions are the same as those in Fig. 3.

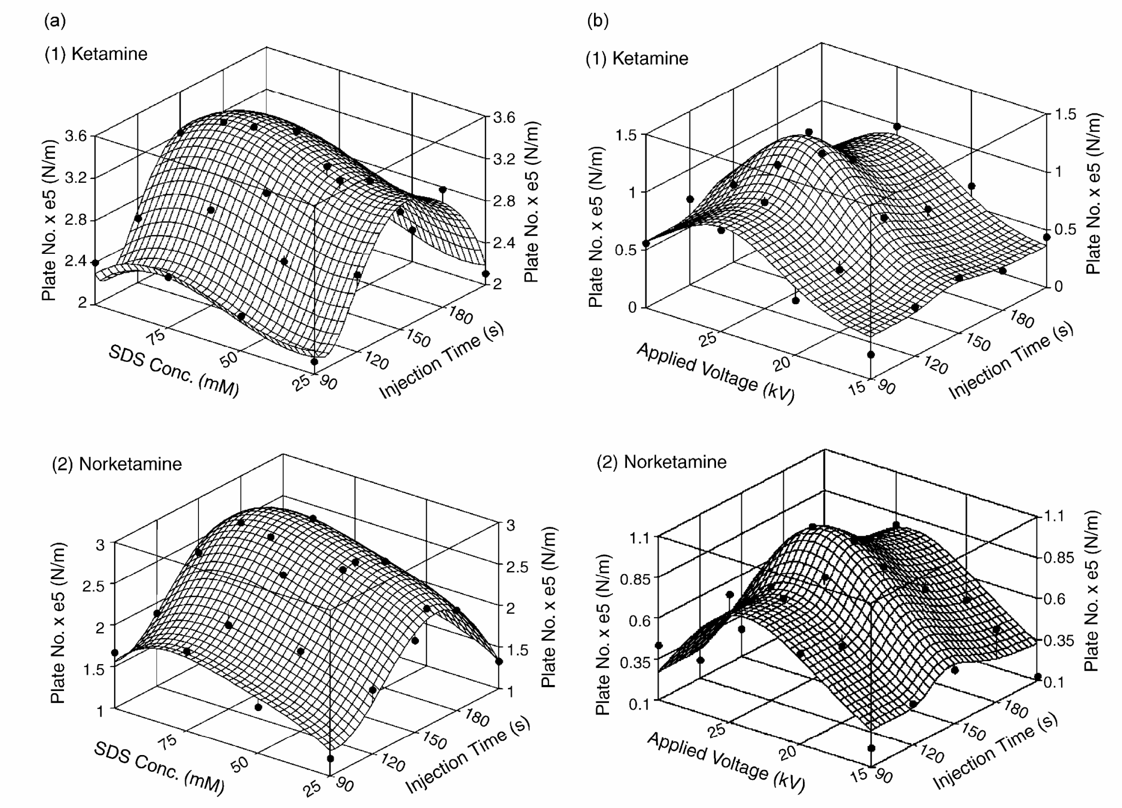


Fig. 5. Three-dimensional representation of the effects that (a) SDS concentration and injection time, (b) applied voltage and injection time have on the number of theoretical plates for (1) ketamine and (2) norketamine.

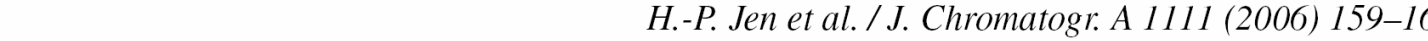
concentration of 75 mM. Fig. 5b illustrates the plate numbers for K and NK, respectively, as a function of injection time and applied voltage. By comparing the sub-figures in Fig. 5, we find that the SDS concentration is the most important condition, more so than the applied voltage or temperature (data not shown), for affecting the plate number of the separation. In comparison, the temperature effect is minimal. We believe that such a three-dimensional representation is useful for determining a range of the optimized conditions for CE separation.

3.3. Comparing MEKC and sweeping MEKC

Fig. 6 depicts the analysis of K and NK by MEKC and sweeping MEKC methods. The concentrations of the analytes K, NK, and K-D_4 were 50, 30, and 20 ppm, respectively in Fig. 6a. However, the sample concentration was diluted 100-fold used in Fig. 6b. Under these conditions, K, NK, and K-D_4 had ca. ∼760-, ∼540-, and ∼800-fold enhancements in their detection sensitivities, respectively, relative to those obtained in Fig. 6a. Table 1 presents values for the range of linearity, coefficient of determination (r^2), limit of detection (LOD), RSD, and the number of theoretical plates for K, NK, and K-D_4 using the MEKC and sweeping MEKC methods; in addition, we compare these values with those obtained when using the GC/MS method. The results indicate that the sweeping MEKC method provides better results than do the other methods for the separation of these analytes.

3.4. Separating and determining of ketamine and norketamine in suspect urine samples

Finally, we have used the sweeping MEKC method combined with SPE, was compared it with the GC/MS method, to analyze real urine samples obtained from suspected K users. First, we attempted to analyze the urine sample without extraction or sweeping, but we could not obtain a signal for K or NK (Fig. 7a). Next, we applied the same conditions as those used to obtain Fig. 7a, but with an injection time of 150 s; the resulting separation remained poor, but peaks for the target of analytes gradually appeared (Fig. 7b). Then, when we utilized SPE in conjunction with sweeping, we were able to clearly distinguish peaks for K, NK, and K-D_4 from the urine sample within 5 min (Fig. 7c). The concentrations of K and NK are 61.2 and 55.4 ppb, respectively. We also compared these results with those obtained by GC/MS for



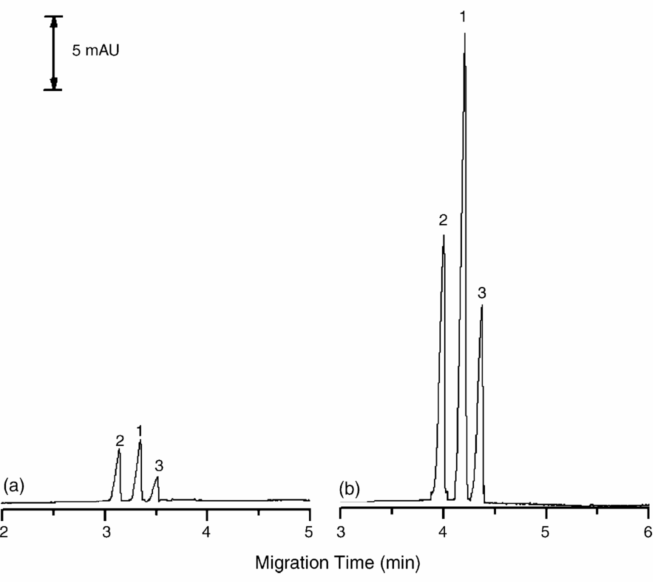


Fig. 6. Analysis of ketamine and norketamine by (a) MEKC and (b) sweeping MEKC methods. Sample concentrations: (a) 50, 30, and 20 ppm for K (peak 1), NK (peak 2), and K-D_4 (peak 3), respectively and (b) 500, 300, and 200 ppb for K (peak 1), NK (peak 2), and K-D_4 (peak 3), respectively. Other conditions are the same as those in Figs. 3 and 4.

Table 1

Values of the range of linearity, coefficient of determination (r^2), limit of detection (LOD), RSD, and the number of theoretical plates for ketamine, norketamine, and ketamine-d_4 during separation by MEKC, sweeping/MEKC and GC/MS, respectively

	Ketamine	Norketamine	Ketamine-d_4
MEKC			
Range of linearity (μg/mL)	5–500	5–500	5–500
Coefficient of determination	$r^2 = 0.9921$	$r^2 = 0.9963$	$r^2 = 0.9938$
LOD (S/N = 3) (μg/mL)	1.1	1.2	1.9
RSD (%; $n = 5$)			
(a) Migration time	3.12	4.74	3.87
(b) Peak area	4.22	3.85	4.66
Number of theoretical plates (N/m)	2.58×10^5	2.45×10^5	2.41×10^5
Sweeping MEKC			
Range of linearity (ng/mL)	5–500	5–500	5–500
Coefficient of determination	$r^2 = 0.9957$	$r^2 = 0.9984$	$r^2 = 0.9961$
LOD (S/N = 3) (ng/mL)	2.8	3.4	3.3
RSD (%; $n = 5$)			
(a) Migration time	2.11	2.03	1.89
(b) Peak area	1.76	1.92	2.04
Number of theoretical plates (N/m)	3.48×10^5	2.81×10^5	3.18×10^5
GC/MS			
Range of linearity (ng/mL)	10–1000	10–1000	10–1000
Coefficient of determination	$r^2 = 0.9992$	$r^2 = 0.9991$	$r^2 = 0.9993$
LOD (S/N = 3) (ng/mL)	5.4	7.1	4.5
RSD (%; $n = 5$)			
(a) Retention time	1.01	1.03	1.0
(b) Peak area	2.11	1.99	2.01

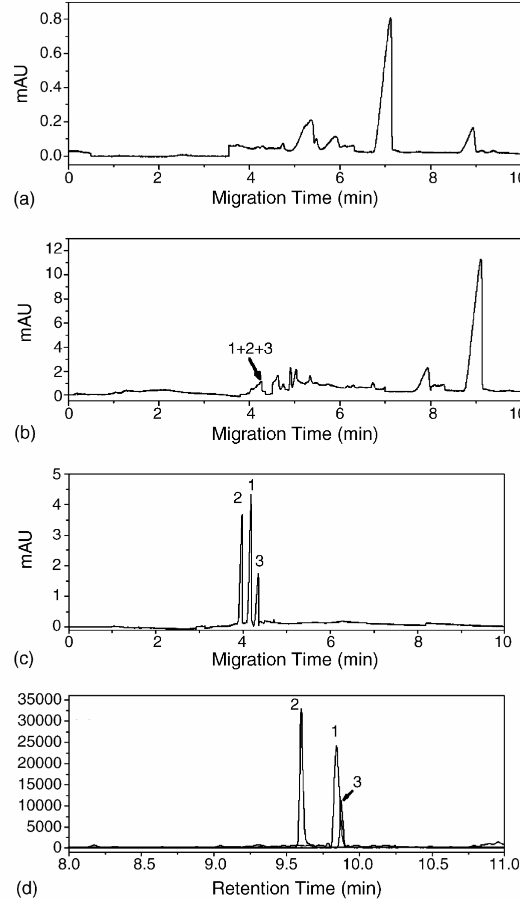


Fig. 7. Electropherograms and GC/MS traces for the analysis of a urine sample of a suspected ketamine user via (a) MEKC; (b) sweeping MEKC; (c) solid-phase extraction and sweeping MEKC; and (d) selective ion current profile measured using GC/MS methods. Conditions are the same as those in Figs. 3 and 4.

the same sample (Fig. 7d). Although the separation remained similarly as that in Fig. 7c, the analysis time was almost twice that required for using the sweeping MEKC technique.

4. Conclusions

In this study, we have demonstrated successfully the use of on-line sample preconcentration for determining the presence of K and NK by sweeping MEKC, which is an easy, rapid, and efficient technique. We have presented our results in a three-dimensional plot to provide a clear depiction of the conditions that effect the optimal separation. Under the optimized separation parameters, the analysis times for K, NK, and K-D_4 were less than 5 min, which is much faster than similar results obtained by GC/MS. The optimized parameters for the sweeping MEKC method were: running buffer, 25 mM citrate/phosphate (pH 2.6); applied voltage, −25 kV; temperature, 25 °C; SDS concentration, 75 mM. The limits of detection were 2.8, 3.4, and 3.3 ng/mL for K, NK, and K-D_4, respectively, and the enrichment factor for each compound fell within the range of 540–800. Accordingly, sweeping in conjunction with MEKC represents a good method that is complementary to GC/MS for use in clinical and forensic analyses.

Acknowledgements

This research was supported by grant (NSC 92-2113-M-009-031) from the National Science Council of Taiwan ROC. We thank the Forensic Science Center (Taiwan Command of the Army Force of Military Police) for donating the urine samples.

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Journal of Chromatography A, 1110 (2006) 240–244

JOURNAL OF CHROMATOGRAPHY A

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Sweeping technique combined with micellar electrokinetic chromatography for the simultaneous determination of flunitrazepam and its major metabolites

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Received 23 July 2005; received in revised form 15 January 2006; accepted 17 January 2006
Available online 17 February 2006

Abstract

A sweeping technique, in conjunction with micellar electrokinetic chromatography, for the simultaneous determination of flunitrazepam and its major metabolites, 7-aminoflunitrazepam and *N*-desmethylflunitrazepam, is described. The optimized conditions for the sweeping and separation were a pH 9.5 buffer, 25 mM borate, 50 mM cetyltrimethylammonium bromide, 30% MeOH (v/v), and a 151-mm injection length. The calibration functions were all linear with the coefficient of determination (r^2) exceeding 0.996 for the three target compounds. Using the sweeping procedure, the limits of detection were determined to be 13.4, 5.6, and 12.0 ng/mL for flunitrazepam, 7-aminoflunitrazepam, and *N*-desmethylflunitrazepam, respectively, and the sensitivity enhancement for each compound was within the range of 110–200 fold. The RSDs for the retention time and the peak area were less than 4.10%. The optimized sweeping method was also used to examine a spiked urine sample. We conclude that sweeping with micellar electrokinetic chromatography has considerable potential use in clinical and forensic analyses of flunitrazepam and its metabolites.
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Keywords: Sweeping; Micellar electrokinetic chromatography; Flunitrazepam; Cetyltrimethylammonium bromide

1. Introduction

Flunitrazepam (Rohypnol), a nitro-containing benzodiazepine, is used as a hypnotic and anesthetic induction agent. It is administered orally or by intravenous injection at doses of 2 mg. It has physiological effects similar to those of other benzodiazepines and has a potency that is ca. 10 times that of benzodiazepine. The illicit use of flunitrazepam usually involves a combination of other drugs, although it may be used alone. It has been used illegally in Asia since the early 1980s. In Taiwan, it appears to be used most frequently in conjunction with alcohol, with which it seems to have a synergistic effect, producing disinhibition and amnesia. This has given flunitrazepam, especially tasteless and odorless solutions, the reputation of being a "date-rape" drug.

Flunitrazepam can be detected in blood, plasma, and urine [1,2]. Because of its low dosage, biotransformation through *N*-demethylation, and the high volume of distribution, flunitrazepam and its metabolites occur at low blood levels after therapeutic administration [3]. Fig. 1 shows the pathway for flunitrazepam metabolism. Two major metabolites of flunitrazepam – 7-aminoflunitrazepam and *N*-desmethylflunitrazepam – can be detected when flunitrazepam is injected or mixed into drinks [4].

Because of the rapid growth in the extent of abuse of flunitrazepam, a simple and consistent method is needed for its determination. Some analytical techniques for detecting flunitrazepam have been reported, including the use of immunoassays [5], high-performance liquid chromatography (HPLC) [6,7], and gas chromatography/mass spectrometry (GC/MS) [8,9]. From the perspective of qualitative analysis, GC/MS provides additional spectral information as well as excellent sensitivity. Although GC/MS is capable of providing reliable data that can usually be used as scientific proof in a court of law, the method has disadvantages in that it involves time-consuming derivatization prior to the GC/MS analysis.

In recent years, capillary electrophoresis (CE) has expanded its scope and range in both instrumentation and applications

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doi:10.1016/j.chroma.2006.01.070

Fig. 1. The major metabolic pathway for the detoxification of flunitrazepam in humans.

[10]. CE has proven to be a powerful analytical tool for separating charged species in diverse samples because of its many advantageous features, which include high column efficiency, rapid analysis times, and small sample volumes. However, the benefits derived by the high separation efficiency of CE can be overshadowed by its low UV detection sensitivity. Thus, using on-line sample preconcentration to overcome the poor sensitivity of CE has been the focus of a number of investigations [11,12]. For example, Quirino and Terabe [13] reported that neutral compounds could be concentrated effectively, when the technique of micellar electrokinetic chromatography (MEKC) combined with stacking was utilized. They later reported a sweeping method that can pick and accumulate neutral or charged analytes into a narrow zone by the pseudostationary phase in MEKC [14–16].

In this paper, we report on an approach involving the use of a sweeping technique combined with MEKC for the simultaneous determination of flunitrazepam and its major metabolites, 7-aminoflunitrazepam and *N*-desmethylflunitrazepam. The effects of the buffer pH, buffer concentration, cationic surfactant, organic modifier, and injection length on the analysis are described. We optimized the sweeping MEKC conditions to enhance the detection sensitivity with satisfactory resolution. We also employed the optimized sweeping MEKC method in an examination of a spiked urine sample.

2. Materials and methods

2.1. Apparatus

CE analysis was performed on a Beckman P/ACE MDQ CE system equipped with a photodiode-array detector (Fullerton, CA, USA). A personal computer, controlled by Beckman Coulter MDQ 32 Karat software was used for data collection. A 60 cm (50 cm to the detector) × 50 μm I.D. fused-silica capillary tube (Polymicro Technologies, Phoenix, AZ, USA) was used. The capillary column was assembled in a cartridge format. The temperature of the capillary tube during electrophoresis was maintained at 25 °C. The electrophoresis separation was performed at an applied voltage of −25 kV. Sample was pressure-injected at 0.5 psi with an extended time. The UV absorption detector was set at 240 nm for sweeping MEKC.

2.2. Chemicals

Flunitrazepam, 7-aminoflunitrazepam, and *N*-desmethylflunitrazepam were purchased from Radian International (Austin, TX, USA). Sodium tetraborate was obtained from Sigma (St. Louis, MO, USA). Cetyltrimethylammonium bromide (CTAB) was purchased from Merck (Hohenbrunn, Ger-

many). All other chemicals were analytical grade. Water was purified using a Milli-Q water system (Millipore, Bedford, MA, USA) and filtered through a 0.22-μm filter.

2.3. Procedure

New capillaries were conditioned prior to separation by washing with methanol, water, 1 M NaOH, and water for 10 min each. The capillary was flushed between runs with 0.1 M NaOH, methanol, and water for 3 min each. For the sweeping MEKC procedure, the stock solutions were diluted with a buffer solution that did not contain CTAB surfactants. The borate background solution (BGS) contained an appropriate amount of CTAB and methanol. The BGS was first passed through the capillary for 3 min and the sample solution was then pressure-injected into the capillary. Finally, voltages were applied at negative polarity. Other experimental conditions are described in the Section 3.

The SPE cartridges (Oasis MCX, 3 mL/60 mg) were conditioned with 2 mL methanol and 2 mL H_2O. A 3-mL urine sample was spiked with 30-μL of a standard solution (10 μg/mL), then mixed with 60 μL pH 5.0 phosphate buffer (1 M) and passed through the cartridge. The cartridge was washed with 2 mL HCl (0.1 M) and 2 mL methanol, and was then dried under a vacuum for 10 min. The analytes were eluted with 3 mL dichloromethane/2-propanol/ammonium hydroxide solution (78:20:2, v/v/v). This organic solution was then evaporated to dryness. The residue was dissolved in 300 μL buffer and was used directly for MEKC.

3. Results and discussion

3.1. Effects of separation conditions for flunitrazepam and its major metabolites

Flunitrazepam and its metabolites (Fig. 1) are hydrophobic substances, with a neutral charge in slightly or strongly basic environments, which could interact with micelles. Thus, a sweeping technique using CTAB was employed to achieve online sample concentration [15,16]. After the voltage was applied with a negative polarity from inlet (cathode), the EOF, under the influence of the cationic CTAB surfactant, moved toward the outlet (anode). Because the velocity of the EOF was higher than that of the CTAB micelle, the analytes stacked at the boundary by the CTAB micelle and moved toward the anode.

When performing the analysis using MEKC, the pH and concentration of the buffer solution were adjusted so as to obtain adequate separation. The migration times of the analytes increased with increasing pH value from pH 9.5 to pH 10.5 with a similar sensitivity enhancement. The peaks also broadened and their heights decreased for electrolyte concentrations lower than 25 mM. The migration time of the analytes was delayed with increasing electrolyte concentration. Taking all of these phenomena into consideration, we conclude that the pH 9.5 buffer with 25 mM electrolyte is the most suitable for the separation.

In the basic buffer solution, flunitrazepam and its metabolites acted as neutral analytes, and migrated with the electroosmotic flow. When they interacted with the positively charged CTAB micelles, however, the decrease in their apparent electrophoretic

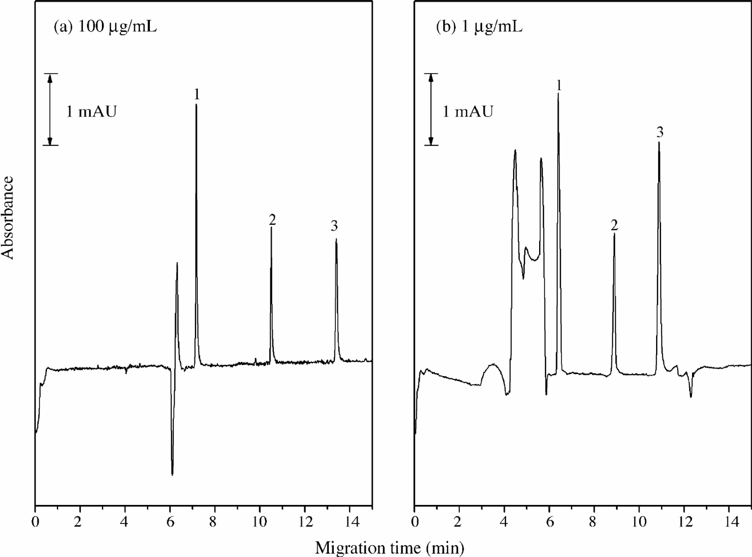

Fig. 2. Normal MEKC and sweeping MEKC analysis of flunitrazepam and its major metabolites. (a) MEKC analysis. Analyte concentration: 100 μg/mL; injection length: 1.51 mm. (b) Sweeping MEKC analysis. Analyte concentration: 1 μg/mL; injection length: 151 mm. Conditions: capillary, 60 cm long (50 cm to detector), 50 μm I.D.; buffer solution: 25 mM borate buffer (pH 9.5), 50 mM CTAB, 30% CH_3OH (v/v), conductivity 7.28 mS/cm; sample matrix: 25 mM borate buffer (pH 9.5); separation voltage: −25 kV; UV detection at 240 nm. Peak identification: peak 1, 7-aminoflunitrazepam; peak 2, flunitrazepam; peak 3, *N*-desmethylflunitrazepam.

Table 1
Calibration lines, coefficient of determination (r^2), limits of detection (LODs), migration times, and values of RSD for flunitrazepam, 7-aminoflunitrazepam, and *N*-desmethylflunitrazepam using the MEKC and sweeping MEKC techniques

	Flunitrazepam	7-Aminoflunitrazepam	*N*-desmethyl-flunitrazepam
MEKC			
Calibration line[a]	$y = 101x + 224$	$y = 130x - 121$	$y = 71.5x - 228$
Coefficient of determination	$r^2 = 0.997$	$r^2 = 0.999$	$r^2 = 0.997$
LOD (S/N = 3, μg/mL)	1.87	0.52	1.74
Migration time (min)	10.54	7.17	13.10
RSD (%; $n = 5$)			
I. Migration time	0.55	0.25	0.62
II. Peak area	2.85	2.04	1.48
Sweeping MEKC			
Calibration line[b]	$y = 11.5x - 682$	$y = 28.2x - 853$	$y = 24.7x - 3.10 \times 10^3$
Coefficient of determination	$r^2 = 0.996$	$r^2 = 0.999$	$r^2 = 0.998$
LOD (S/N = 3, ng/mL)	13.4	5.6	12.0
Migration time (min)	8.48	6.21	10.24
RSD (%; $n = 5$)			
I. Migration time	0.39	0.28	0.51
II. Peak area	1.14	2.01	4.10
SE_{height}[c]	110	140	200

[a] Calibration line (10–200 μg/mL): peak area (arbitrary units) = slope × concentration (μg/mL) + y-intercept.
[b] Calibration line (50–1000 ng/mL): peak area (arbitrary units) = slope × concentration (ng/mL) + y-intercept.
[c] SE_{height} = (peak height obtained with sweeping MEKC/peak height obtained with MEKC)·dilution factor.

velocities caused these analytes to become focused. When the CTAB concentration was increased from 10 to 50 mM, separation and peak height improved, suggesting that the sweep effect became more efficient. Nevertheless, when the CTAB concentrations exceeded 50 mM, the separations became poor. These results suggest that the use of 50 mM CTAB provides the best condition for the separation.

Increasing the percentage of methanol in the buffer had a dramatic influence on the analyte migration time and the peak focusing effect. The results showed that adding an organic solvent to the buffer modified the polarity of the BGS, which further changed the EOF. It also improved the resolution by modifying the partition of the analytes between the solution phase and the micelle phase. The experimental results indicate that adding 30% methanol to the buffer solution provided the best condition for the separation.

In general, prolonging the sample injection length in sweeping MEKC is advantageous, in terms of achieving better sensitivity for a separation. A long sample zone, however, increases the sweeping time and may have a negative influence on the efficiency of the sweeping procedure. Using the optimal conditions discussed above, we found that an injection length of 151 mm is suitable for the complete separation of all the peaks.

3.2. Comparing normal MEKC and sweeping MEKC

Fig. 2 depicts the results of normal MEKC and the sweeping MEKC separation of flunitrazepam, 7-aminoflunitrazepam, and *N*-desmethylflunitrazepam under optimized conditions. Fig. 2a was obtained when the sample solution was the same as the running buffer, but did not contain micelles. The concentration of each analyte was 100 μg/mL. Fig. 2b was obtained in a manner

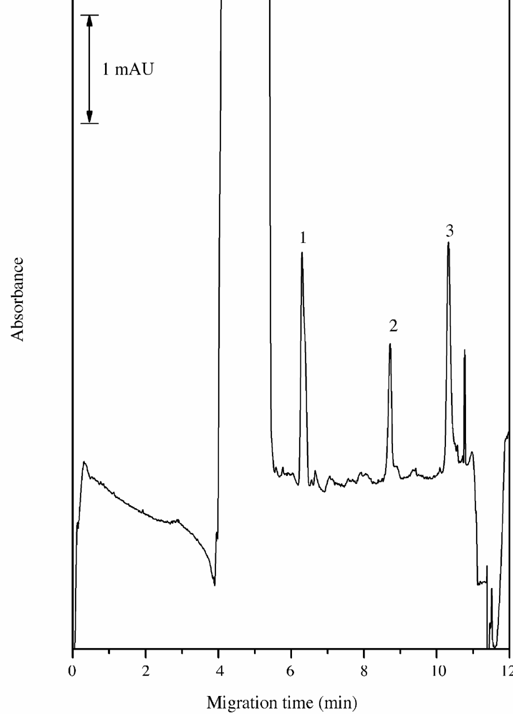

Fig. 3. Sweeping MEKC electropherogram of a spiked urine sample. Analyte concentration: 0.3 μg spiked in a 3-mL urine sample before SPE extraction. The other conditions are the same as in Fig. 2.

similar to that of Fig. 2a, but the injection length was 151 mm and the sample concentration was diluted 100-fold. The sensitivity enhancement in terms of peak heights (SE_{height}) for the three analytes was calculated. Flunitrazepam, 7-aminoflunitrazepam, and *N*-desmethylflunitrazepam had ca. 110-, 140-, and 200-fold enhancements in their detection sensitivities, respectively.

Table 1 presents the calibration lines, coefficient of determination (r^2), limits of detection (LODs), migration times, and RSDs for the three analytes using MEKC and sweeping MEKC techniques. For analyses conducted using the normal MEKC procedure, the LODs were in the low μg/mL range. When the sweeping MEKC procedure was used, the LODs were less than 13.4 ng/mL. Table 1 also presents the reproducibility of the migration times and peak area. The RSD for the migration time was less than 0.62% for either separation procedure. The RSD of the peak area was also less than 4.10%. According to these results, both processes are acceptable separation methods, but the sweeping MEKC procedure is superior to MEKC in detection sensitivity.

Fig. 3 illustrates the use of the sweeping MEKC method in analyzing a urine sample spiked with flunitrazepam and its metabolites. The separation of these analytes in urine was adequate. The total separation time was less than 12 min. Thus, sweeping MEKC can be used as a rapid screening method for the analysis of flunitrazepam and its major metabolites.

In conclusion, we report that a sweeping technique combined with MEKC permits the simultaneous determination of flunitrazepam and its major metabolites through a process that is easily performed, and does not require a derivatization step. The optimized parameters for the sweeping MEKC method were: running buffer, 25 mM borate buffer (pH 9.5); CTAB, 50 mM; organic modifier, 30% MeOH (v/v); injection length, 151 mm. The LODs ranged from 5.6 to 13.4 ng/mL. Accordingly, sweeping in conjunction with MEKC represents an alternative approach with enhanced sensitivity for analyzing flunitrazepam and its major metabolites.

Acknowledgement

This study was supported by grant (NSC 93-2113-M-009-022) from the National Science Council of Taiwan.

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Journal of Chromatography A, 1111 (2006) 267–271

JOURNAL OF CHROMATOGRAPHY A

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Fabrication of microfluidic devices using dry film photoresist for microchip capillary electrophoresis

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Available online 27 December 2005

Abstract

An inexpensive, disposable microfluidic device was fabricated from a dry film photoresist using a combination of photolithographic and hot roll lamination techniques. A microfluidic flow pattern was prefabricated in a dry film photoresist tape using traditional photolithographic methods. This tape became bonded to a poly(methyl methacrylate) (PMMA) sheet with prepouched holes when passed through a hot roll laminator. A copper working electrode and platinum decoupler was readily incorporated within this microchip. The integrated microchip device was then fixed in a laboratory-built Plexiglas holder prior to its use in microchip capillary electrophoresis. The performance of this device with amperometric detection for the separation of dopamine and catechol was examined. The separation was complete within 50 s at an applied potential of 200 V/cm. The relative standard deviations (RSD) of analyte migration times were less than 0.71%, and the theoretical plate numbers for dopamine and catechol were 3.2×10^4 and 4.1×10^4, respectively, based on a 65 mm separation channel.

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Keywords: Microchip capillary electrophoresis; Amperometric detection; Dry film photoresist; Microfluidic devices

1. Introduction

Miniaturized microfluidic devices in which features of photolithographic technologies and capillary electrophoresis (CE) are combined, have recently become a major focus of interest for the preparation of micro total analysis systems (μTAS, also known as lab-on-a-chip systems). Microfluidic devices have been used in electrophoretic separations of a variety of biochemical and chemical analytes [1–4]. These devices have typically been prepared from glass [5–9], quartz, silicon, and a range of polymeric materials [10–16]. Polymeric materials, such as poly(methyl methacrylate) (PMMA) [10–13] and poly(dimethylsiloxane) (PDMS) [14–16], have great potential for the large-scale fabrication of disposable microfluidic device for use in analytical systems, due to their ease of fabrication, low cost, and great versatility. For polymeric devices, a number of processing techniques can be used to create the required network of microchannels, and all of these approaches have been employed and examined for use in the fabrication of polymeric microfluidic devices including photolithography, wire imprinting, hot embossing, powder ablation, laser photoablation, casting, and injection molding [17–23].

In recent years, disposable plastic-based substrates have become extremely popular for use in microfluidic applications owing to the fact that they are easily and inexpensively fabricated. Sudarsan and Ugaz demonstrated the use of printed circuit technology for the fabrication of plastic-based microfluidic devices [24]. do Lago et al. established a dry process for the production of microfluidic devices based on a xerographic process and the lamination of laser-printed polyester films [25].

Although most photoresists are generally considered to be sacrificial materials, liquid-type negative photoresists, such as SU-8, has been used to create microchannels within microfluidic chips [26–28], and these may play an important role as a structural component of a microfluidic device. Concerning the use of a photoresist as a structural material, the thickness of the photoresist determines the depth of the microchannel; therefore, controlling over the thickness of the photoresist is extremely important in this process. Alternatively, the dry film photoresist was originally developed for printed circuit board (PCB) fabrication could be used. Compared to a liquid photoresist, the dry film photoresist offers a variety of advantages, including good conformability, excellent adhesion to other substrates, uniform distribution, no liquid handling, low exposure energy, and short

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doi:10.1016/j.chroma.2005.12.003

processing time [29]. Furthermore, a commercially available dry film photoresist itself has a uniform thickness, since it is used for constructing micro channels with a specified depth.

In the present study, we report on the fabrication and application of a new plastic microchip that features a negative dry film photoresist (an acrylate-based photopolymer) as a structural material laminated in a PMMA sheet. Off-channel amperometric detection was employed to evaluate the performance of the dry film photoresist-based microchip. A platinum wire electrode serving as a decoupler along with a copper working electrode, which was incorporated into the bottom PDMS sheet. Catechol was used to demonstrate the performance of the microchip CE with an amperometric detector. Dopamine was added to the catechol to evaluate the efficiency of separation of the microchip CE.

2. Experimental

2.1. Chemicals

The 75-μm negative dry film photoresist (AF-5075) was obtained from CCP (Hsinchu, Taiwan). PMMA sheets were purchased from a local hardware store. The Sylgard 184 prepolymer and its curing agent were purchased from Dow Corning (Midland, MI, USA). Disodium hydrogenphosphate, sodium dihydrogenphosphate, and other electrophoresis chemicals were obtained from Fluka (Buchs, Switzerland). Catechol and dopamine were purchased from Sigma (St. Louis, MO, USA). All chemicals were of analytical reagent grade; stock solutions were prepared before each experiment and were stored under refrigeration in the dark. Water was purified with a Milli-Q water system (Millipore, Bedford, MA, USA) and solutions were filtered through 0.45-μm MFS-13 filters (Advantec, Dublin, CA, USA).

2.2. Apparatus

The photolithographic procedures involved the use of a Tah-Hsin TCC-6000 hot roller (Taipei, Taiwan) for pressing, a Union EMA-400 UV aligner (Tokyo, Japan) for exposing, and an auto-development machine for developing. The detection system was a CH 8021b electrochemical analyzer (Austin, TX, USA) coupled to the working, auxiliary, and reference electrodes through sockets. The working electrode was a 50 μm 99.99% copper wire and was given as a gift from Yeou-Chuen Wire (Taoyuan, Taiwan). A Major Science MP-5000-250P high-voltage power supply system (Taipei, Taiwan) with adjustable voltage ranging from 0 to +5 kV was used to power the microchip CE separation.

2.3. Microchip fabrication and assembly

The complete procedure used in the chip fabrication is illustrated in Fig. 1. The photolithographic masks were designed using standard computer software (AutoCAD 2000) and transferred to a transparent film. The microchannel on the mask was represented by a 100-μm-wide black line. The PMMA substrate had a size of 30 mm × 85 mm and a thickness of 1 mm. The PMMA substrate was washed with water and ethanol and then dried under a flow of high-pressure air from a compressor. A dry film photolithography fabrication technique was used to create the microfluidic channels in the PMMA-based chip in a yellow-light environment. In a typical run, the PMMA substrate was hot rolled on a 75-μm-thick layer of negative dry film photoresist (30 mm × 85 mm) before being covered with the photomask and exposure to UV light. According to the properties of this negative photoresist, the parts which were covered by the pattern of the mask were rinsed out upon development with a 1% aqueous sodium carbonate solution. The developing conditions were controlled by an automated developing machine. To strengthen the structure of the dry film photoresist, the microchip was subjected to a post bake under a 400-W UV light source for 5 min after its production.

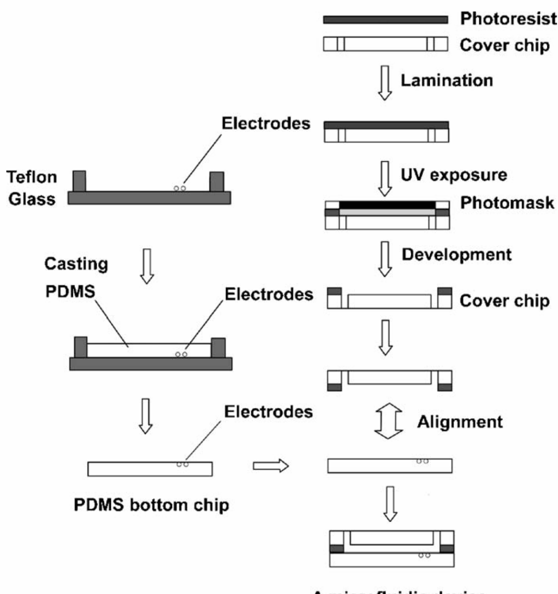
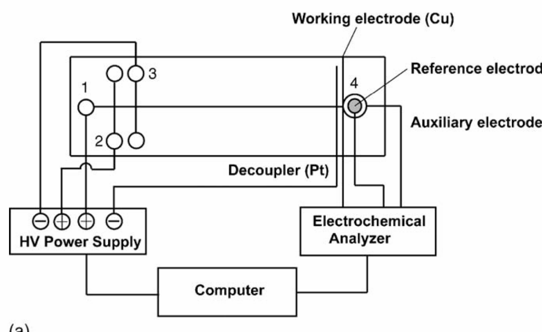


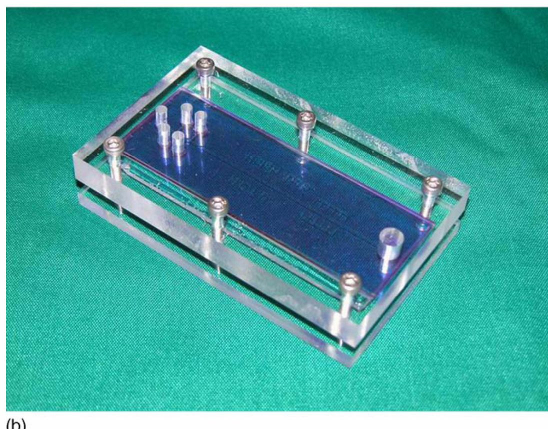
Fig. 1. Schematic diagram of the dry film photolithographic process and lamination procedures for integrating the microchip with an electrochemical detector. The Pt wire decoupler and Cu wire working electrode were embedded in the bottom PDMS sheet.

Electrodes (Pt and Cu wires) were placed 2 mm apart on a laboratory-built Plexiglas mold with two fillisters to fix the electrodes. Before placing electrodes on the mold, they were cleaned with ethanol. A mixture of Sylgard 184 prepolymer and curing agent (10:1) was stirred thoroughly and degassed in a vacuum for 15 min. The prepolymer mixture was then poured onto the Plexiglas mold and cured at 60 °C for 1 h to form a 2-mm thick PDMS sheet (30 mm × 85 mm).

Fig. 2a shows a schematic illustration of a laboratory-built microchip CE with the amperometric detection system used in this study. The effective separation length of microchannel was 65 mm from the injection zone to the working electrode with a double-T injection channel design. As shown in Fig. 2b, the



(a)



(b)

Fig. 2. Schematic diagram and photographs of the microchip CE system. (a) Layout of the microchip CE with electrochemical detection. (b) Photograph of an electrophoresis microchip fixed in a laboratory-built Plexiglas chip holder.

microchip assembly with electrodes was fixed in a laboratory-build Plexiglas chip holder. Six stainless steel screws were used to house the microchip. The cover holder also serves as sample, buffer, and electrode reservoirs with five 3-mm holes and one 6-mm holes. To prevent leakage of the solution, the dry-film-based chip was clipped between two pieces of PDMS; one piece contained the electrode and the other contained six holes corresponding to the reservoirs on the dry-film-based chip. Because of the elasticity of PDMS, when the holder was compressed tightly, the reservoirs were sealed in a manner analogous to the operation of an O-ring. No leakage from the reservoirs or channels was observed. The electrodes were positioned beneath the separation channel in order to allow buffer to flow past the detector. The copper wire functioned as the working electrode and was located 1 mm before the channel outlet. The Pt wire served as the ground electrode as well as the decoupler.

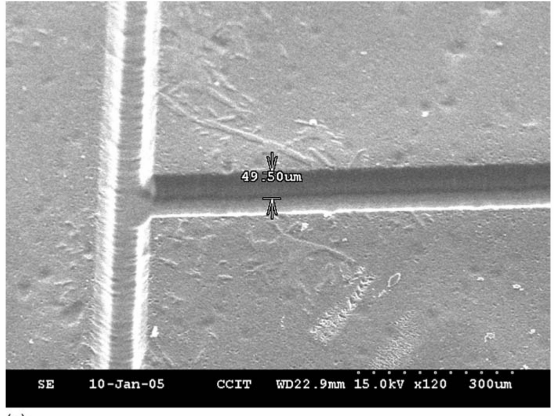
2.4. Procedure

Before conducting each electrophoresis experiment, the channels of the microchip were rinsed with purified water and 1 mM phosphate buffer (pH 7.4) for 10 min each. All standard solutions of analytes (1 mM) were prepared in water, stock solutions were diluted with running buffer to the desired concentrations. The reservoirs were filled with running buffer and the sample and a sample injection potential of 100 V/cm was then applied for 15 s. Separation was then initiated by switching to different voltages across the separation channel.

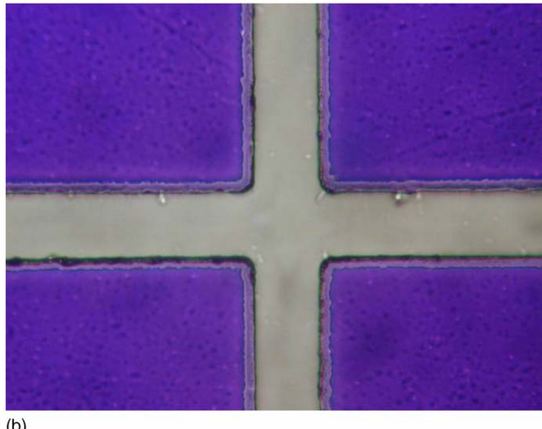
3. Results and discussion

3.1. Fabrication of microchip using dry film photoresist

Prior to the assembly of the microchip, the quality of the microchannels was evaluated by optical microscopy. Fig. 3 shows an SEM image and a photograph of the micro channels on the substrate, produced by dry film photoresist and conventional photolithographic techniques. The observed sidewall



(a)



(b)

Fig. 3. Microscopic images of channels in microchips: (a) SEM image showing a microchannel fabricated in a dry film photoresist and (b) photographic image showing a cross section of microchannels.

images, depicting smooth surfaces and sharp edges, reveal that the microchannel fabricated using dry film photoresist was adequate.

This dry film photolithographic method is a mature and readily accessible technique that can be performed simply and continuously without the need for a restrictive and expensive clean room operating environment. This process can be performed without the use of a spin coating apparatus and does not include time-consuming baking procedures. It is obviously a rapid alternative to the use of a SU-8 liquid type photoresist. Thus, this dry film photolithographic technique could be used for the rapid integration of prototype microchips.

3.2. Performance of microchip CE

After conditioning the microchip in the microchip CE system by running buffer, it was used in the measurement of 500 μM catechol, in order to evaluate the performance of the device. The catechol migrated within 50 s under a 200 V/cm electrical field. The peak width at half height was less than 0.60 s, superior to the same analyte detected using an end-channel detector [30,31]. The sharp analyte peak is probably due to the off-channel detection which eliminates the band-broadening effect [32,33].

To evaluate the performance of the microchip CE device, 50 runs of injection and separation sequences were conducted on 500 μM catechol. Some selected results are presented in Fig. 4. Using a pH 7.4 buffer and 200 V/cm field strength, the average migration time for catechol was 45.8 s. The relative standard deviation (RSD) of the migration time was 1.01% ($n = 5$). We also investigated chip-to-chip reproducibility by running three CE microchips. Each chip led to similar RSD for the migration times. Because migration time is dependent on the distance between the injection zone and the working electrode, the absolute value for migration time should be calibrated for each different microchip.

The calibration curve for catechol was linear from 10 to 1000 μM with a correlation coefficiency (r) that exceeded 0.9996. As calculating from the 10 μM catechol signal, the limit of detection was 730 nM (S/N = 3). These results indicate that the amperometric detector in the microchip displayed a well-defined concentration dependence.

Fig. 4. Five electropherograms of catechol from a total of 50 consecutive runs, showing the first on the bottom and the 50th on the top. Conditions: sample injection: at 150 V/cm for 15 s; separation channel: 65 mm; separation voltage: 200 V/cm; running buffer: phosphate buffer (1 mM, pH 7.4); analyte: 500 μM catechol. Detection voltage of working electrode is +0.3 V (vs. Ag/AgCl).

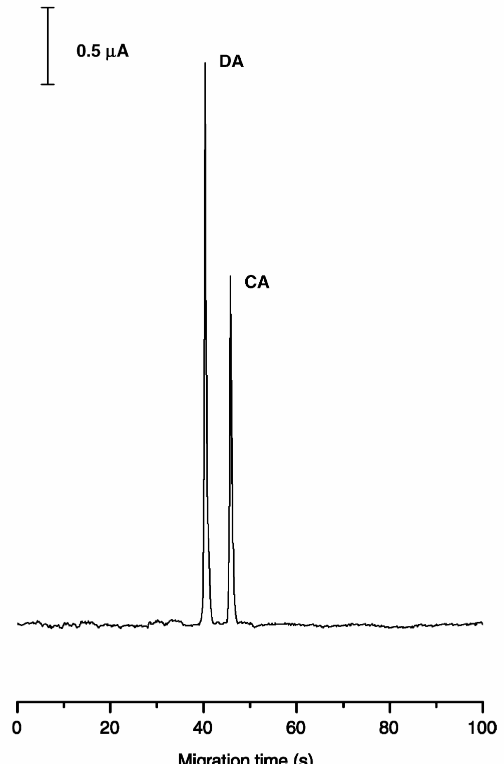


Fig. 5. Electropherogram from the separation of a mixture of dopamine (DA) and catechol (CA). Conditions: sample injection: at 150 V/cm for 15 s; separation channel: 65 mm; separation voltage: 200 V/cm; running buffer: phosphate buffer (1 mM, pH 7.4); analytes concentration: 500 μM. Detection voltage of working electrode is +0.3 V (vs. Ag/AgCl).

3.3. Separation of dopamine and catechol by microchip CE

The separation performance of the microchip CE device was subsequently examined by the separation of dopamine and catechol with the amperometric detector. Fig. 5 shows an electropherogram for the separation of dopamine and catechol by microchip CE. An adequate separation was achieved within 50 s. The average migration times of dopamine and catechol were 30.3 s and 45.8 s ($n = 5$), respectively. The RSD for analyte migration time was less than 0.71% and the RSD for analyte peak height was less than 3.0%. The peak widths at half height were less than 1.0 s for both analytes. The resolution between the two analytes was 2.87. The theoretical plate numbers (N) for dopamine and catechol were 3.2×10^4 and 4.1×10^4 based on a 65 mm separation channel, respectively. The results of the present study clearly demonstrate that this dry film photoresist based microchip can be used successfully in a microchip CE system. Finally, the simple fabrication process of the dry film photoresist-based microchip permits the rapid and convenient replacement of microchips in microchip CE platforms.

4. Conclusions

The dry film photolithographic technique is an alternative and effective method for the manufacture of plastic microchips. The advantages of using this dry film photoresist-based microchip in microchip CE systems include rapid fabrication, low cost, and ease of fit into the chip holders. Thus, other new concepts in the design and fabrication of μTAS microchip can be exploited.

Acknowledgement

This research was supported by grant number NSC 91-2113-M-009-025 from the National Science Council of Taiwan.

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6. **Hsiu-Ping Jen**, Chiung-Wei Huang, Hsiu-Li Su, You-Zung Hsieh*, “Analysis of a wide variety of illicit drugs using cation-selective exhaustive injection/sweeping- micellar electrokinetic chromatography”, ***Journal of Chromatography A.*** (Submitted)
7. **Hsiu-Ping Jen**, Kuan-Wen Lin, Yen-Kai Huang, You-Zung Hsieh*, “On-chip micellar electrokinetic chromatographic separation of amphetamine, methamphetamine, and ephedrine with electrochemical detection”, ***Electrophoresis.*** (Submitted)

Abbreviation

A

1. 6-acetylmorphine：AM
2. Amphetamine：AP

B

1. Background electrolyte：BGE
2. Background solution：BGS
3. Buffer waste：BW

C

1. Capillary electrophoresis：CE
2. Cation-selective exhaustive injection/sweep-micellar electrokinetic chromatography：CSEI-sweep-MEKC
3. Central nervous system：CNS
4. Cetyltrimethylammonium bromide：CTAB
5. Cocaine：CC
6. Codeine：CD

D

1. Deaminonorketamine：DANK
2. 5, 6-dehydronorketamine：DHNK
3. Dry-film-based microchip electrophoresis：DFB-MCE

E

1. Electrochemical：EC
2. Electroosmotic flow：EOF
3. Ephedrine：EP
4. Enzymes multiplied immunoassay technique：EMIT

F

1. Field-amplified sample stacking：FASS

G

1. Gas chromatography/mass spectrometry：GC/MS

H

1. Half-life：$T_{1/2}$
2. Heroin：HR
3. High-conductivity buffer：HCB
4. High-performance liquid chromatography：HPLC
5. Hydrodynamic voltammetric：HDV

I

1. Inside diameter：I.D.
2. Internal standard：I. S.
3. Isotachophoretic sample stacking：ITPSS

K

1. Ketamine：K

L

1. Large-volume sample stacking：LVSS
2. Laser-induced fluorescence：LIF
3. Liquid chromatography/mass spectrometry：LC/MS
4. Liquid-liquid extraction：LLE
5. Limit of detection：LOD
6. Limit of quantitation：LOQ
7. Lysergic acid diethylamide：LSD

M

1. Mass selective detector：MSD
2. Methamphetamine：MA
3. Micrototal analysis system：μ-TAS
4. Micellar electrokinetic chromatography：MEKC
5. Microchip electrophoresis：MCE
6. Morphine：MP

N

1. Norketamine：NK

P

1. Pentafluoropropionic anhydride：PFPA
2. Phencyclidine：PCP
3. Polycarbonate：PC

4. Poly(dimethylsiloxane)：PDMS
5. Poly(ethylenterephthalate)：PET
6. Poly (methylmethacrylate)：PMMA

S

1. Sample waste：SW
2. Selected ion monitoring：SIM
3. Sensitivity enhancement in terms of peak height：SE_{height}
4. Signal-to-noise ratio：S/N
5. Sodium dodecyl sulfate：SDS
6. Solid phase extraction：SPE
7. Solid phase microextraction：SPME

T

1. The effective mobilities：$\mu_{effective}$

Schedules and Items of Controlled Drugs

Schedule Ⅰ Controlled Drugs (including their salts)

Items		Notes
1	Acetorphine	Narcotic
2	Cocaine (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram per milliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	Narcotic
3	Desomorphine	Narcotic
4	Dihydroetorphine	Narcotic
5	Etorphine	Narcotic
6	Heroin (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram per milliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	Narcotic
7	Ketobemidone	Narcotic
8	Opium	Narcotic
9	Morphine [Does not include test kits prepared with organic solvent and with a content less than 1.0 millgram per milliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.]	Narcotic

Schedule Ⅱ Controlled Drugs (including their salts)

Items		Notes
1	Acetyl-alpha-methylfentanyl	Narcotic
2	Acetyldihydrocodeine	Narcotic
3	Acetylmethadol	Narcotic
4	Alpha-methyl-fentanyl	Narcotic
5	Alpha-methadol	Narcotic
6	Alpha-methyl-thiofentanyl	Narcotic
7	Alphaprodine	Narcotic
8	Alfentanyl	Narcotic
9	Allylprodine	Narcotic
10	Alphacetyl-methadol	Narcotic
11	Alphameprodine	Narcotic
12	Amphetamine (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	
13	Anileridine	Narcotic
14	Benzethidine	Narcotic
15	Benzylmorphine	Narcotic
16	Betacetylmethadol	Narcotic
17	Betahydroxyfentanyl	Narcotic
18	Betahydroxy-3 methyl-fentanyl	Narcotic
19	Betameprodine	Narcotic
20	Betamethadol	Narcotic
21	Betaprodine	Narcotic
22	Bezitramide	Narcotic
23	Brolamfetamine	
24	Cannabis (Does not include the mature stems of entire cannabis plants and their products (except resins) and products of the seeds of entire cannabis plans that are not capable of germination.	Narcotic
25	Cannabis resin	Narcotic
26	Cannabis extracts	Narcotic
27	Cannabis tinctures	Narcotic
28	Carfentanyl	Narcotic
29	Cathinone (Does not include test kits prepared with organic	

	solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	
30	Clonitazene	Narcotic
31	Coca	Narcotic
32	Coca leaves	Narcotic
33	Codeine and its preparations with a content more than 5.0grams of codeine per 100 milliliters (or 100 grams).(Does not include test kits prepared with organicsolvent and with a content less than 1.0 milligramper milliliter, packaging less than 1.0 milliliter,and treated with radioactive material, antibodymarkers, or not directly usable in the human body.)	Narcotic
34	Codeine-methyl-bromide	Narcotic
35	Codeine-N-oxide	Narcotic
36	Codoxime	Narcotic
37	Concentrated Poppy straw	Narcotic
38	Cyprenorphine	Narcotic
39	Dexamphetamine (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	
40	Dextromoramide	Narcotic
41	Dextropropoxyphene (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram per milliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	Narcotic
42	Diampromide	Narcotic
43	Diethylthiambutene	Narcotic
44	DET	
45	Difenoxin	Narcotic
46	Dihydrocodeine and its preparation with a content more than 5.0 grams of dihydrocodeine per 100 milliliters (or 100 grams)(Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram per milliliter, packaging less than 1.0 milliliter, and treated with radioactive	Narcotic

	material, antibody markers, or not directly usable in the human body.)	
47	Dihydromorphine	Narcotic
48	Dimenoxadol	Narcotic
49	Dimepheptanol	Narcotic
50	Dimethylthiambutene	Narcotic
51	DMT	
52	Dioxaphetyl butyrate	Narcotic
53	Diphenoxylate	Narcotic
54	Dipipanone	Narcotic
55	DMA	
56	DMHP	
57	DOET	
58	DOM, STP	
59	Drotebanol	Narcotic
60	Ecgonine (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	Narcotic
61	Ecgonine Derivatives (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram per milliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.	Narcotic
62	Ethylmethyl-thiambutene	Narcotic
63	Ethylmorphine (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	Narcotic
64	Eticyclidine	
65	Etonitazene	Narcotic
66	Etoxeridine	Narcotic
67	Fentanyl (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human	Narcotic

	body.)	
68	Fenetylline	
69	Furethidine	Narcotic
70	Hydromorphinol	Narcotic
71	Hydrocodone (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	Narcotic
72	Hydromorphone (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	Narcotic
73	Hydroxypethidine	Narcotic
74	Ibogaine (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	
75	Isomethadone	Narcotic
76	Levamphetamine (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	
77	Levomethorphan	Narcotic
78	Levomoramide	Narcotic
79	Levorphanol (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	Narcotic
80	Levophenacyl-morphan	Narcotic
81	LSD, Lysergide (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in	

	the human body.)	
82	MDA (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram per milliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	
83	MDMA, N-a-dimethyl-3.4-(methylenedioxy)phenethylamine) (Doesnot include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	
84	Mecloqualone	
85	Mescaline (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	
86	Metazocine	Narcotic
87	Methadone (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	Narcotic
88	Methadone-intermediate	Narcotic
89	Methamphetamine (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	
90	Methamphetamine Racemate (Does not include test kits prepared with organic solvent and with a content less than 1.0milligram per milliliter, packaging less than 1.0milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	
91	Methaqualone (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive	

	material, antibody markers, or not directly usable in the human body.)	
92	4-methylaminorex	
93	Methyldesorphine	Narcotic
94	Methyldihydromorphine	Narcotic
95	3-Methylfentanyl (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram per milliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	Narcotic
96	3-Methylthio-fentanyl	Narcotic
97	Metopon	Narcotic
98	MMDA, 2-methoxy-a-methyl-4.5-(methylenedioxy)phenethylamine)	
99	Moramide-intermediate	Narcotic
100	Morphine methobromide	Narcotic
101	Morphine methylsulfonate	Narcotic
102	Morphine-N-oxide and its Derivatives	Narcotic
103	MPPP, 1-methyl4-phenyl-4-piperidinol propionate (ester)	Narcotic
104	Myrophine	Narcotic
105	Nabilone	
106	N-ethyl-amphetamine (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram per milliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	
107	N-ethyl-MDA	
108	N-ethyl-3-piperidyl benzilate	
109	N-hydroxy-MDA	
110	N-methyl-3-piperidyl benzilate	
111	Nicodicodine	Narcotic
112	Nicocodine	Narcotic
113	Nicomorphine	Narcotic
114	N-N-dimethyl-amphetamine	
115	Noracymethadol	Narcotic
116	Norcodeine (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive	Narcotic

	material, antibody markers, or not directly usable in the human body.)	
117	Norlevorphanol	Narcotic
118	Normethadone	Narcotic
119	Normorphine (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	Narcotic
120	Norpipanone	Narcotic
121	Opium Poppy	Narcotic
122	Oxycodone (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	Narcotic
123	Oxymorphone (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	Narcotic
124	Para-fluoro-fentanyl	Narcotic
125	Parahexyl	
126	Phencyclidine (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	
127	Pentazocine (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	
128	PEPAP, 1-phenethyl-4-phenyl-4-piperidinol acetate(ester)	Narcotic
129	Pethidine (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human	Narcotic

	body.)	
130	Pethidine intermediate-A	Narcotic
131	Pethidine intermediate-B	Narcotic
132	Pethidine intermediate-C	Narcotic
133	Peyote	
134	Phenadoxone	Narcotic
135	Phenampromide	Narcotic
136	Phenazocine	Narcotic
137	Phenomorphan	Narcotic
138	Phenoperidine	Narcotic
139	Pholcodine	Narcotic
140	Piritramide	Narcotic
141	PMA	
142	Poppy straw	Narcotic
143	Proheptazine	Narcotic
144	Properidine	Narcotic
145	Propiram	Narcotic
146	Psilocine	
147	Psilocybine	
148	Racemethorphan	Narcotic
149	Racemoramide	Narcotic
150	Racemorphan	Narcotic
151	Rolicyclidine	
152	Sufentanil	Narcotic
153	Tenocyclidine, TCP	
154	TCPy, 1-(1-(2-thienyl) cyclohexyl) pyrrolidine	
155	Tetrahydrocannabinol including isomers and stereoisomers [products made from mature cannabis stems andseeds may not contain more than 10 microgram/gram(10 ppm) tetrahydrocannabinol]　(Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, andtreated with radioactive material, antibody markers, or not directly usable in the human body.)	
156	Thebacon	Narcotic
157	Thebaine (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive	Narcotic

	material, antibody markers, or not directly usable in the human body.)	
158	Thiofentanyl	Narcotic
159	Tilidine	Narcotic
160	TMA	
161	Trimeperidine	Narcotic
162	Morpheridine	Narcotic
163	Piminodine	Narcotic
164	Etryptamine	
165	Levomethamphetamine (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram per milliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	
166	Methcathinone (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	
167	Gammahydroxybutyrate, GHB	

Schedule Ⅲ Controlled Drugs (including their salts)

	Items	Notes
1	Amobarbital (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	
2	Brotizolam	
3	Buprenorphine (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	Narcotic
4	Butalbital (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	
5	Cathine	
6	Cyclobarbital	
7	Glutethimide (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	
8	Methylphenidate [Does not include test kits prepared with organic solventand with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, andtreated with radioactive material, antibody markers,or not directly usable in the human body.]	(3)Revision announcement
9	Nalbuphine	Narcotic
10	Nalorphine (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0	Narcotic

	milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	
11	Pentobarbital (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	
12	Phenmetrazine	
13	Secobarbital (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	
14	Tramadol	
15	Triazolam (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	
16	Codeine preparation with a content more than 1.0 gram and less than 5.0 grams of codeine per 100 milliliters (or 100 grams).	
17	Flunitrazepam (Does not include test kits prepared with organic solvent and with a content less than 1.0 milligram permilliliter, packaging less than 1.0 milliliter, and treated with radioactive material, antibody markers, or not directly usable in the human body.)	
18	Zipeprol	
19	Ketamine	
20	Dihydrocodeine preparation with a content more than 1.0 gram and less than 5.0 grams of dihydrocodeine per 100 milliliters(or 100 grams).	

Schedule Ⅳ Controlled Drugs (including their salts)

Items		Notes
1	Ephedrine	
2	Ergometrine	
3	Ergotamine	
4	Lysergic acid	
5	Methylephedrine	
6	Phenylpropanolamine	
7	Pseudoephedrine