The Detection of Date Rape Drugs by Capillary Electrochromatography

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1. Abstract

Drug facilitated sexual assault is becoming an increasing problem throughout the United States. Victims of this crime often do not report to authorities and when they do, time may have passed to the point where detection of the drug in their system becomes difficult. In addition, many of the drugs used are difficult to detect using standard immunoassay techniques. As a result, more advanced methods such as gas chromatography with nitrogen phosphorus detection must be used, In recent years, a number of forensic laboratories have begun to use capillary electrophoresis (CE) as an alternative to GC/NPD screening for this purpose. The advantages of the capillary electrophoresis over traditional screening tools such as gas chromatography and radio immunoassay are its simplicity, resistance to fouling, and capability to detect highly polar compounds. Using CE, a wide range of drug targets can be analyzed quickly with minimal sample pretreatment.

Unfortunately neutral drugs like benzodiazepines and drugs with very similar structures such as opiates can be difficult to separate using standard CE techniques. Other compounds such as GHB can exist in acidic and neutral forms (GBL) further complicating their analysis. Many of these compounds are implicated in drug facilitated sexual assault. The goal of this proposal was to explore recently developed electrochromatographic methods including micellar electrochromatography, cyclodextrin based inclusion complexes and monolithic stationary phases to improve the detection of these compounds. We used these techniques to develop a number of novel techniques for the detection of drugs implicated in sexual assault including GHB, benzodiazepines, amphetamines, cocaine and heroin. In addition to this work, we also developed new procedures using fluorescence derivatization and in-line extraction to improve the detection of trace levels of these drugs in biological fluids. Lastly, microfluidic approaches were investigated to provide inexpensive implementation of these techniques to smaller forensic laboratories.

2. Executive Summary

In recent years, a number of forensic laboratories have begun to use capillary electrophoresis (CE) as an alternative to GC/NPD screening for this purpose. The advantages of the capillary electrophoresis over traditional screening tools such as gas chromatography and radio immunoassay are its simplicity, resistance to fouling, and capability to detect highly polar compounds. Using CE, a wide range of drug targets can be analyzed quickly with minimal sample pretreatment. The goal of this proposal was to explore recently developed electrochomatographic methods including micellar electrochromatography, cyclodextrin based inclusion complexes and monolithic stationary phases to improve the detection and analysis of drugs used in sexual assault. We also examined a number of techniques to enhance the sensitivity of detection including fluorescence derivatization and in-line extraction. Lastly we experimented with a microfluidics system to explore the potential application of the above procedures in a more portable, disposable format. This report is divided into a series of section describing the different aspects of this research.

Detection of GHB and Benzodiazepines via Micellar Electrokinetic Chromatography

Certain drugs utilized in sexual assault, such as GBL and benzodiazepines, are neutral at physiological pHs. Separations involving CE are generally limited to charged compounds, however, micellar electrokinetic chromatography (MEKC) permits the analysis of uncharged molecules by providing a secondary mode of separation through the addition of a surfactant that forms into micelles (Terabe, 1984). These aggregates interact with the analytes of interest and carry them countercurrent to the electroosmotic flow, enabling greater separating power.

To separate low dose benzodiazepines and GHB, an anionic surfactant, 20 mM to 30 mM. sodium dodecyl sulfate (SDS), was used to create a micellar phase at a concentration sufficient to strongly interact with neutral species yet not interfere with the anionic mobility of GHB. Acetonitrile was added as an organic modifier to enhance the separation of the various benzodiazepines by altering their equilibria beteen the micllar phase and the bulk solution. The electropherograms for two different optimized buffer systems are shown in Figure 1. The 20 mM concentration was preferred, as it is usually a good practice to keep SDS as low as possible to avoid problems with elevated current. Once developed, this method was used to detect GHB and benzodiazepines that were

spiked in a variety of beverages.

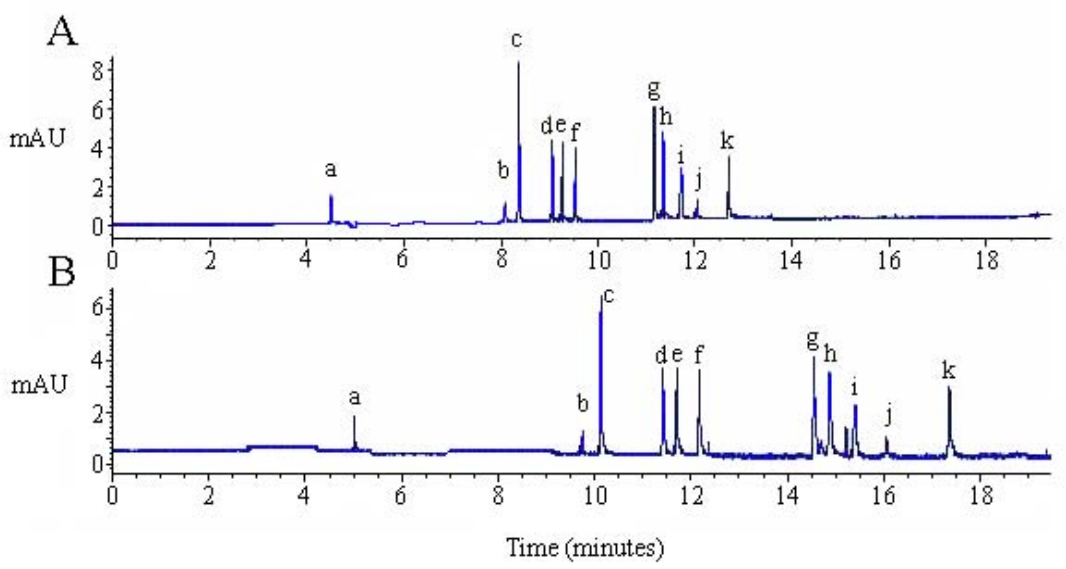


Figure 1. A comparison of the MECC separations using the standard drug mixture. The runs were performed in a 50 µm ID capillary with a total length of 80 cm and an effective length of 72 cm. The electrokinetic injection was for 4 seconds at 30 mbar. In order of elution: a) GBL; b) GHB; c) sulfanilic acid; d) flunitrazepam; e) clonazepam; f) chlordiazepoxide; g) alprazolam; h) lorazepam; i) triazolam; j) midazolam; and k) prazepam. (A) Optimized buffer at 20 mM SDS in borate pH=9.2 with 7% acetonitrile; (B) Alternative buffer at 30 mM SDS in borate pH=9.2 with 10% acetonitrile.

Opiate and Benzylpiperazine Detection using Cyclodextrin Inclusion Complexes and Capillary Electrophoresis

Drugs with very similar structures require a highly selective separation in order to be properly distinguished. Recently a number of applications have appeared in the literature describing the use of cylcodextrins in CE separations (Thorman, 2001). Cyclodextrins are sugar molecules that have the structure of a hollow truncated cone with a hydrophobic cavity. The theoretical basis for separation of neutral and charged species by cyclodextrins arises from the fact that these compounds form inclusion complexes with different drugs and the amount of time spent in these complexes varies with the geometrical shape and solvation characteristics of the drug of interest. This mode of CE analysis has great potential for use in forensic analysis because drugs such as opiates and benzodiazepines have very similar structures. In addition, these and other drugs may be found in combination with phenethyl amines that require chiral separation. Chiral separations can only be performed using cyclodextrins.

The following section describes the application of beta cyclodextrins in the separation of a wide variety of drugs of abuse implicated in sexual assault. We also utilized this technique to investigate the clandestine synthesis of a new class of illicit

drugs, benzylpiperazines. Portions of the project were performed in collaboration with the FDA's Forensic Chemistry Center in Cincinnati, Ohio.

Benzylpiperazines

On September 20, 2002, 1-Benzylpiperazine (BZP) and 1-(3-trifluoromethylphenyl)piperazine (TFMPP), were temporarily placed on the Drug Enforcement Administration's Schedule I of the Controlled Substance Act of 1970.(Fed. Regist, 2002) Over the last few years, these two compounds and their analogs have emerged as "legal" substitutes to the classic amphetamine-type compounds. (de Boer, 2001, Forensic, 2001) At a dose of 125 mg, BZP mimics the physiological effects of d-amphetamine. Additionally, TFMPP has been claimed to exhibit physiological effects similar to 3,4-methylenedioxymethamphetamine (MDMA), or ecstasy. Other documented analogs exist, and there have been several accounts of piperazine tablets being used in combination with gamma-hydroxybutyric acid (GHB) and gamma-butyrolactone (GBL). McGuanicle, 2004, Itsuoda, 2004)) Thus, these compounds may be also present in situations in which date rape and club drugs appear.
Because of the novelty of piperazine-like compounds, there is limited information available on the analysis of these drugs using validated forensic techniques. In the past,

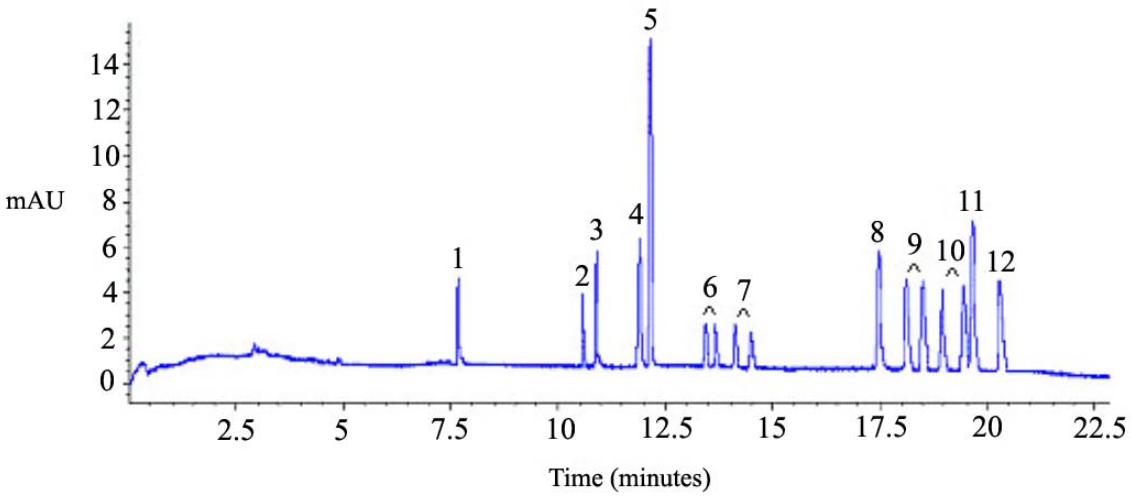


Figure 2. The optimized chiral separation of amphetamine and piperazine compounds at 30 μg/mL. The runs were performed in a 50 μm ID capillary with a total length of 64.5 cm and an effective length of 56 cm. The pressure injection was for 6 seconds at 40 mbar. The capillary temperature was kept at 25°C with a run voltage of 25 kV and a detection wavelength of 210 nm. (1) BZP; (2) phenethylamines (PEA); (3) DBZP; (4) 3-chloroaniline (3-CA); (5) 1,2-MeOPP; (6) D,L- amphetamine; (7) D,L-methamphetamine; (8) 1,4-MeOPP; (9) D,L-MDA; (10) D,L-MDMA; (11) TFMPP; (12) *m*CPP.

chiral capillary electrophoresis (CE) has proven useful in the separation of amphetamine-type compounds. (Itwa, 2002) Because of the possibility of using the piperazine drugs as amphetamine substitutes, CE was explored as a way to simultaneous detect both groups of compounds. By employing the cyclodextrin as a buffer additive in CE, an efficient and versatile separation was developed which permitted separation of various phenethylamines as well as piperazine related drugs. The optimized electropherogram for the analysis of both groups of drugs using a 200 mM sodium phosphate at pH=2.8 with 20 mM HP-β-CD is displayed in Figure 2. Following the development of this procedure, the technique was applied to the analysis of the products of a variety of simulated clandestinely synthesized benzylpiperazines obtained from the FDA's Forensic Chemistry Center. The results showed the method provided useful information towards the identity and quantity of synthesized materials.

Opiates

We have also utilized cyclodextrins to enhance the separation of a series of opiates. These compounds can be difficult to separate using normal CE. We also wanted to develop a technique to enhance the sensitivity of their detection. Using the fact that these drugs are moderately basic, we applied electrokinetic (voltage based injection) to selectively inject the drugs from aqueous matrices. This technique, also known as field amplified sample injection, was then combined with beta cyclodextrin separation techniques to provide high resolution and optimum sensitivity in the detection of opiates by capillary electrophoresis.

In the first stage of this work, we used a buffer containing 50 mM sodium phosphate and 100 mM phosphoric acid at pH 6. Under these conditions, analytes were only partially separated. When ß-CDs were added to the buffer solution, Figure 3, clear separation of the standard mixture of four opiates was achieved. In this case, base-line separation was accomplished with a running buffer composed of 50 mM sodium phosphate (pH 6) and 0.015 M ß-CDs.

Under these conditions, analytes were separated in the following order: normorphine, morphine, 6-AM, codeine, nalorphine, and levallorphan. This order can be attributed to the influence of the hydrophobic cavity of the ß-CDs. The rate that solutes partition into and out of the cavity will vary with their structure, polarity, and size. Concomitantly, the mobility of these solutes will be affected as well. When the solutes partition into the cavities, their velocities are retarded, but when they are in the bulk phase, their mobilities are unaffected. It is this differing partitioning behavior among the

various drugs that leads to greater differences in their mobilities and, therefore, an improved separation when CDs are used.

Field Amplified Injection

Because sensitivity in CE is limited by the short path length of the capillary, it was necessary to explore various methods to enhance sensitivity of these methods, particularly for toxicological applications. Sample stacking by field amplified injection (FAI) provides a way to concentrate the analytes in a thin zone at the boundary between the sample plug and the background buffer, allowing an increase in sensitivity without sacrificing efficiency. In a series of experimental studies, we examined the effect of various solution and matrix parameters on the application of FAI in the analysis of opiates. Different solvents and various specialized injection techniques were examined, however best results were obtained by dissolving an acidified sample in water and using electrokinetic injection, Figure 4. Automated solid phase extraction techniques were combined with this technique to give highly sensitive assays of a wide variety of controlled substances.

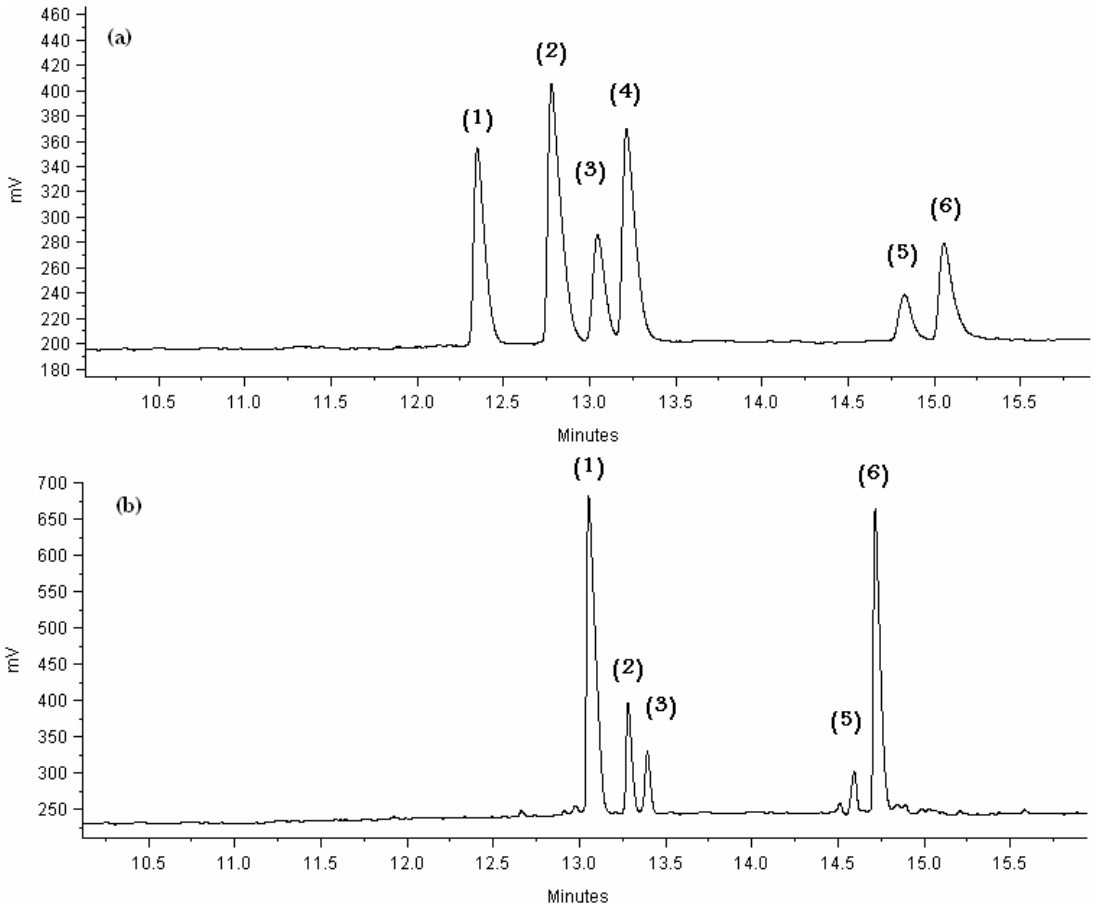


Figure 3: Typical electropherogram of (a) 500 ng/mL mixture of (1) morphine; (2) codeine; (3) thebaine; (4) 1000 ng/mL nalorphine; (5) papaverine and (6) noscapine; (b) prepared opium sample. Conditions: buffer, (pH 6) contained 50 mM sodium phosphate and 0.015 M ß-cyclodextrins, injection, electrically (5 kV) for 10 s, detection, UV absorbance at 214 nm.

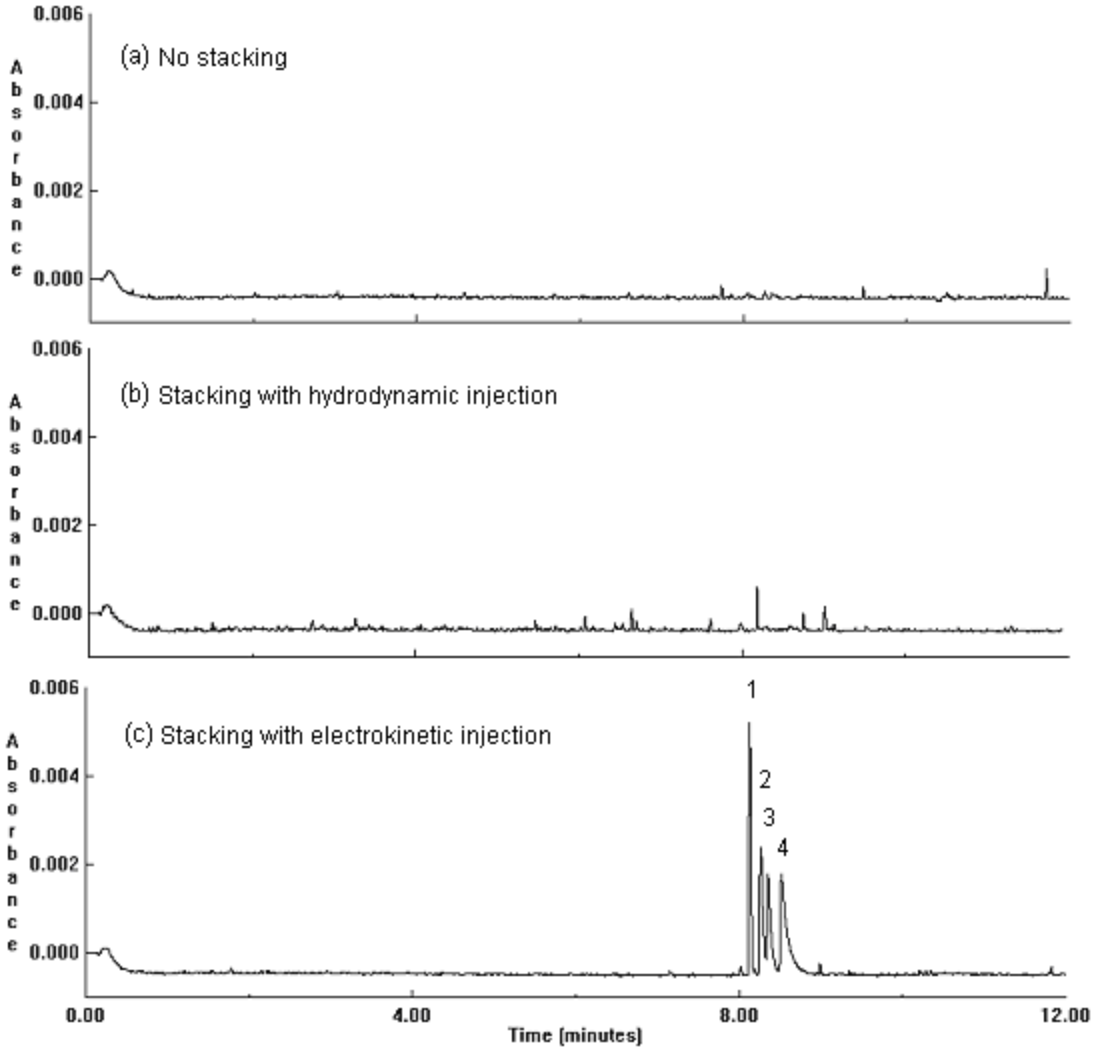


Figure 4 : Electropherograms showing the effect of sample stacking: (a) no stacking (samples dissolved in the running buffer and electrically injected); (b) stacking with hydrodynamic injection and (c) stacking with electrokinetic injection.

Fluorescent Derivatization Techniques for Enhanced Sensitivity

In the detection of drug facilitated sexual assault, sensitivity is an important issue. An alternative method to enhance sensitivity is laser induced fluorescence detection. Unfortunately it is extremely difficult to get many drugs of abuse to fluoresece due to their lack of reactive groups for fluorescent derivatization. The detetion of trace levels of

opiates and other abused drugs with tertiary amine groups was performed in this study by conversion to secondary amines using α-chloroethyl chloroformate. The amine functionality was then covalently bound to fluorescein, producing a highly fluorescent derivative. The reaction worked with most of the illicit drug candidates in our laboratory including codeine, hydrocodone, morphine, hydromorphone, 6-AM, and cocaine. The final separation conditions involved the use of 20 mM borate (pH 9.8) with 10% isopropanol, 10% acetonitrile, and 20 mM β-CDs. As can be seen in Figure 5, the separation of heroin metabolite derivatives is obtained within 10 min using a 47 cm capillary (40 cm to detector). Several drugs of abuse were examined for potential interferences with 6-AM using the above method. These drugs included codeine, hydrocodone, amphetamine, methamphetamine, morphine, hydromorphone. No interferences were found and all compounds were detected at high sensitivity. Detection limits were as low as 50pg/mL. In further studies, extracted urine samples were spiked with heroin metabolites and analyzed by CE with LIF detection. Very few endogenous compounds in the urine were detected, all having non-interfering migration times

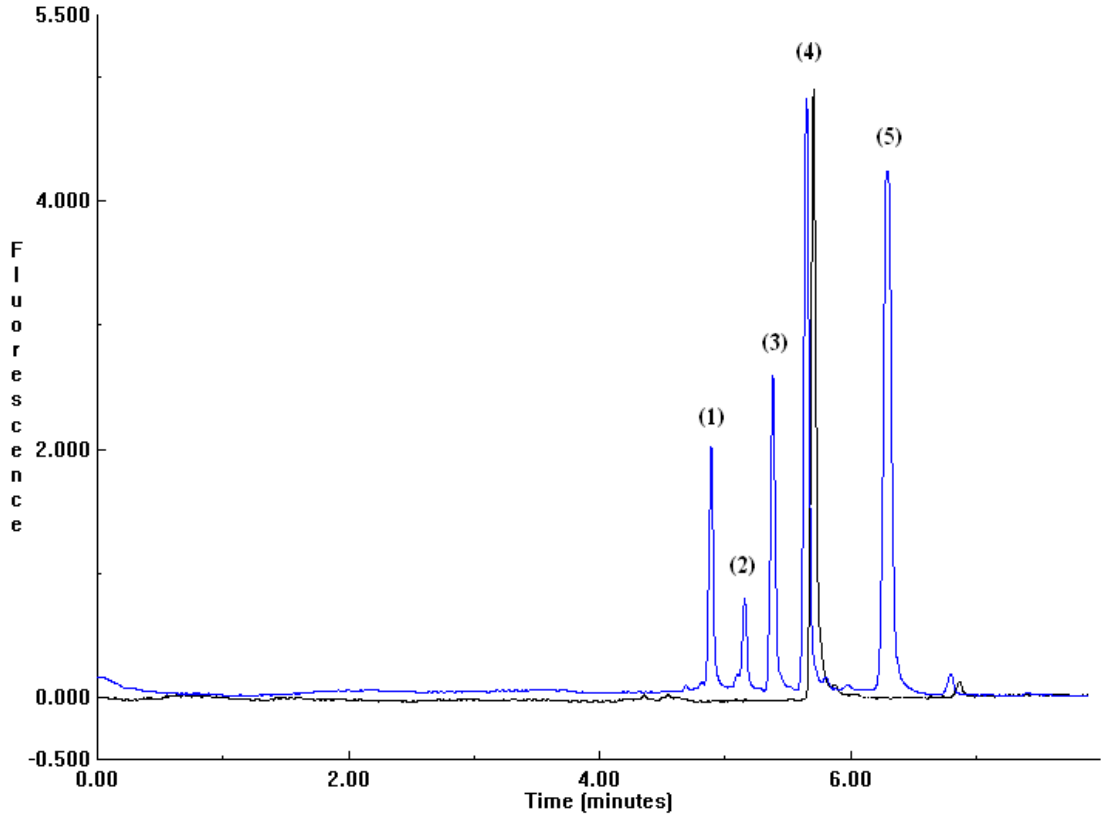


Figure 5: LIF electropherograms of a standard of FITC-labelled drugs (upper electropherogram) and reagent blank (lower electropherogram). Conditions: buffer; 20 mM borate-10% isopropanol-10% acetonitrile-20 mM ß-CDs; capillary, 47 cm x 75 µm (40 cm effective length); injection, 2 s, pressure; applied voltage, 25 kV (~65 µA); detection, LIF fluorescence detection operated at 488 nm excitation wavelength and emission wavelength filter of 520 nm. Peaks: (1) codeine, (2) 6-AM, (3) morphine, (4) FITC, (5) fluorescein (IS).

Diluted concentration of drug 500 ng/mL.

Monolithic (packed) Column Capillary Electrochromatography

Packed column capillary electrochromatography, CEC, offers potential advantages in forensic analysis in that neutral species can be separated without having to resort to pseudostationary phases, such as SDS or cyclodextrins. This makes CEC more compatible with mass spectrometry (MS) than MECC. Despite these advantages, there are several reasons why the technique has been slow to develop. One reason is that column preparation can be very problematic. One way to avoid the problems associated with packed stationary phases would be to use a polymeric stationary phase that adheres to the capillary wall. (Svec, 2000) Such phases would be uniform, cohesive, and free from requiring the use of frits. In this portion of the study several types of so-called monolith polymer columns were explored for use in small-molecule drug separations. The nature of the materials provides a porous, yet rigid, skeletal-type backbone that is attached to the capillary, Figure 6.

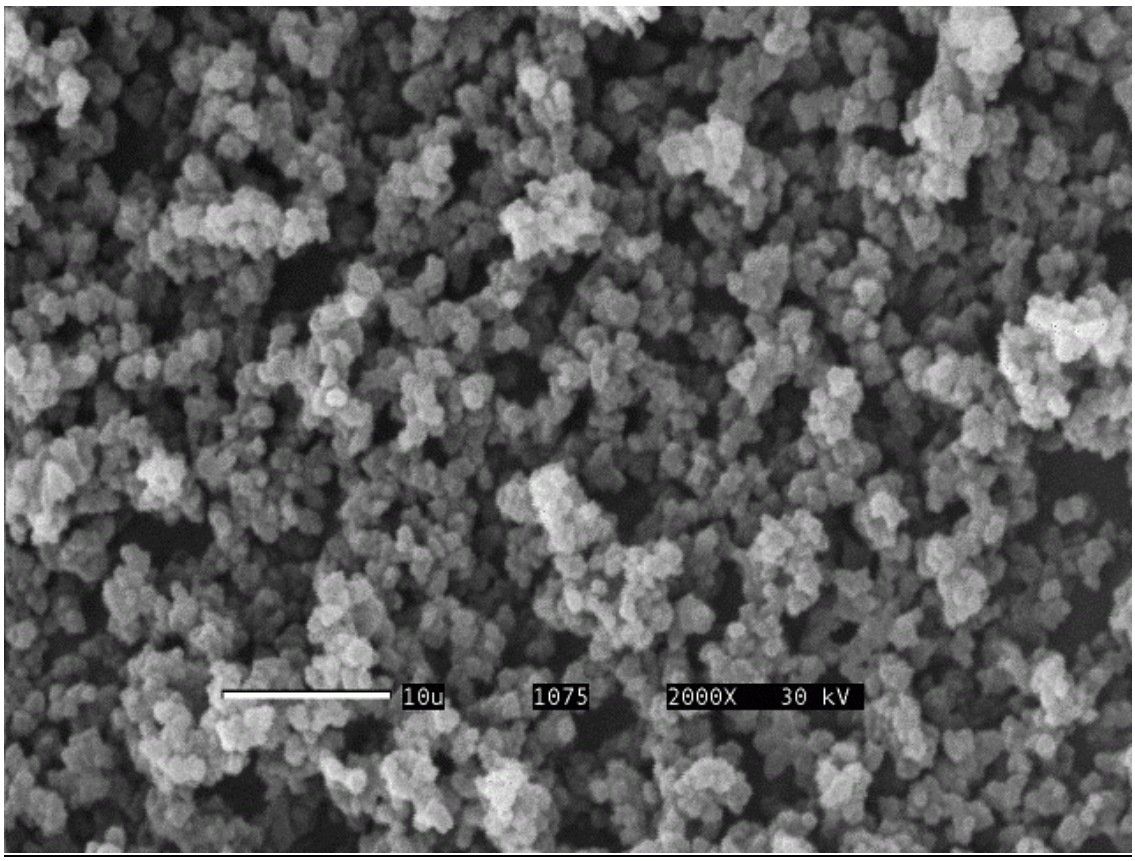


Figure 6: SEM image of poly(butyl methacrylate-co-ethylene dimethacrylate) monolith sample

Monolith experiments were performed with a 17 cm effective length capillary containing poly(butyl methacrylate-*co*-ethylene dimethacrylate) (BMA/EDMA) monoliths. A solution containing 65% 1-propanol 25% 1,4-butanediol, and 10% water was added during polymerization to create pores in the stationary phase. The selectivity of the system is demonstrated by the separation of 3 benzodiazepines, as shown in Figure

7. Unfortunately, results with this polymer were not sufficient for it to be utilized as a stationary phase under these conditions because of poor selectivity, but owing to its high surface area it should prove useful as solid phase extraction media.

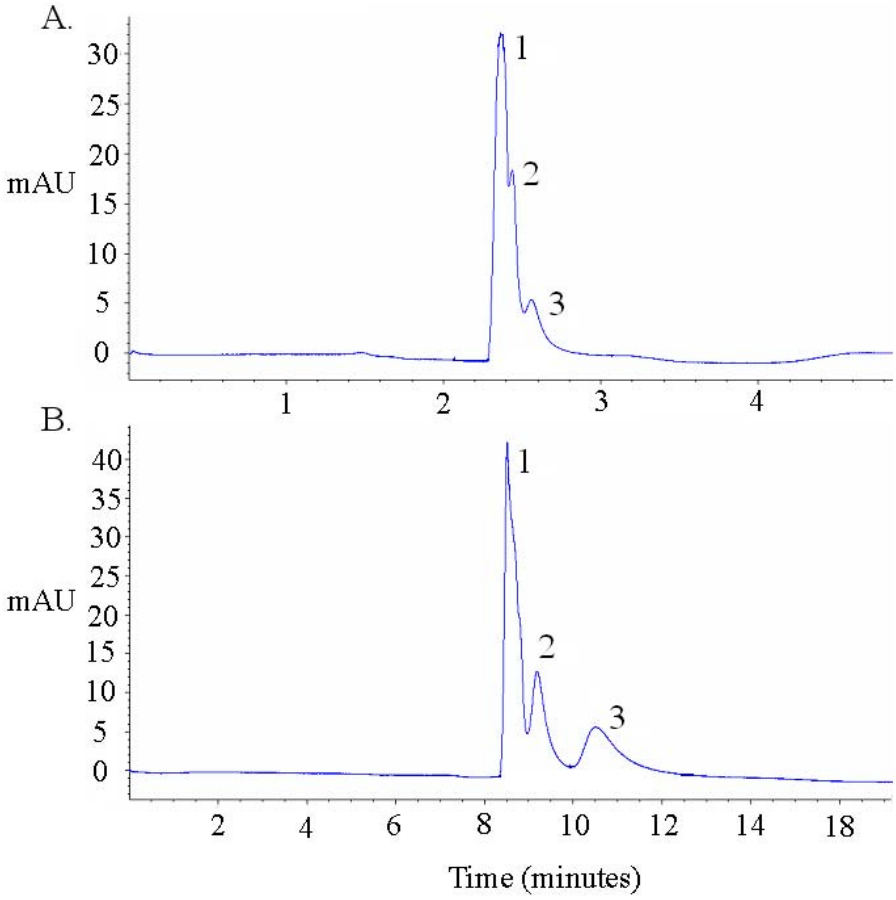


Figure 7. Separation of 3 benzodiazepines on a 17 cm effective length poly(butyl methacrylate-co-ethylene dimethacrylate) monolithic column with 0.3% AMPS and 65% 1-propanol 25% 1,4-butanediol, 10% water porogens. The separation was performed for (A) an 8:2 (v:v) acetonitrile to 5 mM sodium phosphate buffer at pH=7 and a separation voltage of 18 kV and for (B) a 7:3 (v:v) acetonitrile to 5 mM sodium phosphate pH=7 and a separation voltage of 5 kV. For both runs the samples were injected for 5 seconds at 5 kV with a 8 bar pressure on both the inlet and outlet electrodes. (1) Alprazolam; (2) Flunitrazepam; (3) Chlordiazepoxide.

Microfluidic Approaches

In the final stage of this project we began the process of converting the approaches mentioned above towards the development of disposable microfluidic devices for toxicological analysis. Microfluidic approaches have been developed for other areas such as proteomics and genomics, (Sanders, 2000) but there the analysis of small molecules using this technique is limited by the lack of suitable detectors and the necessity to develop separation techniques. The approaches described above using pseudostationary phases and fluorescent derivatization provide a roadmap for the eventual application of microfluidics to drug analysis. The establishment of rapid, portable analytical tools utilizing disposable devices would revolutionize the way in which forensic analysis is performed. Sample contamination would be less of an issue, and throughput would be drastically increased.

For our initial experimental work we utilized a fluorescent assay in combination with a micellar electrokinetic separation for the detection of a series of nitrated benzodiazepines including flunitrazepam which have been implicated in DFSA. The procedure takes advantage of the quenching interaction between the nitro group on these molecules and the fluorescent dye, Cy5. Samples are injected onto a microscope sized glass chip with channels etched into its surface. Benzodiazepines are separated in the glass chip and detected as they quench the fluorescence of the dye in the detector zone. Figure 8.

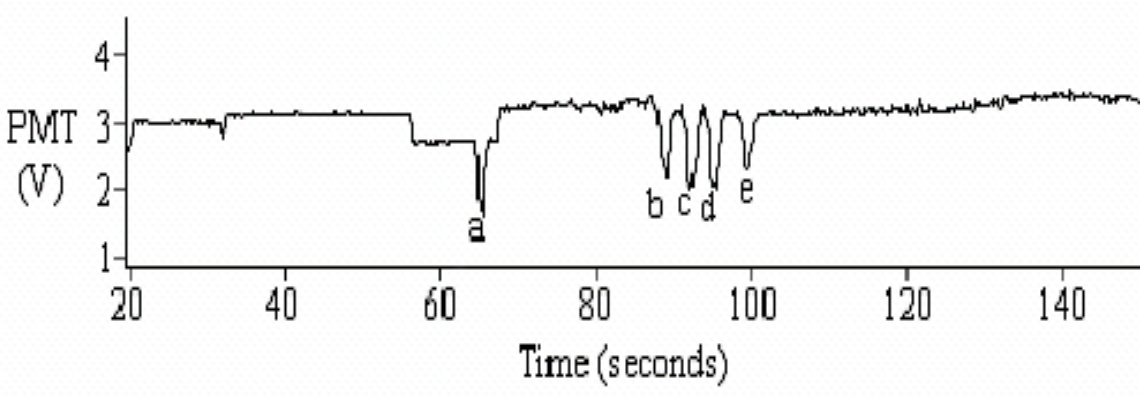


Figure 8. The optimized separation nitrated benzodiazepines using a 15 mM SDS/15 mM boric acid/3 mM sodium tetraborate/2.6 μM Cy5 buffer with 20% methanol. The separation voltage

was 4.0 kV and the effective length from the channel intersection to the detector was at 45 mm. (a) 2,4-DNT (I.S.); (b) desmethylflunitrazepam; (c) flunitrazepam; (d) nitrazepam; (e) clonazepam.

Conclusions

In a series of studies we have demonstrated the promising applications of capillary electrophoresis in the detection of drugs used in sexual assault. Our results show that exceptional separations of charged and neutral compounds are possible using the combination of capillary electrophoresis and chromatography. To achieve these results, we have used both pseudo stationary phases and imbedded stationary phases composed of polymer monoliths. Best results were obtained using beta cyclodextrins combined with organic modifiers. These separations show exceptional selectivity and can be combined with various sensitivity enhancement tools to give detection capabilities as good or better than more commonly used gas chromatographic screening methods. In addition, when used with on-line extraction, the analytical procedure is easy to implement requiring no derivatization Overall, this work demonstrates an exceptional flexibility and range of applications for capillary electrophoresis when utilized for the detection of date rape drugs.

3. The Detection of Date Rape Drugs by Capillary Electrochromatography

Introduction

Drug facilitated sexual assault (DFSA) is defined as the voluntary or involuntary ingestion of a drug by a victim that results in an act of sexual activity without consent. There are different types of DFSA, including situations in which the victim has intentionally taken a recreational drug, combined a prescription medicine with illegal drugs, or unknowingly consumed a drug placed in his/her beverage. (Abarbanel, 2001)

The classic example of the use of date rape drugs involves the application of “mickey-finns” or “knock-out drop” type substances that are surreptitiously spiked into a victim’s beverage. In reality, a study performed by ElSolhy and Salamone indicated that alcohol is by far the most prevalent form of date-rape incapacitation. (ElSohly, 1999) Other studies have demonstrated that over 75% of perpetrators and 50% of the victims have been under the influence of alcohol at the time of the assault. (Pope, 2001) Complicating matters further, investigations have revealed that multiple drug ingestion is common among victims reporting sexual assault. Table 1 displays a list of common DFSA drugs, whether taken willingly by the victim or administered by the perpetrator. (Arbarbanel, 2001) The one statistic that has been common among reports and papers is that many occurrences of sexual assault are overwhelmingly underreported.

During the past few years, there has been the emergence of a new application for illicit drugs. Rave parties involve the use of loud music, flashing lights, and illicit drugs such as MDMA, GHB, and other similar compounds. These so called “club drugs” are sometimes used in drug-facilitated sexual assault. Several tragic cases have been

reported including Hillory Farias, Samantha Reed, and Genevieve Squires that concern the inadvertent ingestion of a GHB spiked drink from a male acquaintance resulting in a fatal overdose. In June 2003, the heir of the Max Factor fortune, Andrew Luster, pled guilty to drugging females with GHB in order to videotape sexual activities. (Wilson, 2003) Another compound, flunitrazepam, or "roofies", is one of many benzodiazepine drugs that are used for their sedative properties to incapacitate victims.

Clinical Analysis of DFSA Drugs

The first step towards the incarceration of individuals who use drugs to overpower victims is to prove the existence of the substance in a victim's body. The presence of the drug is often difficult to establish due to the rapid metabolism of many of these drugs. Ingestion of DFSA drugs can result in an amnesic state of mind, leading to confusion and a concomitant delay in the notification of authorities. This delay in reporting the crime, combined with a fast clearance rate from the victim's body, can result in only trace amounts of the parent drug remaining in a victim's system. In addition, the quantity of sample can be very limited. Therefore, any screening procedures to target these drugs should be highly sensitive and require a low sample volume.

Table 1. List of potential DFSA drugs. (LeBeau, 2001)

Generic Name	Trade Name (if applicable)	Street Name (if applicable)
1-(3-trifluoromethylphenyl)piperazine		TFMPP
1,4-butanediol		diol 14B, thunder nectar, weight belt cleaner
Alprazolam	Xanax	Z bars
Amobarbital	Amytal	
Amphetamine	Adderall	speed, dex, crank
Benzylpiperazine		BZP, piperazine, A2
Chloral hydrate	Notec	knockout drops, Mickey Finn (with alcohol)
Chlordiazepoxide	Librium	
Clonazepam	Klonopin	
Cocaine		crack (freebase), coke, snow, nose candy, dust
Codeine	Nucofed	
Dextromethorophan	Robitussin	DXM, robodrops
Diazepam	Valium	
Diphenhydramine	Benadryl	
Dronabinol, delta-9-THC	Marinol	THC, weed, grass, dope, mary jane
Ethanol		alcohol
Flunitrazepam	Rohypnol	roofies, roche, forget me pills
Gamma-butyrolactone		
Gamma-hydroxybutyric acid	Xyrem	easy lay, grevious body harm, liquid ecstasy
Gamma-valerolactone		
Heroin		dope, junk, smack, horse
Ketamine	Ketalar	K, special K, lady K, vitamin K
Lorazepam	Ativan	
Lysergic acid diethylamide		LSD, acid, blotter, tabs
Methamphetamine	Methedrine	ice (freebase), meth, crystal
Methylenedioxyamphetamine		MDA
Methylenedioxymethamphetamine		MDMA, ecstasy, XTC, X, E, adam
Midazolam	Versed	
Morphine	Roxanol	
Nefazodone	Serzone	
Nitrazepam		
Pentobarbital	Nembural	
Phencyclidine	Sernyl	PCP, crystal, angel dust
Phenobarbital	Luminal	
Prazepam	Centrax	
Secobarbital	Seconal	
Trazodone	Desyrel	
Triazolam	Halcion	
Zolpidem	Ambien	

While flunitrazepam has been the cause of much concern, any low dose benzodiazepine can be utilized in DFSA. In fact, there are many such benzodiazepines that are U.S. Food and Drug Administration (FDA) approved and available with a prescription. A study by Hindmarch and Brinkmann revealed that out of 1033 date-rape specimen samples, only six tested positive for flunitrazepam (0.58%), whereas 12% tested positive for other benzodiazepines.(Hindmarch, 1999) Unfortunately, traditional toxicological screening methods for benzodiazepines target urinary metabolites, such as oxazepam, that have poor cross reactivity with the low-dose benzodiazepines involved in DFSA. (St. Claire, 2001) Owing to the instability of GHB's acid functionality, methods for its analysis involve derivatization steps or esterification to the lactone, GBL. (Marinetti, 2001) Thus, new methods are needed which can provide a more rapid and efficient screening for these compounds.

The goal of this proposal was to explore recently developed electrochomatographic methods including micellar electrochromatography, cyclodextrin based inclusion complexation and monolithic capillary electrochromatography to improve the detection of these compounds. Procedures were also developed to improve detection of trace levels of these drugs in biological fluids. Lastly, microfluidic approaches were investigated to provide inexpensive implementation of these techniques to more laboratories. This report is divided into a series of sections describing the different aspects of this research.

A. Detection of GHB and Benzodiazepines via Micellar Electrokinetic Chromatography

Capillary electrophoresis, with its high efficiency and low sample volumes, is an obvious choice for applications in drug screening. However, drugs such as GBL and benzodiazepines are neutral at physiological pH 7.4. Although CZE is limited in its ability to detect neutral compounds, micellar electrokinetic chromatography (MEKC) permits the analysis of uncharged molecules by providing a secondary mode of separation through the addition of a surfactant that forms into micelles. These aggregates interact with the analytes of interest and carry them countercurrent to the electroosmotic flow, enabling greater separating power. Even though there have been micellar methods for electrokinetic separations of benzodiazepines (Boonkerd, 1996) and GHB, (Dahlen, 2002) the potential for a simultaneous date-rape drug screening for both sets of compounds has not been realized.

For this method, an anionic surfactant, sodium dodecyl sulfate (SDS), was used in the micellar mode to separate a variety of DFSA and club drugs. The buffer system, which also contains an organic modifier, has been proven useful in the alteration of the selectivity by affecting the partition coefficients and capacity factors of the analytes. (Lurie, 1998) A variety of beverages frequently consumed at parties and bars were chosen as media in which GHB and benzodiazepines were detected by this method. There have been several known cases, including the case against Luster, where beverages were used to conceal the administration of date-rape drugs. Not only can the dyes and fillers in beverages interfere with the analysis of the spiked drug, but also in the case of GHB, certain conditions have been known to cause esterification into GBL. (Marinetti,

2001) The alleged spiked drink could also be left at the scene long before it is collected by the authorities and given to the laboratory. By exploring these variables in detail, the proper dilution and optimization steps were determined for the MEKC method in order to properly identify any DFSA drugs that could be present.

Experimental

Reagents and Analytes

GHB was synthesized in-house. (GHB, 2000) The benzodiazepines were purchased from Lipomed (Cambridge, MA). GBL and 1,4-butanediol were purchased from Aldrich (St. Louis, MO). HPLC grade acetonitrile, boric acid and sodium dodecyl sulfate (SDS) were obtained from Fisher Scientific (Pittsburgh, PA). Monobasic and dibasic sodium phosphates along with sodium tetraborate were purchased from Acros (Morris Plains, NJ). The sulfanilic acid internal standard was obtained from J.T. Baker (Phillipsburg, NJ) and was prepared such that the final concentration after all samples and standards were diluted into it would be 1×10^{-4} M. The CE buffer consisted of 5.0 mM sodium tetraborate and 27.2 mM boric acid with SDS. The volume of a standard beverage was estimated at 200 mL of liquid. The drug concentrations were based on a single dose of GHB at 1 g (common 1 to 2.5 g) (Erowid, 2004) and a 1 mg (common doses 0.5 to 2 mg) dose of various benzodiazepines to simulate a prescription tablet. (Brenneisen, 2001) The beverages for the GHB kinetic study were prepared by placing 10 mg of powdered GHB into 2 mL of liquid. All benzodiazepine-spiked beverages were prepared by dissolving 1 mg of drug in 200 mL of liquid. The buffered phosphate consisted of 100 mM sodium phosphate at pH=6.

Instrumentation and Conditions

All separations were performed on an Agilent Capillary Electrophoresis System equipped with a photo-diode array detector. Separations were achieved using 50 μm i.d. capillary from Polymicro (Phoenix, AZ) with approximately 80 cm total length and 72 cm effective length. Corrections for differing capillary lengths were accounted for by using mobility calculations. Typical injections were 30 mbar for 4 seconds for the optimization and 30 mbar for 8 seconds for the calibration and extracted beverages. The capillary cartridge temperature was kept at 35 ^{o}C with a run voltage of 30 kV. Each new capillary was rinsed with a three solution cycle of a) 0.1 M NaOH for 30 minutes, b) 18 MΩ water for 5 minutes, and c) buffer for 10 minutes. Prior to each run, the capillary was rinsed with water for 2 minutes and then rinsed with buffer for 5 minutes. All buffers were placed in an ultrasonic water bath briefly before use.

Method Optimization

The drug standards used for the optimization consisted of 10 μg/mL of the eight benzodiazepines, 100 μg/mL of GHB, and 62.5 μg/mL of GBL in internal standard. During the method optimization of pH, the percent of organic modifier and the concentration of SDS were varied. In these experiments, the SDS concentration was held

at 30 mM, and solutions of borate buffer with pH of 8.2, 8.7, 9.2, and 9.7 were prepared with 0.0, 3.0, 5.0, 7.0, 10.0, 13.0, and 15.0% acetonitrile. For each of the acetonitrile percentages, the SDS concentration was adjusted to 10 mM and 20 mM at a pH of 9.2. Upon review of the data, the most favorable buffer was determined to be borate with 20 mM SDS at a pH=9.2 with a 7.0% acetonitrile organic modifier. This was the buffer that offered the best resolution between closely eluting peaks and distinguishable mobilities for all of the standard components. A second buffer at 30 mM SDS pH=9.2 10.0% was at times necessary to rule out interferences.

Sample Preparation

The concentration of GHB found in a date-rape or recreational dose sample typically is too concentrated for analysis by CE. Therefore, samples were diluted by a factor of ten before analysis. This type of sample preparation is especially compatible with CE because samples are injected by pressure, and when contamination is present, the open tubular capillary can be quickly restored through a hydroxide rinse. Additionally, because this is an aqueous system, derivation of GHB is unnecessary. During the kinetic study, each sample was diluted 1:5 in doubly deionized water. Fifty μL of the diluted sample was further diluted with 50 μL of internal standard to give a total dilution factor of 1:10.

An ethyl acetate liquid-liquid extraction was employed for beverages containing benzodiazepines. To 1.0 mL of beverage sample, 100 μL of buffered phosphate was added. The sample was extracted twice with 0.5 mL ethyl acetate. The top organic layer was transferred and evaporated to dryness by a stream of nitrogen. The sample was reconstituted in 50 μL of deionized water containing 10 % acetonitrile and then diluted 1:1 with 50 μL of internal standard. The solution was then transferred to 100 μL sample vials.

Results and Discussion

In traditional CE, a combination of an applied field and electroosmotic flow (EOF) is the driving force behind migration inside the capillary. The EOF is provided by the electrical double layer that is formed from ionized silanol groups on the capillary wall and the cationic species of the buffer. (Mazzeo, 1997) The pH dictates the abundance of ionized silanol groups and the electric field causes the buffer cations to move towards the cathode, resulting in a bulk electroosmotic flow. Positive, neutral, and negative compounds are separated into zones within the capillary by exploiting their mobility differences. In the case of micellar electrokinetic chromatography (MEKC), micelles provide further separation of these distinct zones. The sodium dodecyl sulfate (SDS) surfactants begin forming aggregates at the critical micelle concentration (cmc) of 8.1 mM, which tends to vary with buffer additives. Being a negatively charged micelle, SDS travels in the opposite direction of the EOF. While moving through the capillary, the SDS interacts strongly with positive compounds, reducing their net mobility. Some neutral analytes will partition between the micelle phase and the buffer phase, increasing migration times in proportion to the amount of time the analyte is bound to the micelles.

The partitioning is based on hydrophobic interactions, hydrogen bonding, and charge effects. Since the bulk flow is generally greater than the micelle velocity, SDS will ultimately reach the detector.

From the optimization data, it was established that lowering the pH decreased migration time and resolution. While a low pH may be sufficient for a faster analysis, without the aid of a mass spectrometer, benzodiazepine identification might be more difficult. Therefore, a pH of 9.2 offered the best baseline separation when relying on relative retention and spectral analysis alone. In this separation, SDS micelles had little to no interaction with the small, neutral compounds, causing GBL and 1,4-butanediol to elute simultaneously at around 4 minutes. This was also the time of the EOF, which was confirmed by the neutral marker dimethylsulfoxide (DMSO). As expected, the anionic micelles did not interact with the negatively charged compounds, GHB and sulfanilic acid, which elute next after the EOF. This was demonstrated by the fact that the mobilities of the compounds were unaffected in an experiment in which SDS was omitted from the electrophoretic buffer.

The relatively bulky neutral compounds can partition in and out of the micelle, which explains why the benzodiazepines had a lower net mobility compared to the negatively charged compounds as shown in Figure 9. With the micelle alone, the structurally similar benzodiazepines co-eluted, because of their comparable hydrophobicities. The three most polar benzodiazepines and the five remaining benzodiazepines did not have distinguishable mobilities. The addition of an organic modifier resolved the overlapping peaks.

The SDS concentration was found to be optimal in the range from 20 mM to 30 mM. The electropherograms for both buffer systems are shown in Error! Reference source not found.. The 20 mM concentration was preferred, as it is usually a good practice to keep SDS as low as possible to avoid problems with elevated current.

GHB Kinetic Study

The conversion of GHB to its lactone form has complicated the quantitative analysis of this compound. Because GHB and GBL coexist in a dynamic state, the two compounds eventually reach equilibrium in their environment. (Martinetti, 2001) In the body, the lactonase enzyme converts all of the GBL to GHB, explaining why the analog is a successful substitute for the parent drug. In matrices, which do not contain lactonase, the interconversion will be pH dependent. GHB spiked beverages including water, fruit juice, soda, beer and wine were monitored on the first day (t=0) and then left uncovered at ambient temperature for 24 and 48 hours to determine if there was any degradation of the sample. There was no observed degradation of GHB in any of the analyzed beverages. The lack of interconversion is supported by an extensive study by Ciolino et al. that examined the stability of GHB and GBL in aqueous solutions as a function of pH and time. (Ciolino, 2001) Throughout their entire 66 day study, GHB exhibited no esterification in four out of the five beverage matrices, while the fifth lost less than 10% GHB by the conclusion of the experiments.

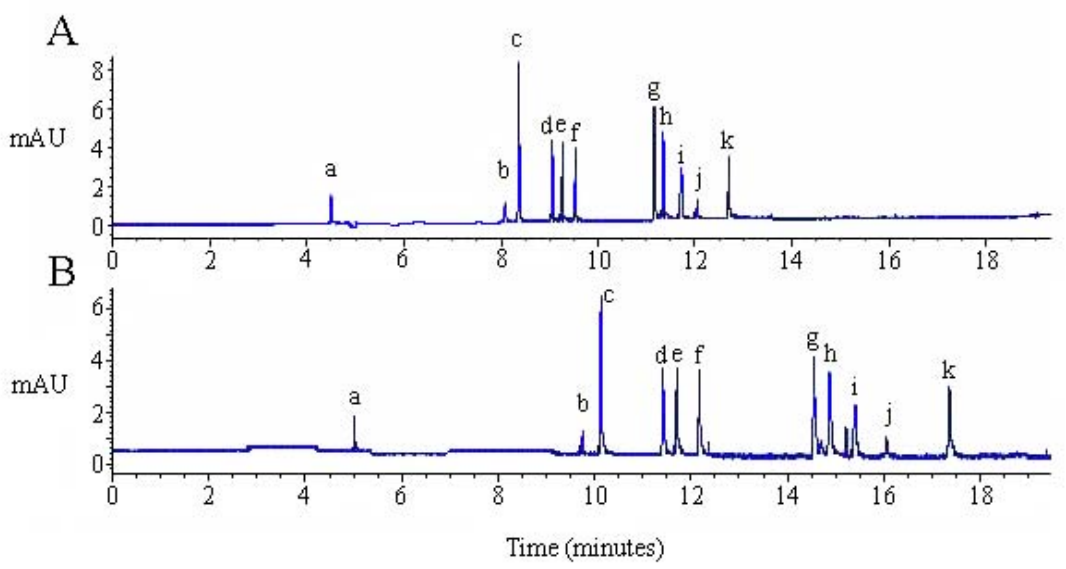


Figure 9: A comparison of the MECC separations. The runs were performed in a 50 m ID capillary with a total length of 80 cm and an effective length of 72 cm. The electrokinetic injection was for 4 seconds at 30 mbar. In order of elution: a) GBL; b) GHB; c) sulfanilic acid; d) flunitrazepam; e) clonazepam; f) chlordiazepoxide; g) alprazolam; h) lorazepam; i) triazolam; j) midazolam; and k) prazepam. (A) Optimized buffer at 20 mM SDS in borate pH=9.2 with 7% acetonitrile; (B) Alternative buffer at 30 mM SDS in borate pH=9.2 with 10% acetonitrile.

Since the typical dose of GHB is 1 g per 200 mL of beverage, it is necessary for the analysis to dilute the sample a factor of 1:10. Following this dilution, most peak contaminants are present at very low levels. A spiked beer sample, however, produced an interfering peak at the migration time of GHB with the 20 mM SDS 7.0% acetonitrile buffer. Using the data obtained from the optimization study, the interfering peak is easily moved out from beneath the GHB peak when the buffer is adjusted to a concentration of 30 mM SDS with 20% acetonitrile.

An interference study was next performed to determine the methods ability to detect other potential drugs that might be present in beverages. By using both optimized buffers, we were able to prove that most interfering drugs have distinguishable mobilities in comparison to the GHB and benzodiazepines. A list of club drugs and their mobilities with both buffer systems is given in Table 1. A typical analysis was accomplished by running the interfering compounds with both the 20 mM SDS 7.0% acetonitrile buffer followed by the 30 mM SDS 10.0% acetonitrile buffer. Ketamine's mobility when using the 20 mM SDS buffer was 3.4×10^{-4} Vcm^2/sec, which was identical to the mobility of clonazepam. When analyzed with the 30 mM SDS buffer, ketamine's mobility became greater than clonazepam by a difference of 4×10^{-3} Vcm^2/sec. By exploiting these mobility differences along with the spectral data, the MECC method can be selectively applied to different analytical problems.

Table 1. Comparison of date-rape and club drug mobilities.[a]

Analyte	(a) Mobility × 10^{-4} (Vcm^2/sec)	Analyte	(b) Mobility × 10^{-4} (Vcm^2/sec)
GHB	3.9	**GHB**	3.3
Sulfanilic Acid	3.7	**Sulfanilic Acid**	3.2
Salicylic Acid	3.6	**Ketamine**	3.1
Flunitrazepam	3.5	**Salicylic Acid**	3.1
Clonazepam	3.4	**Flunitrazepam**	2.8
Ketamine	3.4	**Heroin**	2.8
Chlordiazepoxide	3.3	**Clonazepam**	2.7
Heroin	3.1	**Chlordiazepoxide**	2.6
Benzylpiperazine	3.0	**Benzylpiperazine**	2.6
Alprazolam	2.8	**Alprazolam**	2.2
Lorazepam	2.8	**Cocaine**	2.2
Triazolam	2.7	**Lorazepam**	2.2
Midazolam	2.6	**Methamphetamine**	2.1
Methamphetamine	2.6	**Triazolam**	2.1
MDMA	2.5	**MDMA**	2.1
Cocaine	2.5	**Midazolam**	2.0
Prazepam	2.4	**Prazepam**	1.9
Dextramethoraphan	2.4	**Dextromethorphan**	1.7

[a]Samples were run in borate buffer at pH=9.2 with (a) 20 mM SDS with 7% acetonitrile and (b) 30 mM SDS with 10% acetonitrile. All other conditions were the same as in **Error! Reference source not found.**.

Conclusions

The SDS/borate buffer MECC method has proven useful in the analysis of unknown beverage samples. The method shows good separation of all benzodiazepines, as well as GHB, and provides a rapid screening method for many of the common sexual assault drugs and other club drugs. By using the optimized 20 mM SDS in borate buffer with 7.0% acetonitrile we were able to identify GHB in a variety of different beverages. Our results indicate that there is no apparent interconversion of GHB into GBL after the drug has been allowed to stand in the beverage for 2 days. To analyze for benzodiazepines and other drugs of interest, an extraction using ethyl acetate is necessary to detect quantities that would be present in a single dose. A list of potential interfering compounds has been provided with an alternative buffer of 30 mM SDS in borate with 10.0% acetonitrile to be used in situations when the analyte of interest is in question. The optimization conditions have proven to be reliable in order to distinguish DFSA drugs from other drug interferences. This method can provide an excellent overall screening tool for the detection of date-rape drugs.

B. Opiate and Benzylpiperazine Detection using Cyclodextrin Inclusion Complexes and Capillary Electrophoresis

Cyclodextrins are sugar molecules that have the structure of a hollow truncated cone with a hydrophobic cavity. These compounds, as shown in Figure 10, consist of six, seven, or eight glucopyranose units attached by (1→4) linkages (α, β, and γ, respectively). They contain a hydrophilic outer rim and a hydrophobic inner core, as depicted in Figure 10b. Run buffers containing CDs have proven invaluable for forensic drug applications, including the analysis of enantiomers (Lurie, 1994) and diastereomers. (Lurie, 1998) This mode of CE analysis is particularly attractive because certain drugs, such as opiates and benzodiazepines, have very similar structures. In addition, these and other drugs may be found in combination with phenethyl amines that require chiral separation.

The theoretical basis for separation of neutral and charged species by cyclodextrins arises from the fact that these compounds form inclusion complexes with different drugs and the amount of time spent in these complexes varies with the geometrical shape and solvation characteristics of the drug of interest. Thus, by exploiting the fact that mobility varies depending on the equilibrium constants for the complexation of the drug with cyclodextrins, enhanced resolution can be obtained even for very similar compounds.

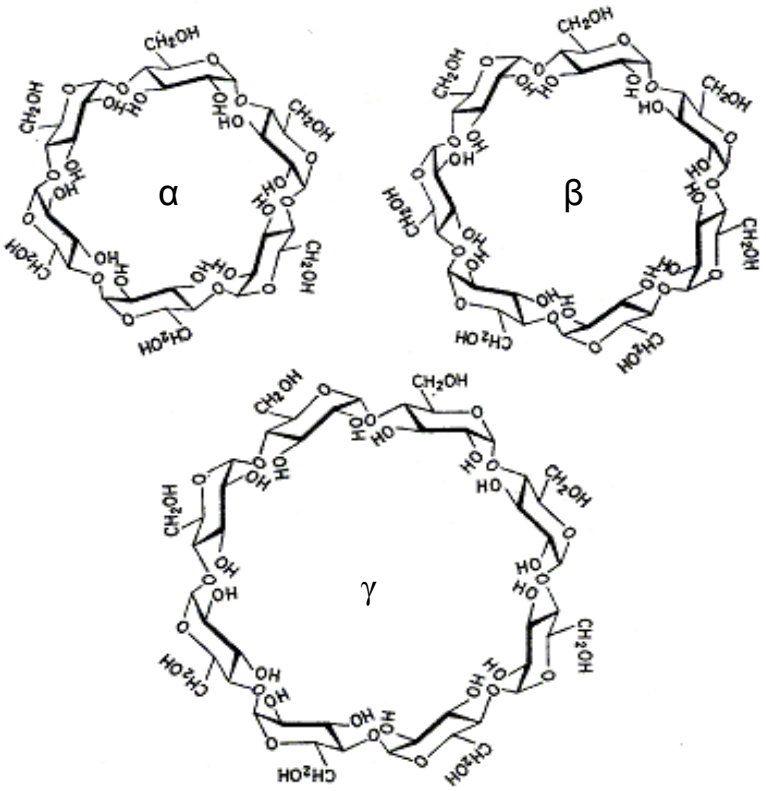
The following section describes the application of beta cyclodextrins in the separation of a wide variety of drugs of abuse implicated in sexual assault. We also utilized this technique to investigate the clandestine synthesis of a new class of illicit drugs, benzylpiperazines. This portion of the project was performed in collaboration with the FDA's Forensic Chemistry Center in Cincinnati, Ohio.

Benzylpiperazines

On September 20, 2002, two piperazine compounds were temporarily placed on the Drug Enforcement Administration's Schedule I of the Controlled Substance Act of 1970. (Fed. Regist, 2002) 1-Benzylpiperazine (BZP) and 1-(3-trifluoromethylphenyl)piperazine (TFMPP), will remain on the emergency schedule list until further rulings can be made regarding their hazard to public safety. Over the last few years, these two compounds and their analogs have emerged as "legal" substitutes to the classic amphetamine-type compounds. (deBoer, 2001) At a dose of 125 mg, BZP mimics the physiological effects of d-amphetamine. Additionally, TFMPP has been claimed to exhibit physiological effects similar to 3,4-methylenedioxymethamphetamine (MDMA), or ecstasy. Other documented analogs with a potential for abuse include 1-[4-methoxyphenyl]-piperazine (*p*MeOPP), 1-[2-methoxyphenyl]-piperazine (*o*MeOPP), and 1-[3-chlorophenyl]-piperazine (*m*CPP).

Reports of piperazine tablet seizures have appeared frequently in the Drug Enforcement Administration's 2003 and 2004 Microgram Bulletins. (US DEA, 2003, 2004)) There have also been accounts of piperazine tablets being used in combination with gamma-hydroxybutyric acid (GHB) and gamma-butyrolactone (GBL). (Forensic, 2001, Itsuoda, 2004) Thus, these compounds may be also present in situations in which date rape and club drugs appear.

(a)



(b)

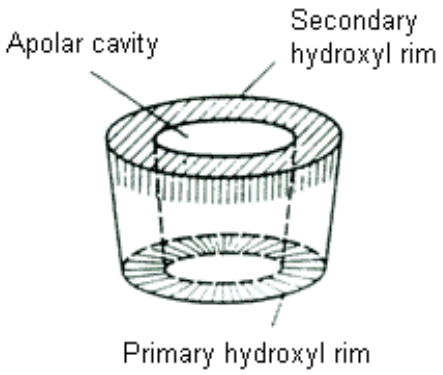


Figure 10: (a) Native cyclodextrin α, β, and γ. (b) basic feature of the three native cyclodextrins. The primary hydroxyl rim is made up of hydroxyls attached to the C6 carbons, and the secondary hydroxyl rim is made up of hydroxyl groups attached to the C2 and C3 carbon atoms (Rogan, 1993).

Because of the novelty of piperazine-like compounds, there is limited information available on the analysis of these drugs using validated forensic techniques. Staack and co-workers have published work involving toxicological studies and quantitation of piperazine compounds by GC-MS. (Staack, 2002) De Boer et al. suggested a variety of analytical strategies for detection of BZP in capsules, (deBoer, 2001) but the potential for a capillary electrophoretic method has not been realized. In the past, chiral capillary electrophoresis (CE) has proven useful in the separation of amphetamine-type compounds. (Varesio, 1995, Tagliaro, 1998)) Because of the possibility of using the piperazine drugs as amphetamine substitutes, CE was explored as a way to simultaneous detect both groups of compounds. Since the reported effects of these two classes of drugs are strikingly similar and often used interchangeably, this method will greatly benefit laboratory analyses where the abused or seized substance is in question. . A chemically modified hydroxypropyl-β-cyclodextrin (HP-β-CD) was utilized in this study as an inclusion type chiral selector. By employing the cyclodextrin as a buffer additive in CE, an efficient and versatile separation was developed which permitted separation of various phenethylamines as well as piperazine related drugs.

Experimental

Chemicals and Reagents

1-Benzylpiperazine was purchased in its liquid form from Sigma-Aldrich (St. Louis, MI) and crystallized at ambient temperature to yield a BZP solid. All other compounds described herein were used without further purification. Also obtained from Sigma-Aldrich were 1-(4-methoxyphenyl)piperazine, 1-(2-methoxyphenyl)piperazine, 1,4- dibenzylpiperazine, and DL-3,4-methylenedioxy-methamphetamine (MDMA). 1(3-trifluoromethylphenyl)piperazine was obtained from Alfa Aesar (Ward Hill, MA). Both 1-(3-chlorophenyl)piperazine and the internal standard 3-chloroaniline were purchased from TCI America (Portland, OR). DL-Methamphetamine was purchased from Radian (Austin, TX) while DL-Amphetamine and DL-MDA were obtained from Cerilliant (Austin, TX). A 1-BZP dihydrochloride salt and a dibenzylpiperazine analogue were prepared in the laboratory. The starting materials and chemicals used to synthesize these two compounds were obtained from Sigma-Aldrich and were of high purity. Sodium phosphate was obtained from Acros (New Jersey, NJ) and 85% phosphoric acid was obtained from Spectrum Quality Products Inc. (Gardena, CA). Hydroxypropyl-β-cyclodextrin (HP-β-CD) with a substitution rate: 4.9 was obtained from the eCAP™ Chiral Methods Development Kit (Beckman Instruments, Inc., Fullerton, CA).

The chiral CE buffer was prepared using a 200 mM phosphate buffer. A final pH of 2.8 was reached after adjustment with 1 M NaOH. The chiral selector hydroxypropyl-β-cyclodextrin was added at a concentration of 20 mM.

Instrumentation

Separations were carried out on an Agilent Capillary Electrophoresis System equipped with a photo-diode array detector. The capillary was obtained from Polymicro (Phoenix, AZ) with a total length of 64.5 cm, an effective length of 56 cm, and an internal diameter of 50 μm. Typical injections were 40 mbar for 4 seconds for the optimization studies and 40 mbar for 6 seconds for the calibration and extracted samples. The capillary temperature was kept at 25 ^{o}C with a run voltage of 25 kV. Each new capillary was rinsed with 0.1 M NaOH for 15 minutes and doubly-deionized water for 10 minutes. Prior to each run, the capillary was rinsed with 0.1 M NaOH for 2 minutes, and then 18 Ω water for 2 minutes, and then sodium phosphate β-CD buffer for 5 minutes. The detection wavelength was 210 nm.

Synthesis of Benzylpiperazine and Its Analog Compounds

In collaboration with the U.S. FDA Forensic Chemistry Center in Cincinnati, OH a series of BZP samples were synthesized and characterized by various analytical methods. Our lab took part in CE and proton NMR (^{1}H-NMR) characterization, while chemists at the FDA performed the synthesis and all other techniques. To generate a series of clandestine BZP diHCl samples, synthetic procedures obtained from the Internet were used to manufacture BZP diHCl. The main goal was to determine what types of products would be generated from a synthetic procedure obtained via a non-literature source. The synthetic procedure was also modified to investigate other types of products, which could be generated as a result of the modifications to the synthesis. The products generated were characterized using Fourier transform infrared (FT-IR) spectroscopy, proton NMR (H^{1}NMR), gas chromatography with mass spectral detection (GC-MS), and liquid chromatography with mass spectral detection (LC-MS).

The first synthesis (BZP synthesis #1) involved mixing equal molar amounts of piperazine hexahydrate, piperazine diHCl monohydrate, and benzyl chloride. 1-BZP diHCl was the predominant product with a small amount of an additional compound, 1,4 dibenzylpiperazine. The amount of 1,4-dibenzylpiperazine was not quantitated by LC-MS as no standard was available at that time.

The second synthesis (BZP synthesis # 2) involved mixing equal molar amounts of piperazine hexahydrate and benzyl chloride. The predominant compound made from BZP synthesis # 2 was 1,4-dibenzylpiperazine. A small amount of 1-BZP diHCl was also observed in synthesis # 2. The two synthetic methods are shown in Figure 11.

Discussion

The goal of this analytical method was to achieve a screening procedure for both piperazines and amphetamines owing to a high probability of their joint application. Even though piperazines are not chiral compounds, our CE buffer contained a cyclodextrin to achieve baseline separation of the amphetamine-like compounds that are occasionally associated with these drugs. Careful optimization of the composition of the buffer and the analysis conditions was required in order to find a procedure that provided full resolution of all compounds of interest.

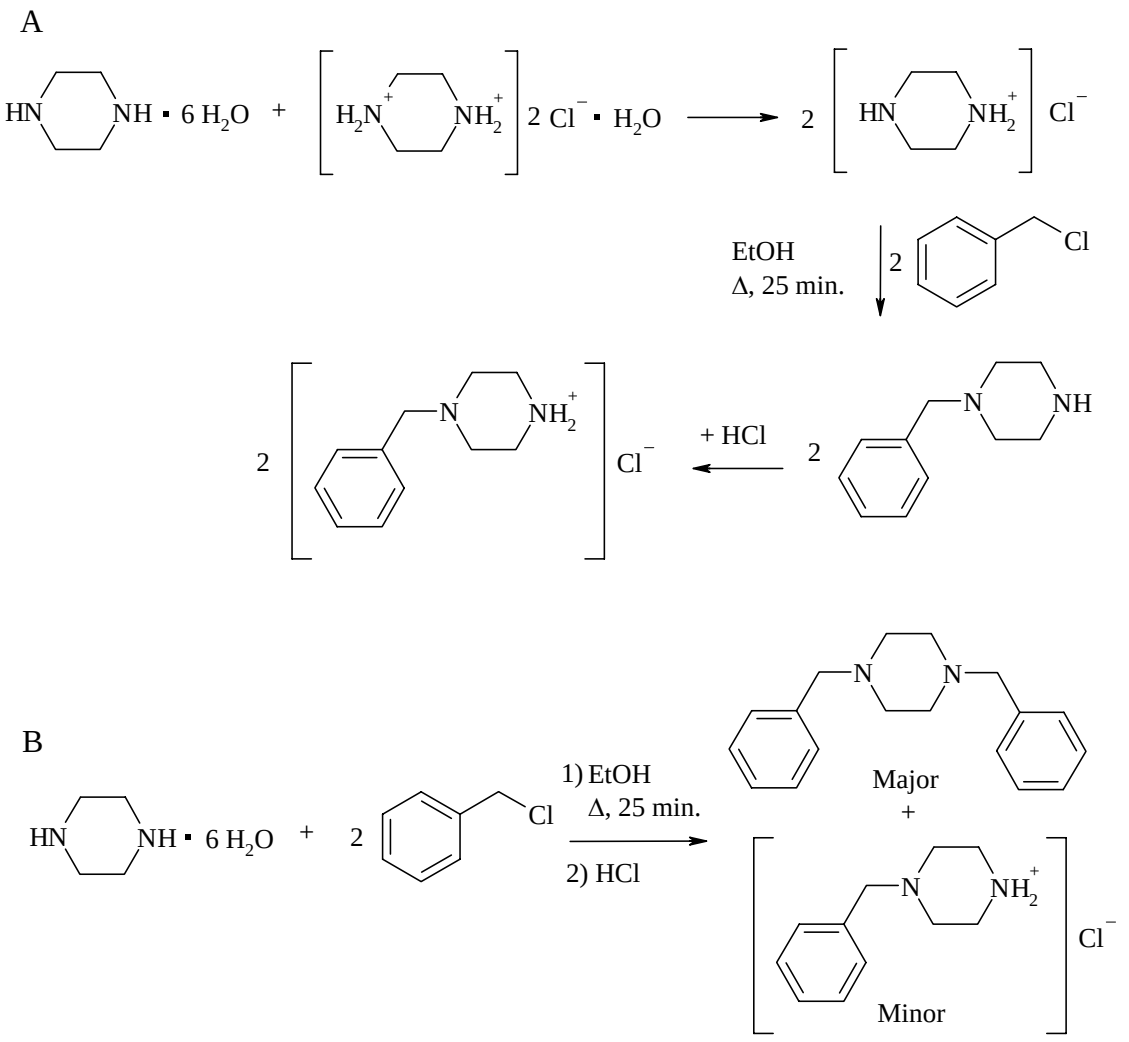


Figure 11. Clandestine Synthesis of piperazines. (A) Synthesis #1: equal molar amounts of piperazine hexahydrate, piperazine diHCl monohydrate, and benzyl chloride; (B) Synthesis #2: equal molar amounts of piperazine hexahydrate and benzyl chloride.

The optimization steps included the determination of the most favorable sodium phosphate concentration, hydroxypropyl-β-cyclodextrin (HP-β-CD) concentration, pH, and temperature. The choice of cyclodextrin type can be difficult and compound specific. Previous studies have also shown that the concentration of the cyclodextrin can affect both the analyte resolution and the migration time. Temperature can also have an effect on the selectivity of cyclodextrins. The optimized electropherogram for the analysis of both groups of drugs using a 200 mM sodium phosphate at pH=2.8 with 20 mM HP-β-CD is displayed in Figure 12. The spectral data generated from the piperazine compounds is shown in Figure 13.

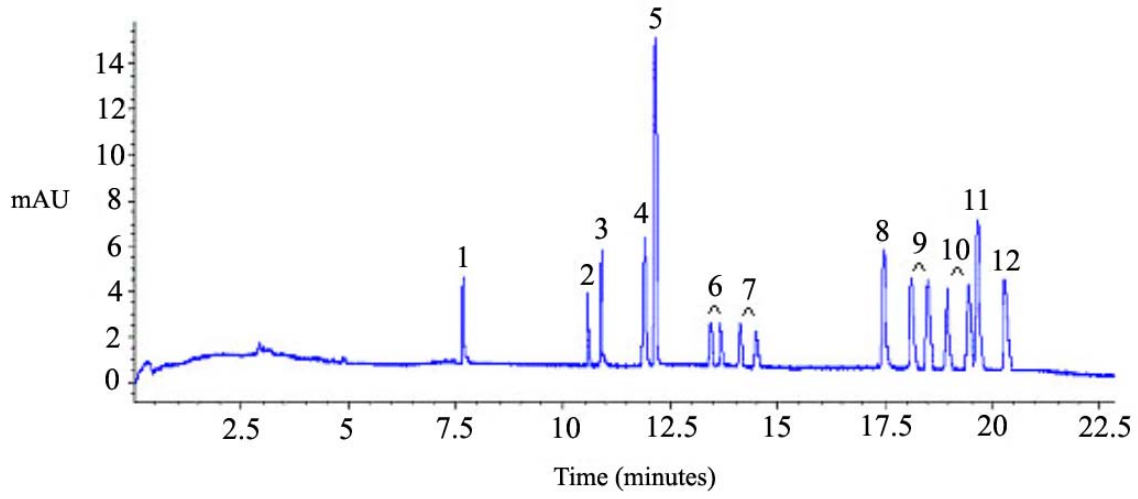


Figure 12: The optimized chiral separation of amphetamine and piperazine compounds using the standard drug mixture at 30 mg/mL. The runs were performed in a 50 m ID capillary with a total length of 64.5 cm and an effective length of 56 cm. The pressure injection was for 6 seconds at 40 mbar. The capillary temperature was kept at 25 C with a run voltage of 25 kV and a detection wavelength of 210 nm. (1) BZP; (2) phenethylamines (PEA); (3) DBZP; (4) 3-chloroaniline (3-CA); (5) 1,2-MeOPP; (6) D,L-amphetamine; (7) D,L-methamphetamine; (8) 1,4-MeOPP; (9) D,L-MDA; (10) D,L-MDMA; (11) TFMPP; (12) mCPP.

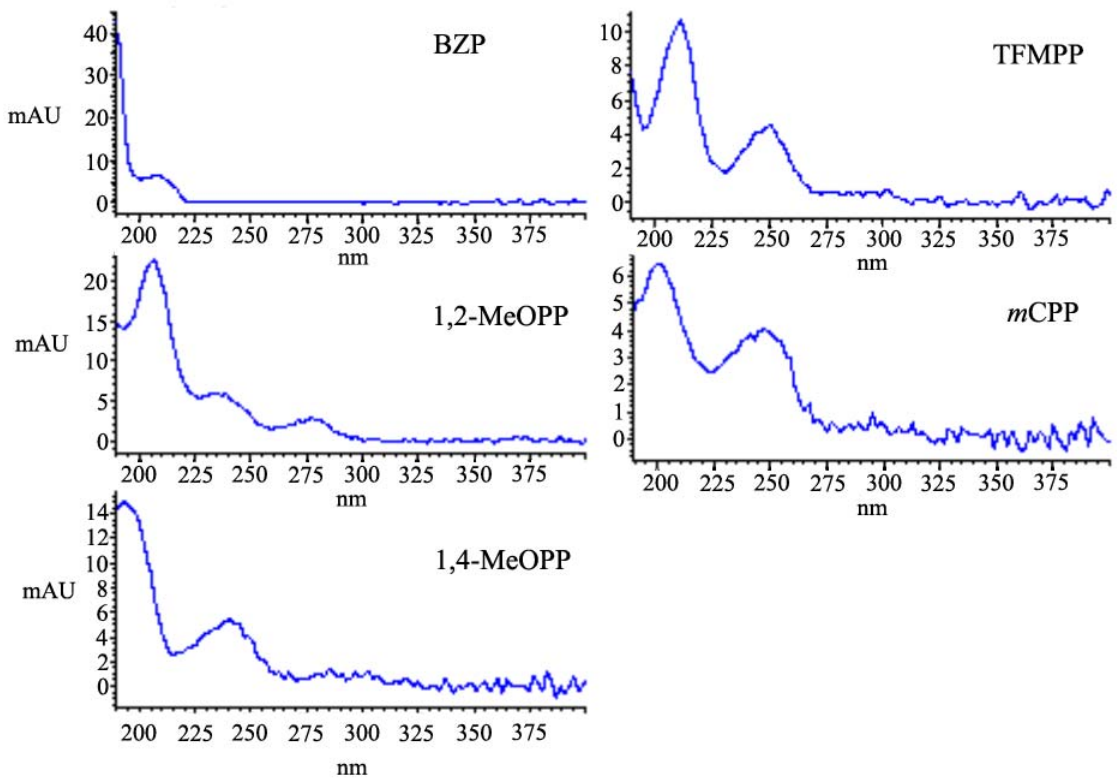


Figure 13. UV-spectral data for the piperazine-like compounds taken from the standard mixture.

Synthesized Samples

Following the optimization of the method, a series of samples obtained from the FDA's Forensic Chemistry Center were analyzed to assess the application of the method towards detecting different procedures for clandestine synthesis of these compounds. Examples of the electropherograms from samples of piperazine synthesis #1 and #2 are shown in Figure 14. Synthesis #1 (with piperazine diHCl monohydrate) produced BZP at a yield of 72.6%. The product also contained 8.7% DBZP with no other piperazine peaks. Synthesis #2 (without the monohydrate) contained traces of BZP and 14.9% yield for DBZP. No other piperazine peaks were present. The results showed the method provided useful information towards the identity and quantity of synthesized materials.

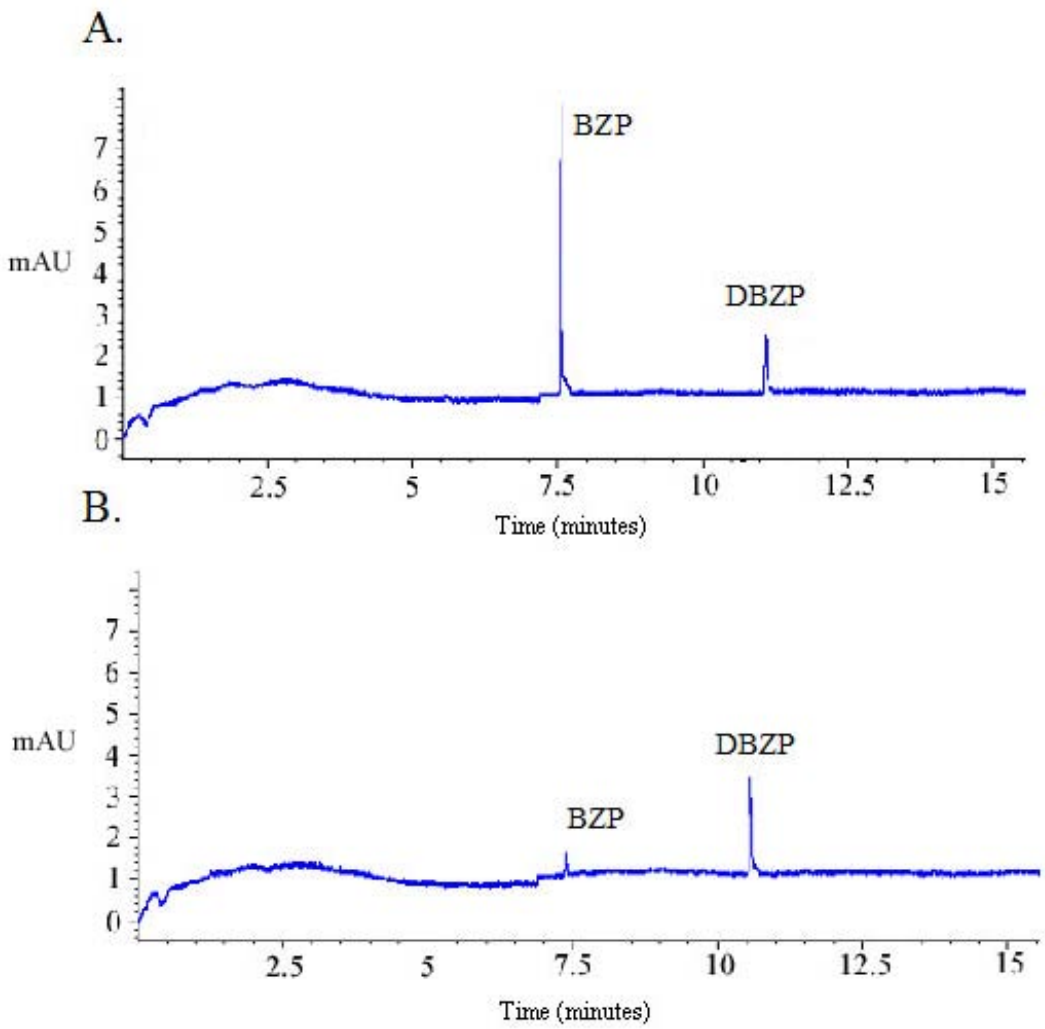


Figure 14. CE analysis of synthesized piperazines. (A) Analysis of synthesis #1 that generated BZP as the primary product with small amounts of the DBZP; (B) Analysis of synthesis #2 that generated DBZP as the primary product with trace amounts of BZP. Conditions are the same as in Figure 12.

Opiates

In this work we were interested in applying cyclodextrins to enhance the separation of this class of compounds which can be difficult to separate due to the similar structures of the different drug analogs. We also wanted to develop technique to enhance the sensitivity of detection. Using the fact that these drugs are moderately basic, we can utilize electrokinetic (voltage based injection) to selectively inject the drugs from aqueous matrices. This technique, known as field amplified sample injection was then combined with beta cyclodextrin separation to provide high resolution and optimum sensitivity in the detection of opiates by capillary electrophoresis.

In the first stage of this work, we used a buffer containing 50 mM sodium phosphate and 100 mM phosphoric acid at pH 6. A typical electropherogram for a mixture of heroin metabolites is shown in Figure 15. Under these conditions, analytes were only partially separated in the following order: normorphine + codeine, morphine, nalorphine (internal standard), and 6-AM. The lack of resolution may be attributed to the fact that all analytes migrate according to their charge-to-size ratios in CE, which in this case are very similar. To enhance this separation, further experiments were performed by changing the pH and the buffer concentration. However, no improvements were seen in the results.

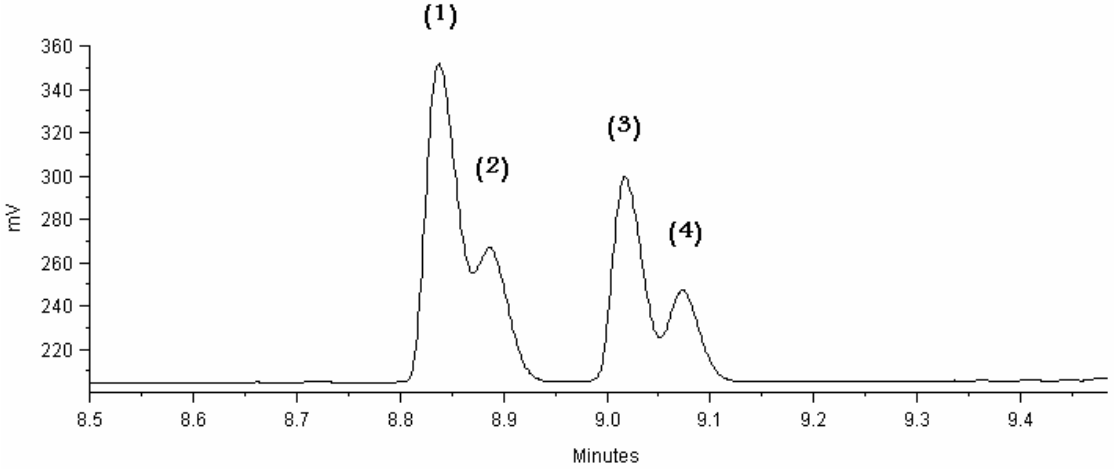


Figure 15: Typical electropherogram of 500 ng/mL mixture of heroin metabolites: (1) normorphine, codeine; (2) morphine; (3)1000 ng/mL nalorphine and (4) 6-AM. Conditions: injection, electrically (5 kV) for 10 s; capillary, uncoated (50 µm I.D.) fused-silica capillary, 77 cm long, and containing the detector window 10 cm from the outlet end; buffer, (pH 6) contained 50 mM sodium phosphate; potential 25 kV; detection, UV absorbance at 214 nm.

When ß-CDs were added to the buffer solution, Figure 16, clear separation of the standard mixture of four opiates was achieved. In this case, base-line separation was accomplished with a running buffer composed of 50 mM sodium phosphate (pH 6) and 0.015 M ß-CDs. Under these conditions, analytes were separated in the following order: normorphine, morphine, 6-AM, codeine, nalorphine, and levallorphan. This order can be attributed to the influence of the hydrophobic cavity of the ß-CDs. The rate that solutes partition into and out of the cavity will vary with their structure, polarity, and size. Concomitantly, the mobility of these solutes will be affected as well. When the solutes partition into the cavities, their velocities are retarded, but when they are in the bulk phase, their mobilities are unaffected. It is this differing partitioning behavior among the various drugs that leads to greater differences in their mobilities and, therefore, an improved separation when CDs are used.

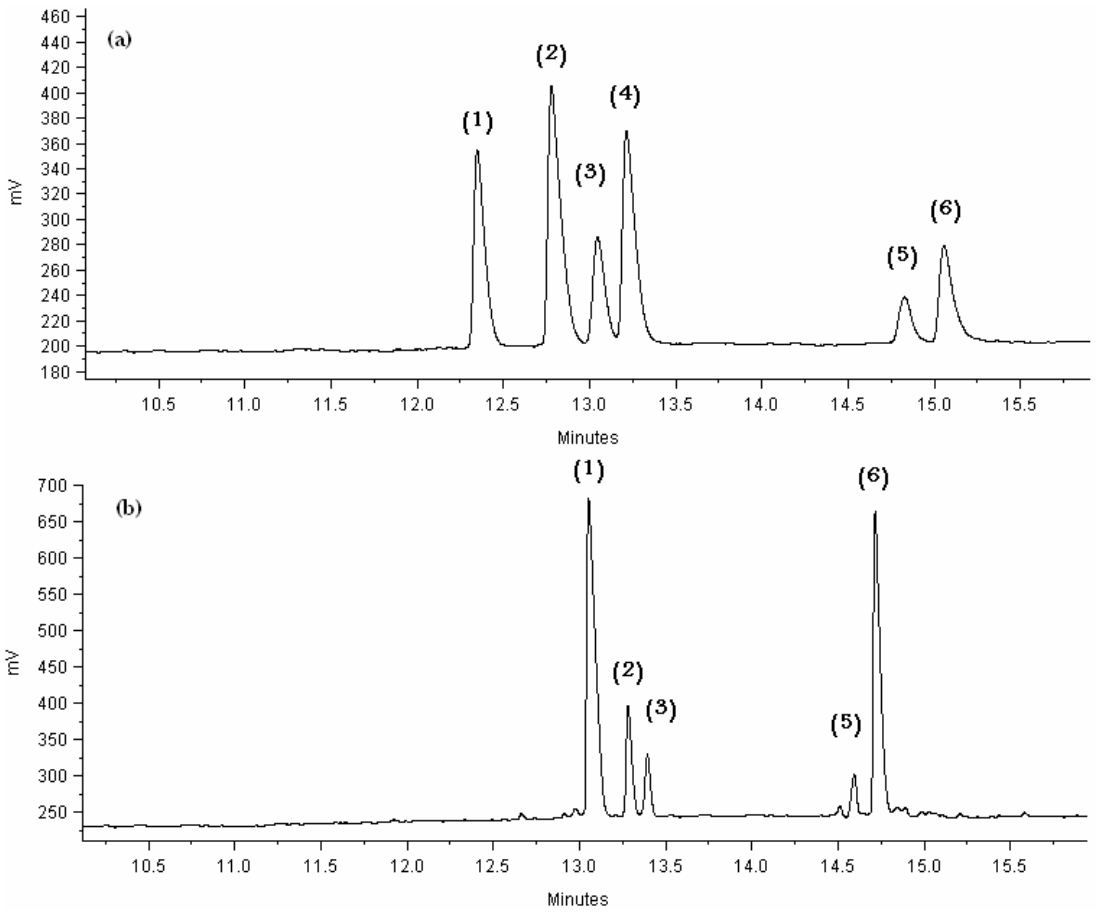


Figure 16: Typical electropherogram of (a) 500 ng/mL mixture of (1) morphine; (2) codeine; (3) thebaine; (4) 1000 ng/mL nalorphine; (5) papaverine and (6) noscapine; (b) prepared opium sample. Conditions: buffer, (pH 6) contained 50 mM sodium phosphate and 0.015 M ß-cyclodextrins, injection, electrically (5 kV) for 10 s, detection, UV absorbance at 214 nm.

In studies comparing the mobility of heroin metabolites versus ß-CD concentration, it was apparent that mobility decreases as ß-CDs concentration increases until the limit of 0.015 M, above which point the solubility of the cyclodextrins becomes a problem. Interestingly, while ß-CDs were effective in providing an increase in resolution, α- and γ-CDs did not. Since these molecules only differ in the dimensions of their inner cavities, these results emphasize the importance of analyte complexation inside the cyclodextrin cavities. Buffer pH also has a significant effect on electroosmotic flow because it changes the degree of surface charge on the capillary wall. As pH increases, the dissociation of Si-OH to Si-O^- on the inner capillary wall increases and, consequently, the electroosmotic velocity increases. At lower pH values, there is less surface ionization and lower electroosmotic velocity. On the other hand, the pH of the buffer will influence the degree of ionization of the drug under study. At values 2 units above and below the pK_a, the largest changes in migration times are expected because the solutes change from the free base to ionized form. In this study, at low pH values the opiates are all protonated and their relative mobilities are constant. As the pH is raised,

the buffer approaches the pK_a of the different drugs (8.0-10) and the selectivity increases. However, reproducibility becomes an issue as small changes in pH will have dramatic effects on resolution. We found that all four opiates can be resolved at pH 6 and that separation was most reproducible at this pH value.

Field Amplified Injection

It is well known that, because of the limited volume of the capillary, CE separation is greatly affected by the length of the injection plug. Sample stacking by field amplified injection (FAI) provides a way to concentrate the analytes in a thin zone at the boundary between the sample plug and the background buffer, allowing an increase of injection time without sacrificing efficiency. Using electrokinetic injection, both electrophoretic migration of charged sample ions and electroosmotic flow of the sample solution contribute to the introduction of the sample into the capillary, consequently increasing the introduction of charged drugs.[11] The quantity injected, Q_{inj}, is given by:

$$Q_{inj} = V\pi\, ctr^2\, (\mu_{EP} + \mu_{EOF})/\, L \qquad (5\text{-}4)$$

where V is the voltage, c is the sample concentration, t is the time duration the voltage is applied, r is the capillary radius, μ_{EP} is the electrophoretic mobility of the solute, and μ_{EOF} is the electroosmotic mobility.

In these studies, we examined the effect of various solution and matrix parameters on the application of FAI in the analysis of opiates. Different solvents and various specialized injection techniques were examined, however best results were obtained by simply dissolving the sample in water and using voltage injection, Figure 17. The injection time was also investigated for further optimization of the FAI conditions. Injection for longer time (99 s) allowed a large number of opiate drug molecules to enter the capillary at a high velocity and stack at the interface between the high and low conductivity zones. However, as injection time increases, band broadening begins to occur. Thus, a 10 s injection time was chosen as the optimum.

Analytical Characterization

To evaluate the linearity of this method, standard curves were prepared by analyzing six different concentrations of a mixture of four opiates in the range of 100-500 ng/mL with a constant amount of nalorphine (1000 ng/mL). Linear regression analyses were performed using the ratios of the peak areas of drugs to the internal standard (nalorphine) against the respective drugs concentrations. The linear regression equations for the normorphine, morphine, 6- AM, and codeine standard curves were $y = 0.0011x + 0.3511 (r = 0.9960)$,

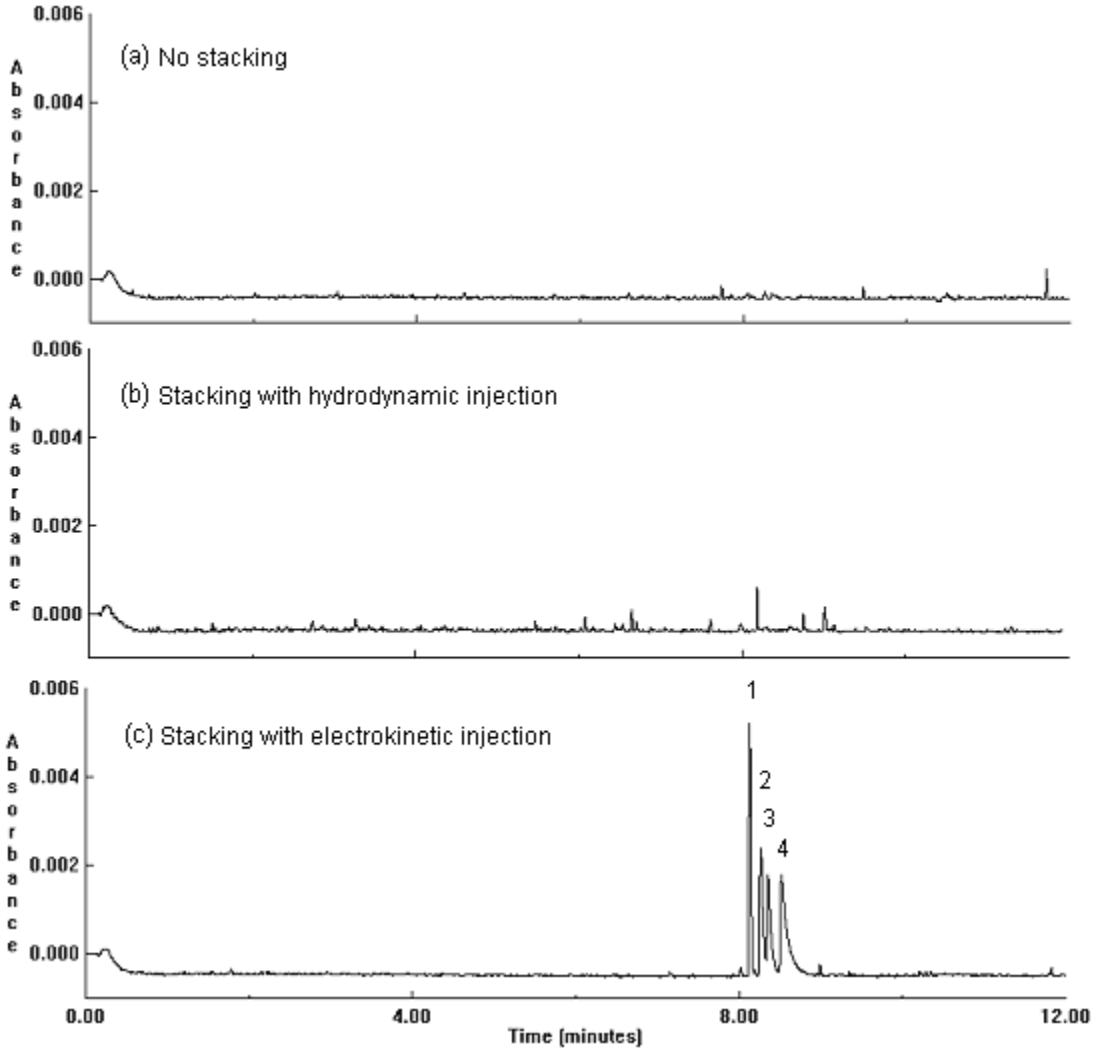


Figure 17: Electropherograms show the effect of sample stacking and the injection methods employed on the sensitivity of the CE system for drugs analysis: (a) no stacking (samples dissolved in the running buffer and electrically injected); (b) stacking with hydrodynamic injection (samples dissolved in water); and (c) stacking with electrokinetic injection.

$y = 0.0007x + 0.2652$ ($r = 0.9977$), $y = 0.0005x + 0.1687$ ($r = 0.9985$) and $y = 0.001x + 0.3715$ ($r = 0.9971$), respectively.

The intra-day and overall accuracy and precision of the calibration curves were determined by analyzing three different concentrations of a mixture of standards containing normorphine, morphine, 6-AM, codeine, and a constant amount of nalorphine on three separate days. Three replicate determinations were made at each concentration level. The intra-day relative standard deviations (RSDs) for migration times were <0.22%, and the overall precision was <0.14%. The RSDs for peak area were <4.7%. The

internal standard (nalorphine) was introduced to minimize variation resulting from the electrokinetic injection used.

The detection limits (signal-to-noise ratio of 3) were in range from 30-40 ng/mL using the conditions specified in the optimized assay method. This method gave a 100 fold improvement in the sensitivity as compared to the conventional CE methods and at least a 10 fold improvement over the CE methods that utilized sample stacking with hydrodynamic injection (500-700 ng/mL). (Weinberger, 1993) These low detection limits are more than adequate for the usual analytical requirements for controlled drugs analysis in forensic laboratories (the concentration of 6-AM results from heroin metabolites range from 10-5000 ng/mL). (Kerrigan, 2003, Telepchak, 2004)

Application to Toxicological Samples

In this study, mixed-mode SPE was used to enrich analytes and to clean-up samples prior to CE analysis. This relatively new technology offers superior clean-up and selectivity when extracting basic and zwitterionic compounds. When compared directly against a standard C_{18} column, mixed-mode SPE produced significantly less background (potentially reducing misleading peak responses), column back pressure, and ion suppression during the subsequent analysis. This can be attributed to the combination of strong ionic bonds and hydrophobic retention which allows the use of stronger wash solvents (i.e., 100% methanol) that prematurely elute the compounds on standard single mode chemistries (i.e., C_{18}).

Figure 18 shows a representative electropherogram of urine spiked with heroin metabolites and levallorphan (IS_2) and including nalorphine (IS_1) after mixed-mode SPE and electrophoresis with detection at 210 nm. It is evident that very few endogenous compounds in the urine are being extracted and applied to the capillary under the electrokinetic conditions used. The endogenous species detected have migration times shorter than 6-AM.

Conclusions

In the present work, our attention was focused on the optimization of sample enrichment offered by a field-amplified sample injection. The results show excellent resolution, separation efficiency, and analytical precision. The limit of detection was more than sufficient to determine some of the major opiates at physiological concentrations. Enrichment up to 100 orders of magnitude was achieved by taking advantage of differences in the conductivity of the sample and background electrolyte.

In addition, the experimental work was directed towards investigating the effect of ß-cyclodextrins as a complexing agent for improving the separation selectivity of capillary electrophoresis. It was demonstrated that ß-CDs, usually applied for chiral separations, also improves the resolution of closely related non-chiral substances. This work further demonstrates that pH adjustment is very important for obtaining selectivity. All four opiates can be resolved at pH 6, and that separation was most reproducible at this pH value.

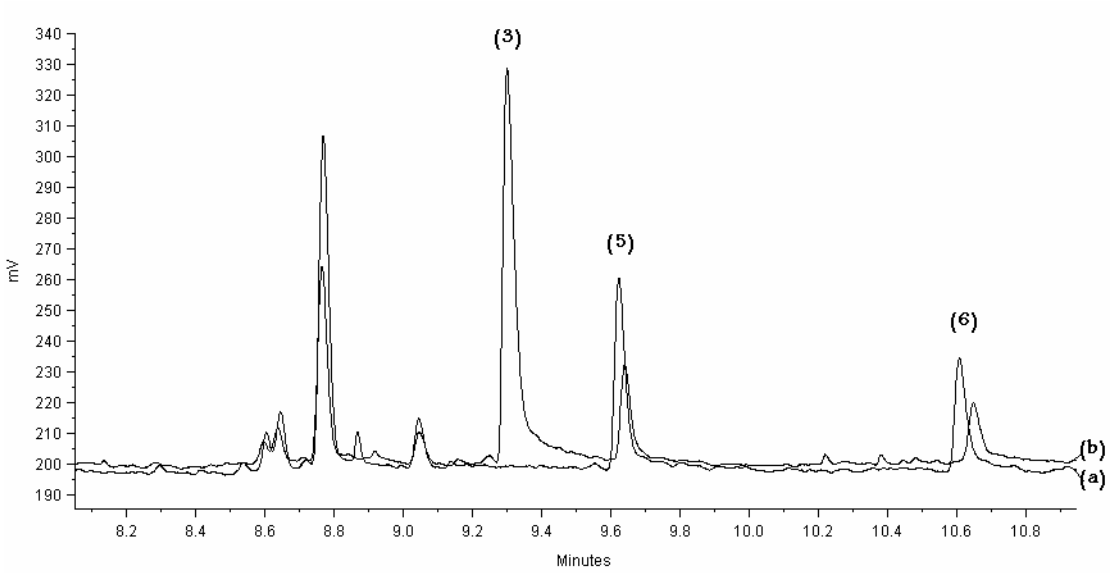


Figure 18: Typical electropherogram of (a) human urine spiked with 500 ng/mL 6-AM and 1000 ng/mL levallorphan (second internal standard used for SPE); and (b) blank urine sample spiked with levallorphan. The nalorphine (internal standard for calibration) was added after extraction.

C. Fluorescent Derivatization Techniques for Enhanced Sensitivity

In the detection of drug facilitated sexual assault, sensitivity is an important issue when detecting drugs in biological fluids. In this section of the report, we discuss our experiments examining the potential of CE coupled with fluorescence detection for the determination of multiple drugs (mainly heroin metabolites: normorphine, morphine, 6-AM, and codeine) in biological fluids. Chemical derivatizations are necessary when the drug itself is non-fluorescent or exhibits fluorescence of insufficient intensity. In this study, where native fluorescence was insufficient for the detection of trace levels of 6-AM (used for distinguishing between the presence of morphine in biological samples due to poppy seed ingestion versus heroin abuse), more sensitive measurements were made possible using simple derivatization reaction.

Abused drugs with tertiary amine groups can be easily converted to secondary amines in a high yield by reaction of amino groups with α-chloroethyl chloroformate, followed by warming the intermediate carbamate in methanol. The reaction worked with most of the illicit drug candidates in our laboratory including codeine, hydrocodone, morphine, hydromorphone, 6-AM, and cocaine. The *N*-demethylated reactions gave a single product for each drug when analysed by CE-UV. The yields of the demethylated reactions were better than 90% for all opiates tested.

The most obvious advantages of the ACE-Cl method are its simplicity and the moderate reaction conditions. ACE-Cl reacts with drugs containing tertiary amines by forming a carbamate that is easily hydrolysed to the desired product simply by heating with methanol. Other chloroformate reactions have been utilized in the dealkylation of

opiate and alkaloid drugs, (Rice, 1975, Motzka, 1974) but these reactions often require a long hydrolysis under harsh conditions. In addition, the demethylated products do not require a purification step, reducing the total derivatization time.

The next step was to derivatize the demethylated drug with fluorescein isothiocyanate, FITC. FITC reacts with primary and secondary amines like phenyl isothiocyanate under alkaline conditions to form fluorescein thiocarbamyl derivatives. These derivatives exhibit strong fluorescence with an excitation wavelength that matches the 488 nm light provided by an argon laser that is used in many CE system with LIF detection. The conditions for the FITC reaction were optimized using a standard solution of normorphine (1 µg/mL). Several parameters affecting the FITC reaction were studied, including the FITC concentration, the pH of the buffer, the proportion of organic solvents, the reaction time, and the temperature. In a series of optimization studies, 20 mM carbonate buffer (pH 8.5) gave the best results. The best reaction conditions involved using a temperature of 80 ºC for 30min, Figure 19.

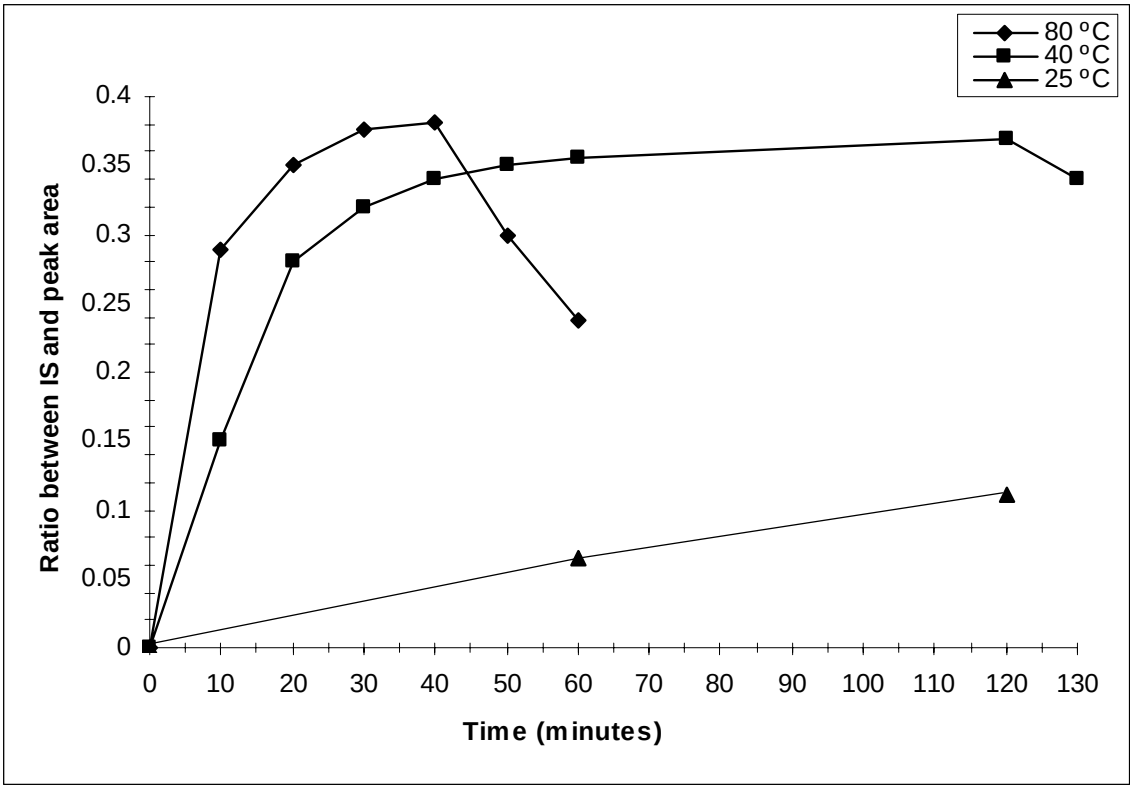


Figure 19: Effect of reaction temperature and time on the fluorescence derivatization of morphine with FITC.

Optimization of the separation conditions

An optimization study was undertaken by examining the migration behavior of a derivatized mixture of codeine, 6-AM, morphine, and fluorescein (IS). Various

parameters such as buffer concentration, pH, β-CD concentration, and organic solvent content were examined in order to optimize the separation, sensitivity, and analysis time.

In the first stage of our work, we used a buffer containing 20 mM sodium borate at pH 9.5 and an applied voltage of 25 kV. Under these conditions, the analytes were only partially separated. This may be attributed to the fact that all analytes migrate according to their charge-to-size ratio in CE, which in this case are very similar (FITC-derivatives may appear more similar in size and charge than underivatized drugs due to the large structure of the fluorescence tag). Further experiments were performed by changing the pH and the buffer concentration; however, the results did not provide an acceptable separation. Instead, 20 mM β-CDs were added to the electrolyte buffer but the resolution of the FITC-derivatized drugs was still unsatisfactory. However, by adding a mixture of 10% isopropanol and 10 % acetonitrile to the buffer complete separation was obtained. The final separation conditions involved the use of 20 mM borate (pH 9.8) with 10% isopropanol, 10% acetonitrile, and 20 mM β-CDs. As can be seen in Figure 20, the separation of heroin metabolite derivatives is obtained within 10 min using a 47 cm capillary (40 cm to detector).

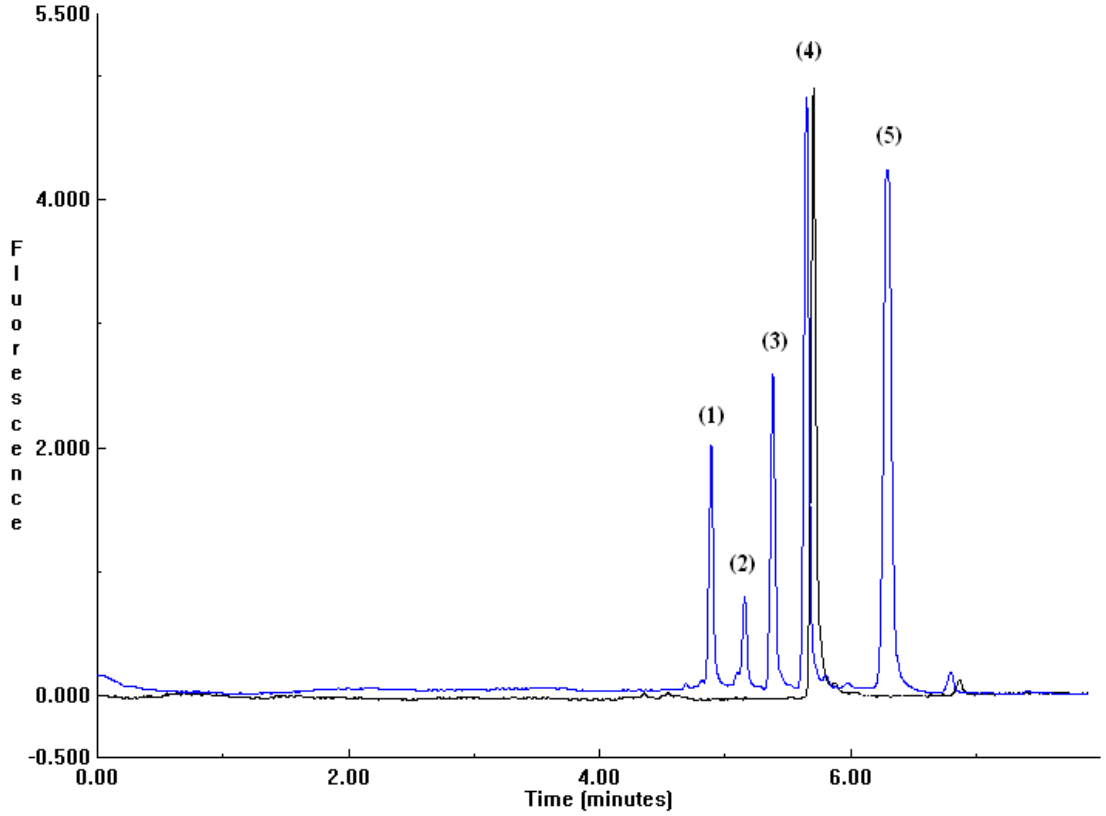


Figure 20: LIF electropherograms of a standard of FITC-labelled drugs (upper electropherogram) and reagent blank (lower electropherogram). Conditions: buffer; 20 mM borate-10% isopropanol-10% acetonitrile-20 mM ß-CDs; capillary, 47 cm x 75 µm (40 cm effective length); injection, 2 s, pressure; applied voltage, 25 kV (~65 µA); detection, LIF

fluorescence detection operated at 488 nm excitation wavelength and emission wavelength filter of 520 nm. Peaks: (1) codeine, (2) 6-AM, (3) morphine, (4) FITC, (5) fluorescein (IS). Diluted concentration of drug 500 ng/mL.

Analytical characterization

Under the conditions already mentioned, analytes were separated in the following order: codeine, 6-AM, morphine, and fluorescein (IS) within 10 min. The LODs (signal-to-noise ratio of 3) for heroin metabolite derivatives are in a range of 50-100 pg/mL using the conditions specified in the assay method. These low detection limits are more than adequate for the usual analytical requirements for controlled drugs analysis in forensic laboratories. The proposed CE method provides equivalent or better detectability than what can be obtained by HPLC or GC (Table 2). In addition, by increasing the injection time, the detection limits of the developed procedure can be lowered even further.

The intra-day and day-to-day accuracy and precision of the method were determined by running three replicates of the standard (500 ng/mL). Each FITC-derivatized nor-drug showed high reproducibility in terms of peak areas and migration times. The RSDs of the peak areas were between 0.09% and 0.74%. As for the reproducibility of migration time, RSDs were <0.03%. The day-to-day reproducibility was < 3.1%.

Applications

Several drugs of abuse were examined for potential interferences with 6-AM using the above method. These drugs included codeine, hydrocodone, amphetamine, methamphetamine, morphine, hydromorphone. No interferences were found, Figure 21. Extracted urine samples spiked with heroin metabolites and derivatized using the above procedure were analyzed by CE with LIF detection. Representative electropherograms of these samples are displayed in Figure 22. It is evident that very few endogenous compounds in the urine are being extracted, derivatized, and injected into the capillary under the conditions used. In addition, the endogenous species that are detected have migration times shorter than 6-AM and the other major heroin metabolites (morphine and codeine). Furthermore, results from the extraction of urine samples were found to be very reproducible (RSDs < 2.4).

Conclusions

The present fluorimetric CE methods give an exceptional sensitivity for the determination of opiates and other abused drugs in biological fluids. Using FITC derivatives, the sensitivity of 50 pg/mL is superior to most published procedures. As a result, this method shows good promise for application to the detection of trace levels of abused drugs in forensic analysis or as a complementary technique to traditional methodologies.

Table 2: Comparison of the detection limits reported for drugs of abuse.

Separation method	Sample pre-treatment	Detection	Limit of detection
GC	TMS-derivatized	Mass spectrometry[139]	1 ng/mL
HPLC		Native fluorescence[140]	5 ng/mL
		Mass spectrometry[141]	1-5 ng/mL
		UV absorbance[87]	10 ng/mL
	Dansyl-Cl	Fluorescence[117]	10 ng/mL
	Dimerization	Fluorescence[118]	142 ng/mL
		Coulometry[87]	0.5 ng/mL
CE			
		Amperometry[142]	285 ng/mL
		UV absorbance[57]	30-40 ng/mL
	Permanganate-derivatized	Chemiluminescence[45]	30 ng/mL
	FITC-derivatized	Fluorescence (Current work)	50 pg/mL

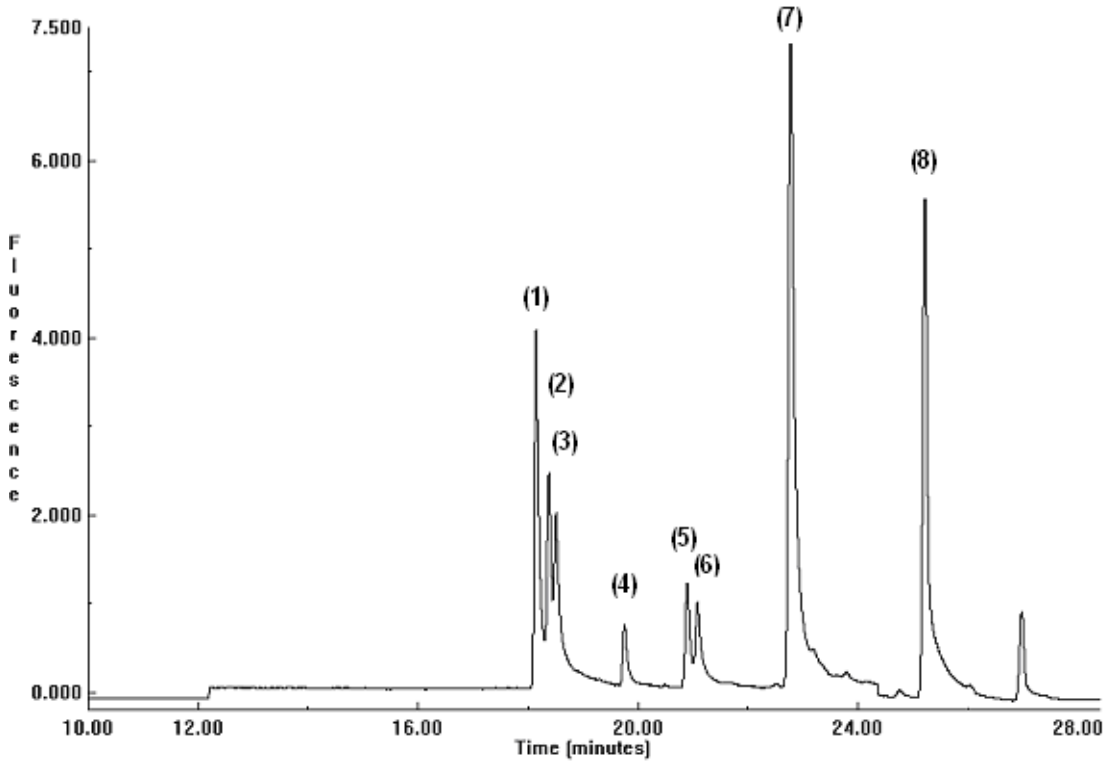


Figure 21: Typical electropherogram of multiple drugs of abuse derivatized using FITC reaction and analyzed by CE-LIF detection. Conditions: buffer; 20 mM borate-20% isopropanol-20 mM ß-CD; other condition as in Figure 6-6. Peaks: (1) codeine and hydrocodone, (2) amphetamine (3) methamphetamine (4) 6-AM, (5) morphine, (6) hydromorphone, (7) FITC and (8) fluorescein (IS).

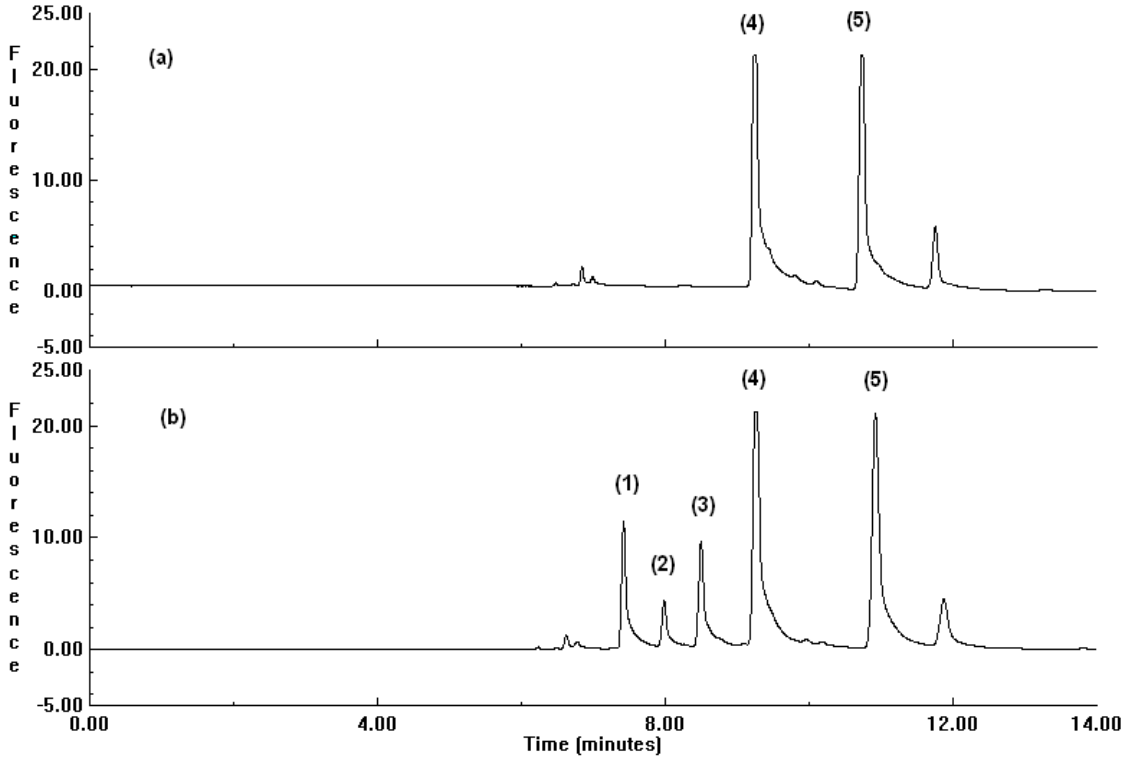


Figure 22: Typical electropherogram of (a) blank urine sample; (b) urine sample spiked with 500 ng/mL heroin metabolites derivatized and analyzed by CE-LIF detection.

D. Sensitivity Enhancement via In-line Extraction

In-line Extraction

An automated interface for coupling SPE with capillary electrophoresis has been developed and tested. This arrangement allows for the sample preparation capabilities of SPE to be combined with the separation and detection capabilities provided by CE. To demonstrate the usefulness of such integration, an automated methodology has been developed for at-line extraction, pre-concentration, and separation of twenty basic drugs of potential forensic interest in biological samples. Separation was accomplished by using a selective buffer consisting of 100 mM phosphate (pH 6), 20 mM ß-CDs, 5% acetonitrile, and 20% isopropanol with an applied voltage of 25 kV. Separation conditions, analytical characterization, method optimization, and validation were reported. Also, the influence of the automated procedure on CE sensitivity was investigated. The detection limits were in the range of 0.5-25 ng/mL using UV detector operated at 214 nm. This detection limit is about 40 times better than conventional CE analysis. Also, the method was found to yield good reproducibility, precision, accuracy,

and high recovery and a comparison was made of the proposed method with other extraction techniques such as off-line SPE and SPME.

Method Development

Initially, our attention was focused on developing an extraction technique that could be performed directly on the CE capillary. Early experiments involved the use of on-line SPE-CE, as this technique does not require any modification of the CE instrument. Several groups have demonstrated the enhancement of CE sensitivity using on-line SPE. (Strausbauch, 1996, Sentellas, 1995) However, it was our experience that CE performance was compromised. In these experiments, the use of on-line SPE resulted in reduced analyte resolution, broader peaks, and substantial component tailing. These observations can be attributed, at least in part, to increased analyte-analyte and analyte-wall interactions that can occur in the CE capillary. (Tomlinson, 1996)

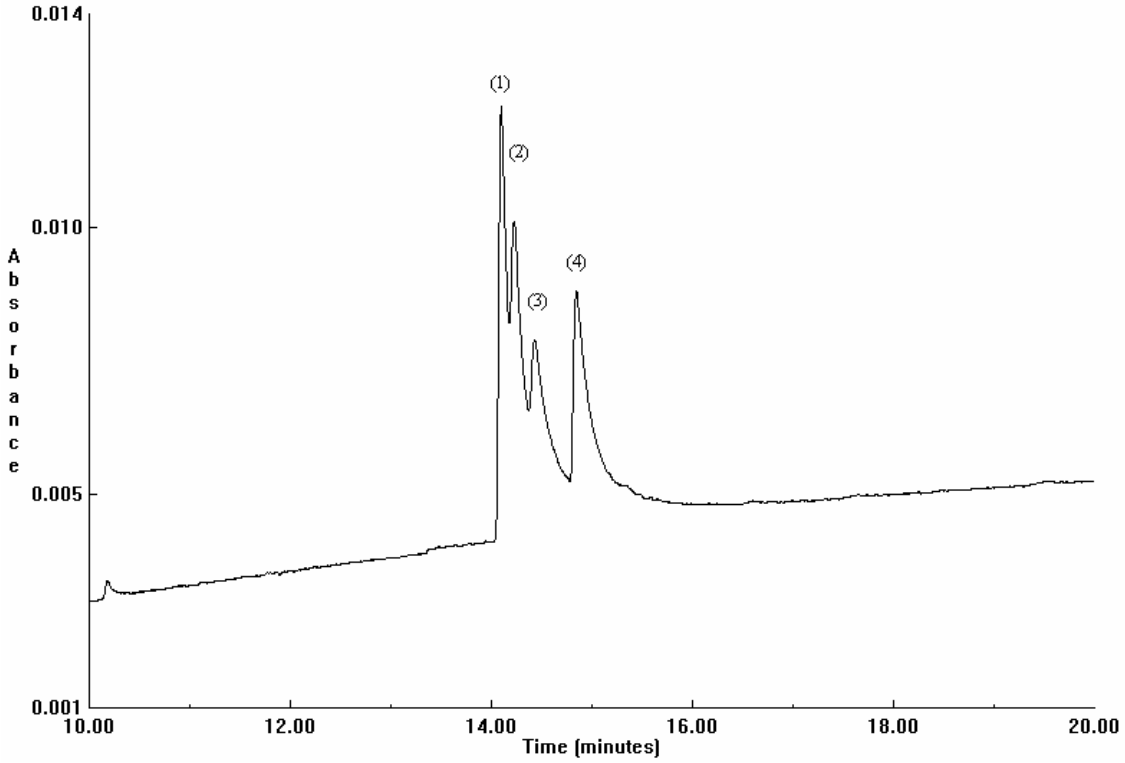


Figure 23: Electropherogram of a mixture of heroin metabolites (1) normorphine, (2) morphine, (3) 6-AM, and (4) codeine. Analysis was performed using mPC. Extraction and pre-concentration conditions as previously described.

The use of a membrane based technique slightly improved the CE performance, Figure 23; however, the resolution of the technique was somewhat limited in our application and the capillary was prone to clogging, especially when urine samples were analyzed.

One possibility for overcoming the previously mentioned problems is to couple SPE at-line with CE--a method developed in our laboratory for drug analysis. In this

procedure, an external C_{18} column was connected to the CE system by means of a switching valve. Liquid chromatography pumps were used to deliver the samples and the reagents through the C_{18} column. Once samples were loaded onto the column they could be cleaned with an organic solvent and then the analytes could be eluted and transferred directly to the CE system for analysis. This technique provided a way to automate the extraction and pre-concentration processes without affecting the electrophoresis performance.

Optimization of the automated SPE-CE conditions

The pre-concentration and clean-up steps were carried out on a C_{18} sorbent phase that was previously flushed with methanol and water. Methanol helps to wet and activate bonded functional groups to ensure consistent interactions between the sorbent and analyte, and water (adjusted to pH 9.5) helps to maximize the reverse-phase retention prior to sample loading. Two min (ca.1.6 mL) was found to be enough time to condition and equilibrate the C_{18} column. Urine samples (or working standards) adjusted to pH 9.5 were introduced into the system and an appropriate volume was passed through the C_{18} column in order to retain adequate amounts of the compounds and, therefore, obtain intense CE signals. The non-polar groups on the drugs are attached by Van Der Waal's or dispersive forces to the sorbent until a more favorable solvent will carry the analytes off the column and directly into the CE system. A 4 mL urine sample was found to be optimal.

Liquid chromatography pumps were used to push samples and reagents through the SPE column. This technique increases flow rate reproducibility (relative to the vacuum manifold) and yields more precise analytical results. An optimum flow rate was found to be 0.8 mL/min. The highest SPE recoveries were obtained using precise flow rates for each step in the extraction method.

Washing of the urine sample was initially performed using only deionized water for 2 min. However, large interferences resulting from the urine matrix were obtained. This problem could be solved by introducing another wash step containing a low percentage of organic solvent. 20% methanol was found to be optimal for this method. Finally, the elution step was carried out using 85% methanol for 40 s (about 100 µl).

Optimization of the separation conditions

Optimization of the electrophoretic separation was achieved by testing the migration behavior of twenty basic drugs (Table 3). The effect of various parameters, such as buffer concentration, pH, β-CD concentration, organic additives, applied voltage, and length of the capillary, were examined in order to determine the best separation conditions.

Initially, experiments were performed using a 100 mM phosphate buffer at pH 6 and a constant field strength of 373 V/cm. Under these conditions, the resolution of a basic drug standard was unsatisfactory. To enhance this separation, additional experiments were performed utilizing 20 mM β-CDs in the buffer solution. While the separation was slightly improved, this system also did not provide an acceptable separation, Figure 24. A complete separation of the twenty basic drugs was achieved only after the addition of 5% acetonitrile and 20% isopropanol to the buffer system in the presence of β-CDs, Figure 25.

Table 3: Peak identification for drug separations using in-line extraction.

Peak No.	Compounds	pK_a
1	Amphetamine	9.8
2	Methamphetamine	9.5
3	Ephedrine	9.6
4	Psilocin	*
5	Cocaine	8.4
6	Cocaethylene	*
7	Methadone	8.3
8	Pentachlorophenol (PCP)	4.8
9	Pheniramine	4.2, 9.3
10	Diphenhydramine	9.0
11	Oxycodone	8.5
12	Thebaine	8.2
13	Fentanyl	8.4
14	Codeine	7.9
15	Morphine	8.0, 9.6
16	6-AM	*
17	Heroin	7.8
18	Noscapine	6.2
19	Papaverine	5.9
20	Morphine-3-glucuronide (M-3-G)	*

*Not reported

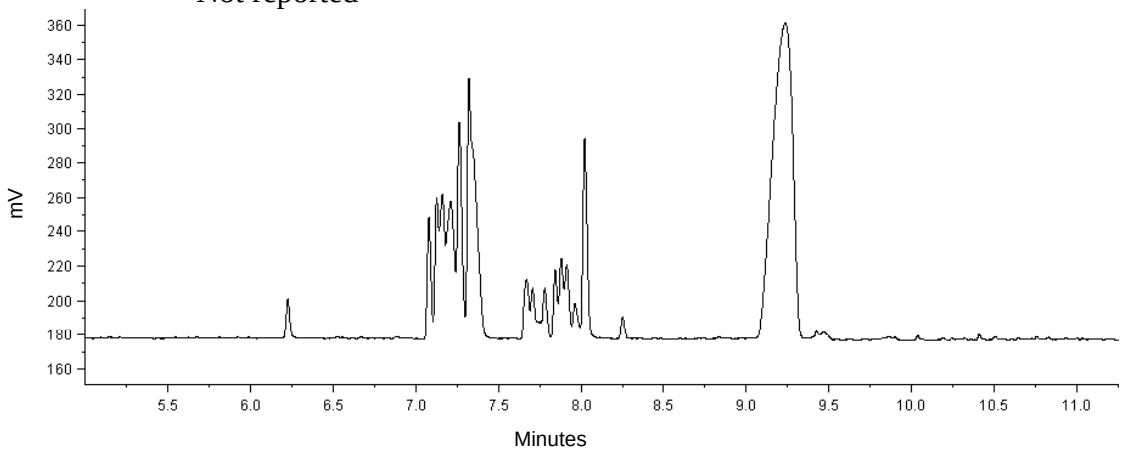


Figure 24: Electropherogram of mixture of twenty basic drugs before the addition of an organic modifier. Conditions: buffer: 100 mM phosphate, pH 6 and 20 mM β-CDs; capillary: 51 µm I.D., 60 cm long to the detector; detection: UV 210 nm; injection: electrokinetic, 15 s; temperature: 25 °C; separation voltage: 25 kV.

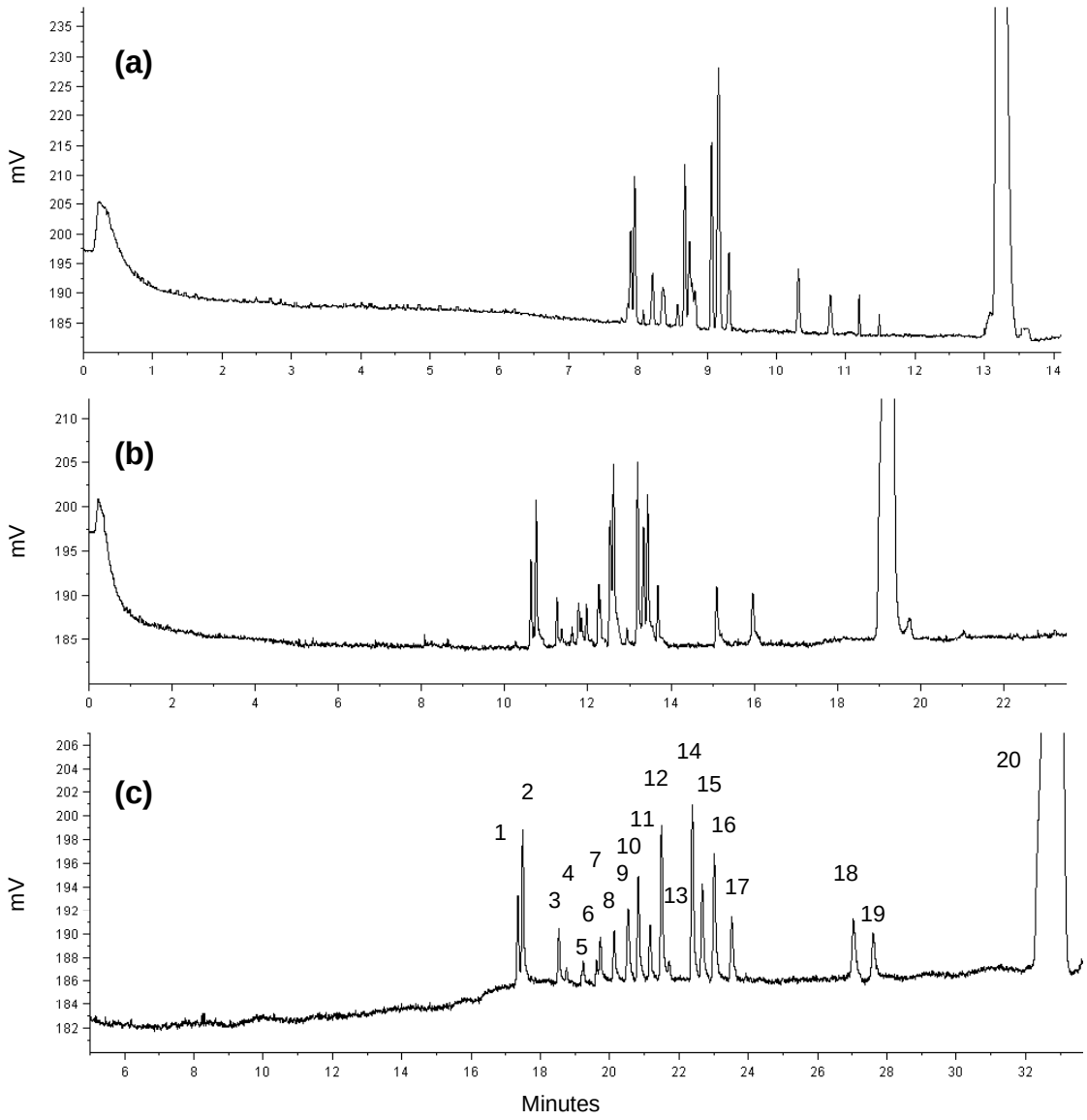


Figure 25: Electropherograms showing the effect of the addition of an organic solvent to the buffer on the resolution and migration times: (a) 25% acetonitrile; (b) 25% acetone; (c) 25% isopropanol. Conditions: buffer: 100 mM phosphate, pH 6, 20 mM β-CDs, organic solvent as indicated.

As can be seen in Figure 25, the complete separation of twenty basic drugs of different classes and different polarities was achieved within 30 min. In this separation, all tested drugs showed migration times of 15 to 23 min except M-3-G, which eluted at 28 min.

Performance of the standard CE method

Calibration plots were obtained by using standard solutions with drug concentrations ranging from 0.5-6 µg/mL. The response of all tested drugs was linear throughout this range. The RSD values of the peak areas ranged between 0.77% and 8.40%. Relatively stable migration times (RSDs less than 0.13%) could be obtained when the capillary was rinsed with sodium hydroxide after each run. Therefore, it appears to be better to rely on migration times for peak identification because of their greater reproducibility. The internal standard (quinine) was introduced to minimize variation resulting from the electrokinetic injection used.

Signal enhancement by automated SPE-CE

Figure 26 shows a comparison between a standard CE and an automated SPE-CE for the analysis of twenty basic drugs. Table 4 summarizes the characteristics of the proposed method for the pre-concentration and clean-up of standard solutions. As can be seen in the figure, automated SPE-CE provides a tremendous sensitivity enhancement over standard CE for most abused drugs and especially opiates including codeine, morphine, 6-AM, heroin, and oxycodone (this may be attributed to the stronger hydrophobic interactions of these compounds with the C_{18} column at pH 9.5 since opiates have pK_a values ranging from 7.8-8.5). This increase in sensitivity resulted from the large volume (4 mL) of sample that can be injected and pre-concentrated into the head of the C_{18} column prior to electrophoresis. This method allowed the determination of basic drugs at low concentrations (below 0.5-25 ng/mL), which is an appropriate range for the analysis of real urine samples. This detection limit is about 40 times lower than a conventional CE system. Furthermore, the proposed CE method provides equivalent or better detectability than that which is obtained by HPLC and GC without the need for derivatization or an expensive LIF detection. In addition, the method showed a high sample-to-sample reproducibility. The RSD values were between 0.8% and 2.5%. The recovery was also very high ranging from 63% to 99%.

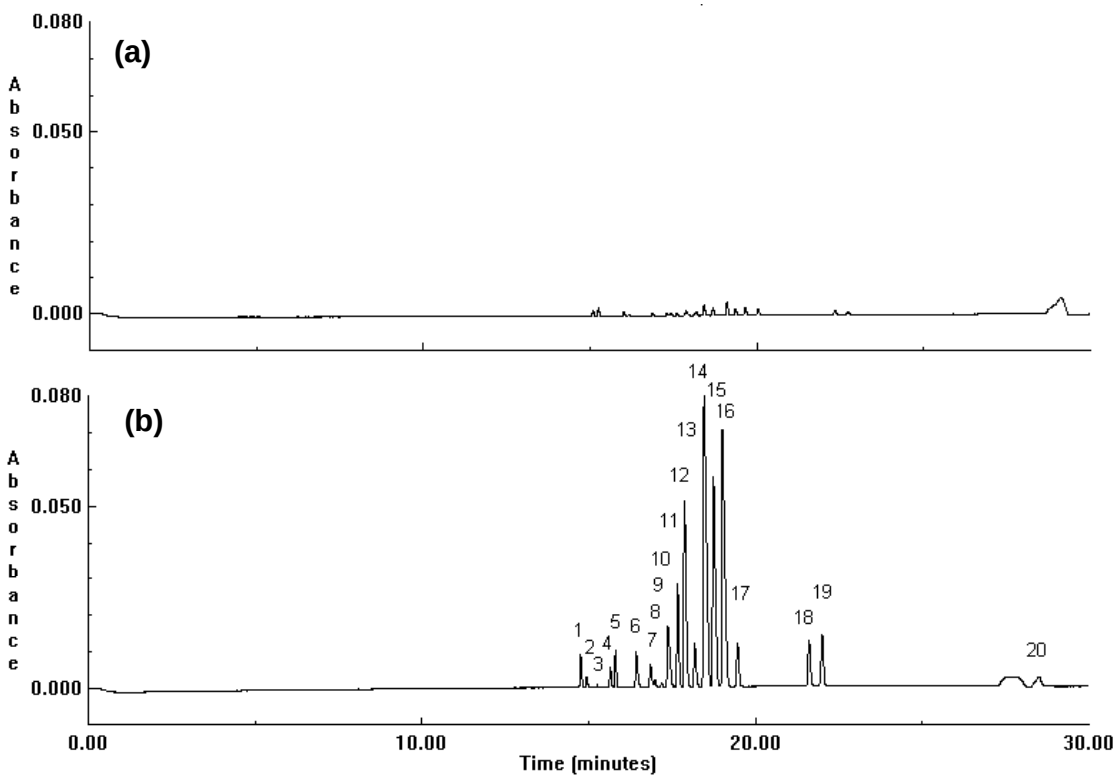


Figure 26: Comparison of (a) standard CE and (b) automated SPE-CE for the analysis of basic drugs.

Table 4: Characteristic of the automated SPE-CE with standard solutions.

Drug	Actual concentration (µg/mL)	Calculated concentration (µg/mL)	RSD (%)	Recovery (%)	LOD (ng/mL)
Amphetamine	2.00	1.531	1.5	76.55	10
Methamphetamine	2.00	1.415	1.3	70.75	10
Ephedrine	2.00	1.258	1.3	62.90	10
Psilocin	0.20	0.168	1.2	84.00	5
Cocaine	3.00	2.472	1.7	82.40	10
Cocaethylene	2.00	1.611	0.8	80.55	10
Methadone	1.00	0.691	2.5	69.10	10
PCP	2.00	1.243	2.3	62.15	30
Pheniramine	2.00	1.392	1.5	69.60	20
Diphenhydramine	2.00	1.693	1.3	84.65	3
Oxycodone	1.00	0.981	1.1	98.10	3
Thebaine	2.00	1.915	1.4	95.75	3
Fentanyl	0.50	0.411	1.6	82.20	10
Codeine	2.00	1.976	1.8	98.80	0.5
Morphine	2.00	1.963	1.5	98.15	1
6-AM	2.00	1.932	1.6	96.60	0.5
Heroine	2.00	1.572	1.4	78.60	10
Noscapine	2.00	1.622	1.2	81.10	10
Papaverine	2.00	1.666	1.1	83.30	10

Applications

Since the off-line mixed-mode SPE method is commonly used for the routine analysis of abused drugs, its accuracy and precision were also determined and compared to that of the proposed automated method. We found that the off-line SPE has slightly better recovery for all tested drugs (ranging from 88% to 102%). This may be due to the use of mixed-mode technology which allows a strong wash solvent to be utilized without the problem of losing analytes. In automated SPE, in which a C_{18} column was used, 20% methanol was found to be important to minimize the interferences resulting from the urine matrix. However, this solvent can also elute basic compounds of interest prematurely. On the other hand, the precision of the off-line SPE was found to be lower than that of the automated procedure (the RSDs were between 1.1% and 7.6%). This can be attributed to the minimal sample and reagent handling that occurs when using the automated method.

Conclusions

As CE is used for more diverse applications in the forensic and pharmaceutical sciences, the ability to enhance sample loading capacity will become more important. The present study demonstrates that dilute drug samples can be analyzed effectively by automated SPE-CE methods with lower detection limit than is currently possible with conventional techniques. The automated SPE system allows the continuous pre-concentration and clean-up of analytes, while the CE system affords highly sensitive separations over broad concentration ranges. The coupling of both systems allows the expeditious, reproducible, sensitive, and inexpensive determination of abused drugs in human urine. The proposed method also overcomes most of the problems that are encountered with on-line techniques. Finally, the proposed methodology may be an effective alternative to GC and HPLC for the analysis of these compounds.

E. Packed Column Capillary Electrophoresis

Packed column capillary electrochromatography, CEC, offers potential advantages in forensic analysis in that neutral species can be separated without having to resort to pseudostationary phases, such as SDS or cyclodextrins. This makes CEC more compatible with mass spectrometry (MS) than MECC. The high SDS and β-CD concentrations, which are typically used in MECC, overwhelm the spectrometer rendering it incapable of proper analysis. In packed column CEC, these mobile-phase additives are replaced by stationary phases similar to those used in LC and LC-MS.

Despite these advantages, there are several reasons why the technique has been slow to develop. (Svec, 2000) One reason is that column preparation can be very problematic. Not only does it require a great deal of skill to pack a capillary column with silica particles, but once completed, they are quite fragile. Another reason is that occasionally, particles from the stationary phase become dislodged, causing voids, and loss of electrical continuity. Additionally, frits that are designed to keep the silica uniformly packed inside the column create restrictions that lead to bubble formation and subsequent reproducibility problems. One way to avoid the last two problems would be to use a polymeric stationary phase that adheres to the capillary wall. Such phases would

be uniform, cohesive, and free from requiring the use of frits. Several types of so-called monolith polymer columns will be explored for use in small-molecule drug separations. The nature of the materials provides a porous, yet rigid, skeletal-type backbone that is attached to the capillary.

In this section, both traditional CEC silica packing and monolithic polymer phases were examined with respect to their utility as separation systems for small molecule separations. Sufficient background regarding the polymer chemistry behind the monoliths will also be provided as a guide for future experiments. In particular, the problems involved in developing materials capable of providing sufficient chromatographic efficiency and EOF for drug separations will be discussed.

Modified Silica Phases

The theoretical basis of CEC selectivity is similar to that of reversed phase LC. In both techniques, the hydrophobic selectivity of the stationary phase can be improved by increasing the carbon-chain length of the modified stationary phase. (Johnson, 2001) Unfortunately with CEC, this increase in carbon loading will reduce the number of free silanol groups causing a concomitant decrease in the EOF. When separating neutral drug molecules, careful attention to EOF generation must take place, or overly long separations will result. In addition, because of the necessity to use somewhat exposed silica stationary phases to generate EOF, neutral compounds give well-defined peak shapes in CEC. Basic compounds that separate better with HPLC stationary phases tend to have poor peak shape in CEC due to strong interactions with the poorly end-capped stationary phases.

Separations of aryl compounds using thiourea as a neutral EOF marker have been previously studied and are often used as a test of individual column performance. (Smith, 2000) Typically, in order to maintain a high EOF, buffer pH is kept high. At a high pH, acidic compounds are charged and therefore migrate away from the detector. Unlike MECC and chiral CE separations, which possess extremely high bulk flow that eventually carries most components to the detector end, EOF in CEC is not as strong. However, several groups have demonstrated that it is possible to use a sufficiently low pH where acidic compounds are still neutral and have an EOF adequate enough to allow for detection. Separation mechanisms for basic compounds are still under investigation and development.

Monolithic Polymer Phases

Monolithic polymers offer an alternative to traditional silica packing materials. Monoliths are created *in situ* resulting in a porous polymer capillary matrix. Some of the first monolithic columns were prepared in the 1970s for gas and liquid chromatography from open-pore polyurethane foams. Hjerten and coworkers performed initial work on acrylamide-based CEC stationary phases, but because of their limited chromatographic properties, alternative polymers have been developed. (Ericson, 1997) Among the most promising of these new methods was the involvement of methacrylate ester monomers, which were originally developed for molded, rigid monolithic HPLC columns.

Monolithic polymer columns possess several advantages over silica gel packed columns. The most important difference is the elimination of CEC column frits due to the rigid monolithic polymer structure that adheres to the capillary wall. As previously mentioned, frits are normally used to keep the silica gel uniformly packed in the columns, but can often lead to severe band broadening and reproducibility problems. Monolithic columns are relatively inexpensive and can be just as efficient as packed columns. With optimum conditions, some groups have reported that separation efficiencies of up to 140,000 plates/m are possible. Another advantage to these polymers is the simplicity of column preparation along with the numerous combinations of surface functionalities that can be prepared from a variety of monolith components.

Advocates of monolithic materials claim that the problem with utilizing the previously described HPLC-type phases for CEC is that these particular phases cater to partitioning alone. CEC-type phases need to fulfill a dual role that involves interaction with analytes, as well as being able to provide a proper environment to generate sufficient EOF. One way some researchers have been able to combat this problem is by performing pressure assisted CEC (p-CEC). As its name implies, p-CEC provides pressure from the inlet electrode, pushing the separation towards the detector. This practice is similar to µ-HPLC, with the exception that a slightly lower pressure can be used, since voltage inherent to electrophoresis is also applied. The addition of pressure for p-CEC imparts a third contributor to the separation, complicating the separation mechanism. It is probable that p-CEC was created by researchers who could not achieve high enough EOF to permit analytes to reach the detector in a reasonable amount of time. While some interesting results have been attained using this technique, in general, the ability to achieve high theoretical plate numbers and Gaussian peak shapes is lost. Therefore, experiments performed in the following work focuses on the development of monolithic phases capable of separating small molecules without the use of pressure.

The Polymer Chemistry Behind Complex Monoliths

In order to appreciate the complex interactions involved in the creation of monolithic stationary phases, an understanding must first be achieved of the manner in which the polymer components interact. A polymer is a large macromolecule, constructed from a repetition of smaller chemical units, the monomers. A copolymer is a polymer synthesized with more than one type of monomer. The monomer distribution, for example could be random, constructed in blocks, or grafted. (Svec, 2003)

Monolithic polymer stationary phases are prepared by mixing an initiator with one or more monomers causing a random chain polymerization. Also included in the mixture are one or more porogens, which are small molecules that intercalate within the polymer matrix to create pores. The resulting mixture of components, while not easily miscible, can be forced into a homogenous solution and used to create a unique polymeric structure. At the conclusion of the polymerization, the porogens are washed free of the monolith. The choice of solvent used in the polymerization process dictates the porous properties of the monolith without affecting its chemical composition. Pore sizes are manipulated through a process known as phase separation, or nucleation. Certain solvents can compete in the solvation of the polymer chains, effectively orienting and controlling the size and distribution of pores in the monolith. On occasion, only one

solvent is chosen as a porogen, while often a combination of two or more porogens in different ratios are used to achieve the most desirable range of pore sizes. Another way to influence size is to vary the amount of cross-linker in the monomer mixture. Unlike the choice of solvents, this manipulation will in fact alter the chemical composition of the finished stationary phase.

Ideally, a useful stationary phase must contain interaction sites such that analytes of interest will partition out of the mobile phase and into the stationary phase. As explained previously, other surface functionalities must also be used to generate and maintain the EOF. These types of porous phases will only be able to provide proper interactions if there is sufficient access between the sites of interest and the analytes. Accurate pore size data is crucial in the development of a viable polymer matrix.

Experimental

Supplies and Reagents

Butyl methacrylate and N-dimethyloctylamine were obtained from Acros (Morris Plains, NJ). Ethylene dimethcrylate was obtained from Sartomer (Exton, PA). N-octylmethacrylate was purchased from Polysciences, Inc. (Warrington, PA). A vinylbenzyl chloride (VBC) sample was obtained from Dow Chemical (Midland, MI). 3-(Trimethoxysilyl)-propyl methacrylate (98%), cyclohexanol (99%), 1-decanol (98%), 1,4-butanediol (99%), methanol, and 2,2'-azobisisobutyronitrile (98% AIBN), divinyl benzene (DVB), and 2,2-dimethoxy-2-phenyl-acetophenone (DMPA) were purchased from Aldrich. Basic alumina and 2-(4-Morpholino)ethanesulfonic acid (MES) were obtained from Acros (Fairlawn, New Jersey).

Instruments and Conditions

All silica columns were purchased from Unimicro Technologies, Inc. (Pleasanton, CA). The C8, C18, and phenyl columns all had an internal diameter (I.D.) of 100µm, 25 cm of packed material that had a particle size of 3 µm.

The capillary for the monoliths was purchased from Polymicro Technologies (Phoenix, AZ). Clear, UV-transparent capillary (TSU100375) with an I.D. of 100 µm was used for the DMPA initiated polymers while standard polyimide coated capillaries, also with a 100 µm I.D., were used for the thermal AIBN initiation.

A temperature controlled polymerization light box was designed and manufactured in-house. A temperature controlled water bath was used for the thermal polymers. Capillaries conditioned outside the CE were rinsed with the BS-8000 Programmable Syringe Pump from Braintree Scientific (Braintree, MA) equipped with 1.00 mL syringes from Hamilton (Reno, NV) and micro-fittings from Upchurch Scientific (Oak Harbor, WA).

All electrophoretic experiments were performed on an Agilent Capillary Electrophoresis System. Mercury Intrusion Studies were performed at the Materials Sciences Division E.O. Lawrence Berkeley National Laboratory of Dr. Frantisek Svec at the University of California, Berkeley. Instrumentation included a Micromeritics

AutoPore IV Mercury Porosimeter for the MIP measurements and the ASAP 2020 Accelerated Surface Area and Porosimetry Analyzer for gas sorption techniques.

Column and Buffer Preparation

Silica Columns

For the treatment of the commercial silica columns, the pre-analysis column conditioning procedure provided by the manufacturer was used. Once in the CE, the columns were prepared for analysis by rinsing with buffer for 10 minutes at high pressure (typically 5.0-12.0 bar) at the inlet electrode inducing a forward rinse. High pressure was then applied at both ends of the capillary for 10 minutes to establish equilibrium. Voltage was increased by 5 kV every 10 minutes for the next 40 minutes to achieve a constant baseline and a steady current. The buffer made for the separation of barbiturates and benzodiazepines was 4:3:3 (v:v:v) acetonitrile (ACN):2-(4-morpholino)ethanesulfonic acid (MES):H_2O.

Monolithic Polymers

Methacrylate Monoliths

The polymerization procedure for the methcrylate polymers was adapted from protocols originally developed by Svec and coworkers. (Svec, 2000) The monolith polymers were prepared in three steps: surface modification, polymerization, and washing. Surface modification helped secure the monolithic polymers to the wall of the capillary and was performed in bulk, usually about 3 to 5 yards at a time. This step could be omitted; however the resulting polymer structure was more susceptible to collapsing under high pressure.

The capillary was rinsed for 15 minutes with acetone at 0.50 mL/hour using a syringe pump. This was followed by 0.20 M NaOH for 1 hour at 0.5 mL/hour, a brief rinse with doubly-deionized water, and 0.20 M HCl for 1 hour at 0.5 mL/hour. There was a second water rinse at 0.50 mL/hour until pH paper indicates neutrality. The capillary was rinsed briefly with acetone a second time followed by conditioning with 20% 3-(trimethoxysilyl)propyl methacrylate in acetone overnight at approximately 50 μL/hour. This coupling agent was used to ensure proper grafting sites between the silica and forming polymer. The washing procedure ended with another short acetone rinse. The capillary was then purged with a stream of nitrogen running in the same direction as the conditioning to remove all excess acetone. It was then cut into the desired column length.

For the polymerization step, the desired stationary phase monomer solution was prepared in disposable black top glass vials. The solution was purged gently with nitrogen for 10 minutes through a syringe needle. Using capillary action, a few centimeters more than the desired capillary was filled with monomer solution. The capillaries could be filled to the desired active length and then polymerized by plugging both ends with rubber stoppers. The capillaries were then subjected to either a hot water bath at 70°C or UV irradiation.

The polymerized capillary column was rinsed free of porogenic material by using the syringe pump to flush with MeOH, acetonitrile, and then the desired buffer. The syringe pump was set at a low dispensing volume of 20 µL/hour until all air bubbles were removed. Pump pressure was steadily increased to approximately 80 µL/hour. The capillary was then installed into the Agilent CE cartridge with the ceramic interface to protect the more fragile UV-transparent capillaries.

Results and Discussion

Silica Columns

CEC Hypersil C18

Open capillaries are usually maintained through a 1 to 5 minute buffer flush between runs, however, CEC preparation required more time and effort. Preparing a column involved a steady ramping of current from 0 to 20 kV, as described in the experimental section, in order to achieve suitable EOF and a steady current. Owing to the decreased amount of ionic strength and high concentration of organic solvent, currents in CEC are much lower in comparison. Experiments were performed on silica CEC columns as a way to better understand the chromatographic interactions of the small drug molecules, specifically those utilized in DFSA. Results could be directly compared to monolithic type phases to speculate on possible mechanisms of retention and migration.

Euerby et al. performed a barbiturate separation on a variety of different silica columns. (Euerby, 1999) They concluded that in the ion suppression mode, CEC was a useful tool for the separation of weakly acidic analytes. The buffer used in their study was a 6:2:2 (v:v:v) acetonitrile:50 mM MES buffer at pH 6.1:H_2O. When studied using a C18 column, the best results were achieved with a 4:3:3 (v:v:v) ACN:MES:H_2O buffer. Using these conditions, the method was capable of separating 4 barbiturates along with an additional 3 benzodiazepines, as shown in Figure 27. It was interesting to note the peak broadening and loss of Gaussian peak shape. Separations such as these might be aided by the use of gradient CEC. Usually, barbiturate and benzodiazepine screenings were two separate procedures normally performed by forensic toxicology laboratories. An optimized CEC procedure combining these two drug classes would be very useful to reduce screening time.

Monolithic Polymer Columns

Column Preparation

Column preparation procedures were adapted from publications of the Svec group. (Hilder, 2004, Yu, 2002) An inhibitor, 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH), was added at the 0.01% (w/v) to a 50% (v/v) solution of the silanizing agent in dimethylformamide (DMF).

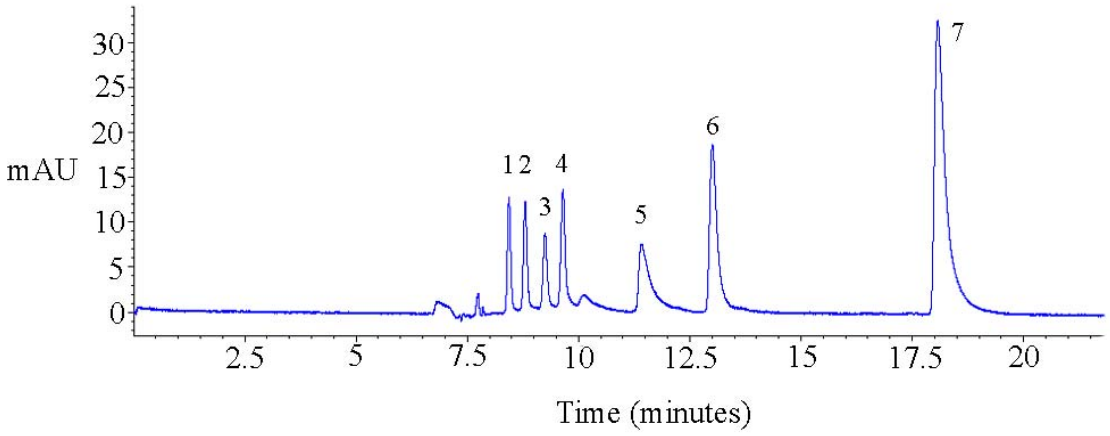


Figure 27: Separation of benzodizepines and barbiturates using a C18 column with 25 cm of packed silica using a buffer of 4:3:3 (v:v:v) ACN:MES:H2O. (1) Phenobarbital; (2) Pentobarbital; (3) Chlordiazepoxide; (4) Secobarbital; (5) Alprazolam; (6) Diazepam; (7) Triazolam.

The capillary was rinsed with ten-column volumes of this solution, sealed, and then heated at 120 °C for 6 hours. Scanning electron micrograph (SEM) images of columns made with and without reactions containing DPPH showed gaps between the capillary wall and the polymer clearly appeared in non-inhibited columns.

A major drawback to filling the column by capillary action was the unfavorable monomer/air interface that was created. The interface led to gel formation and uneven edge polymerization, which caused column blockage. Protective sleeves, made from HPLC tubing secured with black tape, were used to mask portions of an overfilled column where active phase was not desired. This procedure generated a polymer/monomer solution interface that prevented inconsistent polymerization, which appeared to have increased the success of useable columns. Unfortunately, this same interface existed with thermal initiation; however columns were less prone to problems with the interface when subjected to this type of polymerization.

When using UV-light initiation it is important make sure that the area surrounding the columns was temperature regulated. This was because free radicals could also be thermally generated using azobisisobutyronitrile (AIBN). Purging the polymerization chamber at a constant temperature assures that the polymerization mechanism occurring in that environment was UV-mediated. In later experiments, some of the polymerization mixtures used 2,2-dimethoxy-2-phenyl-acetophenone (DMPA) as it was found to be a more efficient UV-light initiator.

Despite the lack of frits in monolithic packings, there was still bubble formation inside some of the capillaries. Other groups observed that certain instances of bubble formation was caused by the interface between the stationary phase and the open capillary. (Euerby, 1997, Smith, 2000) More specifically, the bubbles formed due to the increase of EOF once the stationary phase edge was traversed. Pressurizing the inlet and outlet electrodes during separation helped to avoid these problems. The same

conditioning method described for the silica columns was used for the monoliths. An SEM image of the 75% 1-propanol poly(butyl methacrylate-co-ethylene dimethacrylate) monolith sample "75A" preparation is shown in Figure 28.

Chromatographic Results

An important aspect of CEC discovered after the conclusions of these experiments was the importance of packed capillary length. To reduce the effects of band broadening due to the analytes traveling from a CEC type environment to a CE-type environment, columns should be made 25 cm in length for long CEC columns and 8.5 cm for reverse end CEC columns. These lengths equaled the effective capillary length from the inlet tip to right before the detector interface on an Agilent CE. Adjustments should be made accordingly for different vendors. Unfortunately, this polymer was not suitable as a stationary phase under these conditions because of poor selectivity, but due to is high surface area, it might prove useful as solid phase extraction media.

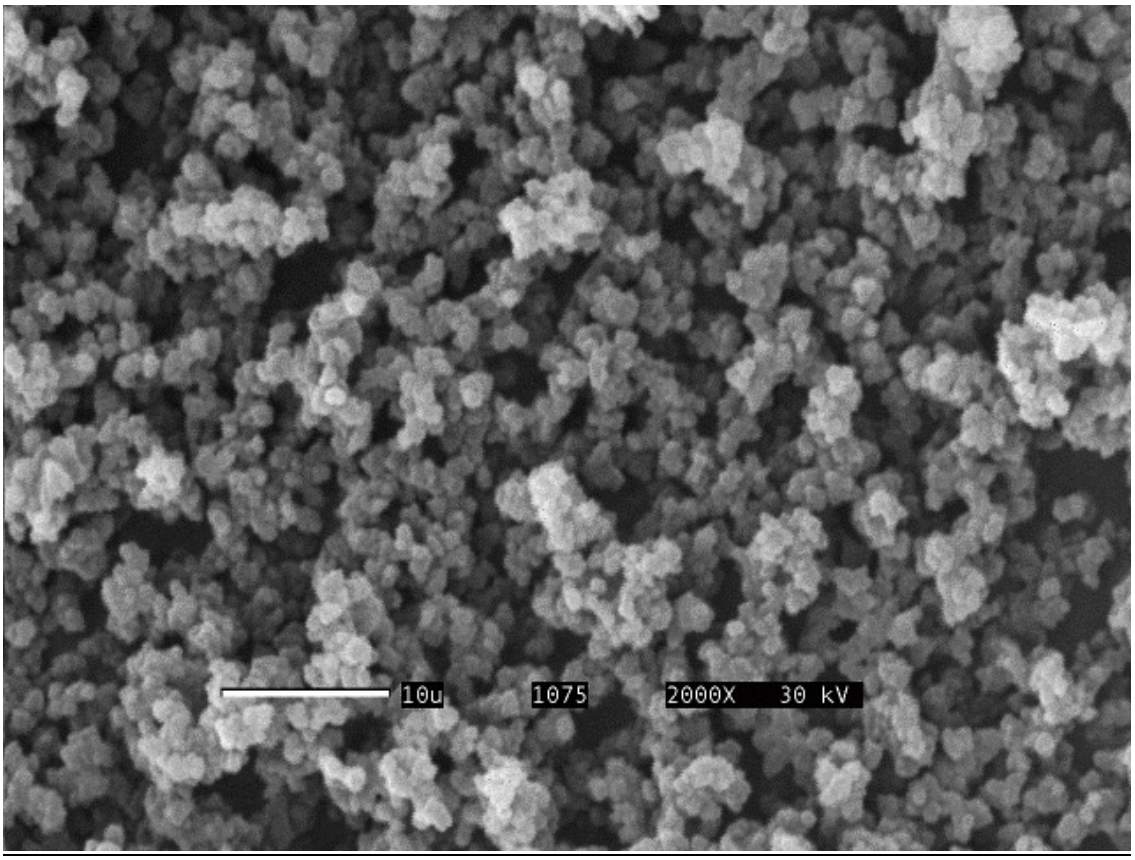


Figure 28: SEM image of poly(butyl methacrylate-co-ethylene dimethacrylate) monolith sample "75A"

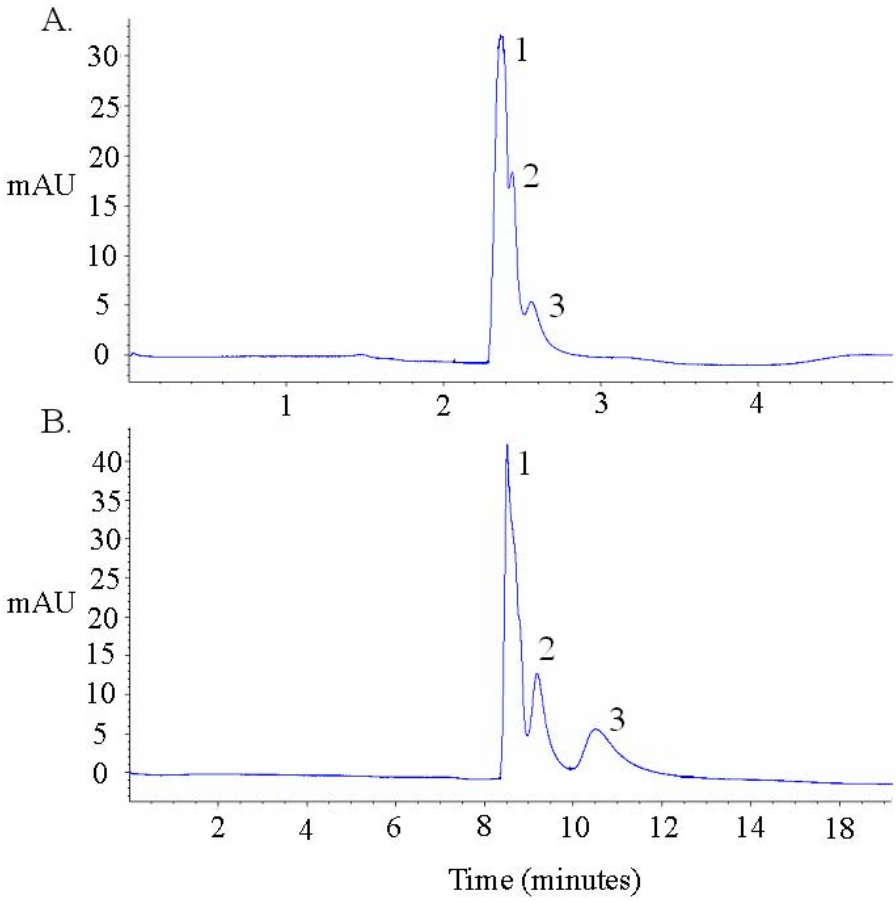


Figure 29. Separation of 3 benzodiazepines on a 17 cm effective length poly(butyl methacrylate-co-ethylene dimethacrylate) monolithic column with 0.3% AMPS and 65% 1-propanol 25% 1,4-butanediol, 10% water porogens. The separation was performed for (A) an 8:2 (v:v) acetonitrile to 5 mM sodium phosphate buffer at pH=7 and a separation voltage of 18 kV and for (B) a 7:3 (v:v) acetonitrile to 5 mM sodium phosphate pH=7 and a separation voltage of 5 kV. For both runs the samples were injected for 5 seconds at 5 kV with a 8 bar pressure on both the inlet and outlet electrodes. (1) Alprazolam; (2) Flunitrazepam; (3) Chlordiazepoxide.

The selectivity of the system was demonstrated by the methcrylate column for the separation of 3 benzodiazepines, as shown in Figure 29. The BMA/EDMA co-polymer column was 17 cm in effective length. The AMPS percentage was 0.3% with 65% 1-propanol 25% 1,4-butanediol, and 10% water porogens. The column exhibited the best selectivity with a 7:3 ratio of acetonitrile to 5 mM sodium phosphate at pH 7. The change in run time in Figure 29 (B) was the result of a 5 kV separation voltage. Thiourea was omitted from the separation because of its co-elution with the 3 drugs.

F. Microfluidic Approaches

Microfluidic systems have great potential as rapid, disposable devices for forensic analysis. Over the past decade, capillary electrophoresis (CE) performed on microfabricated devices has been the subject of several books and review articles. (Kopp, 1997, Verpoorte, 2002, Rossier, 2002) While the field of DNA analysis has seen respectable progress, (Sanders, 2000) advances in small molecule drug detection have been limited.

Like other electrophoretic techniques, CE microfluidics separate compounds on the basis of charge by applying a potential difference across buffer filled channels. For any liquid-based microfluidic or chip analysis, such channels are embedded into glass or plastic slides that also come with small reservoirs which are able to house electrodes. While commercial markets have been focused in other areas such as proteomics and genomics, the application of microfluidics systems for forensic drug analysis is still unachieved. The establishment of rapid, portable analytical tools utilizing disposable devices would revolutionize the way in which forensic analysis is performed. Sample contamination would be less of an issue, and throughput would be drastically increased.

Detection Methods

Basic and acidic drug methods that were developed for traditional CE instrumentation,(Thormann, 2001) can be adapted to microfluidic approaches, however there are certain changes in approach that must be made. Currently, the most popular detection method for traditional CE is UV-absorbance. Microfluidic devices, however, utilize laser induced fluorescence for detection due to the strong UV adsorption of the glass substrates that are used. Laser induced fluorescence (LIF) involves the excitation of a fluorescent compound which emits radiation that can be collected by a photomultiplier. Diode LIF was combined with CE in 1992 and further developed by Yeung and coworkers for numerous applications including direct and indirect (ID) detection techniques. (Yeung, 1989) Another advantage of optical on-chip detection is its increase in sensitivity and selectivity when compared to UV detection. (Mank, 1995) Unfortunately, while most drugs have chromophores that can be detected using UV adsorption, only a small number exhibit native fluorescence. Derivatization techniques must be developed to render classes of drugs suitable for LIF detection.

In one recent report, Nagaraj et al. used Cy5.29.OSu for the pre-column derivatization of amantadine detected using traditional CE.(Nagaraj , 1998) A Cy5 dye was also used by Jiang et al. for use with a confocal microscope for a microchip capillary electrophoresis assay for the protein biological threat agent stimulant, ovalbumin.(Jiang, 2000) This method involved using an affinity protected labeling procedure to tag the anti-ovalbumin enabling detection using a borate buffer pH 8.5. Wallenborg et al. made use of 4-fluoro-7-nitrobenzofurazane (NBD-F) to directly label amphetamine-like compounds to detect on-chip with a 488 nm argon ion laser. (Wallenborg, 2000)

An alternative to developing direct protocols is to take advantage of indirect detection. Indirect techniques are designed to accommodate analytes that are not capable

of responding to a chosen mode of detection. For this to occur, the detection baseline is elevated and a signal reduction indicates the presence of a particular analyte. However for valid ID techniques the reduction must be the result of a predictable, quantitative measure of change. This is done by means of a transfer ratio (TR), described as the number of probe molecules displaced or replaced by an analyte. (Yeung, 1989) The background signal stability is of extreme importance in indirect techniques because of its effect on the limit of detection (C_{LOD}). The dynamic reserve (DR) is defined as the ratio of background signal to background noise and is related to detection limit by Equation 9, where C_m is the concentration of the mobile phase component.

$$C_{LOD} = \frac{C_m}{(TR \times DR)} \qquad (9)$$

Even though this is not as sensitive as the direct approach because of the problem of distinguishing signal in the presence of enhanced background noise, indirect methods appear to be more practical for widespread drug screening because complex derivatization does not have to take place prior to or subsequent to the analysis.

Indirect laser-induced fluorescence (IDLIF) is a type of indirect detection that has received much attention. In this variation, a fluorophore added to the buffer can be displaced either because of the necessity for maintenance of charge neutrality in the detector zone or as a result of quenching resulting in a negative detector response. The resulting negative peaks can then be transformed and integrated using appropriate adjustments to the software. IDLIF has advantages over indirect absorption detection for the same reasons as direct florescence. By increasing the detector sensitivity when the path length or analyte concentration is decreased, the dynamic reserve can be maintained or improved.

Indirect Detection of Nitrated Benzodiazepines

Recently, a method was developed for the indirect fluorescent detection of explosive compounds using MECC. This procedure, developed by Bailey and Wallenborg involves a quenching interaction between the nitro groups on the explosives and a fluorescent probe molecule. (Wallenborg, 2000) Since several of the more potent benzodiazepines involved in DFSA (e.g. flunitrazepam and clonazepam, as shown in Figure 30) are nitrated compounds, an investigation was initiated to determine if a similar quenching interaction would occur.

In order to assess the potential of these interactions for the benzodiazepine compounds, a standard mix of eight explosive compounds was prepared to calibrate the instrumentation. When IDLIF is combined with MECC, a variety of different interactions can occur. In some cases there is a potential for increased fluorescence intensity when the fluorophore is partitioned into the micelle. During detection, the analyte can disturb the fluorophore-micelle complex in favor of its own complex with the micelle. Thus, there will be a decrease in the observed fluorescence because of the fluorophore's displacement into the aqueous environment, where the quantum yield is lower. Additional intensity decreases can be caused by charge-transfer interactions between the fluorophore and the analyte resulting in quenching.

In this study, Cy5 N-hydroxysuccinimide ester was employed as the visualizing fluorophore. Because the quantum yield of Cy dyes are lower inside the micelle, (Bailey, 2000) forming the fluorophore-SDS complex did not cause an increase in the intensity. Instead, fluorescent quenching was determined the main contributor to the indirect detection mechanism. Bailey confirmed this hypothesis in his separations by demonstrating the inability of the method to detect nitramine explosives.

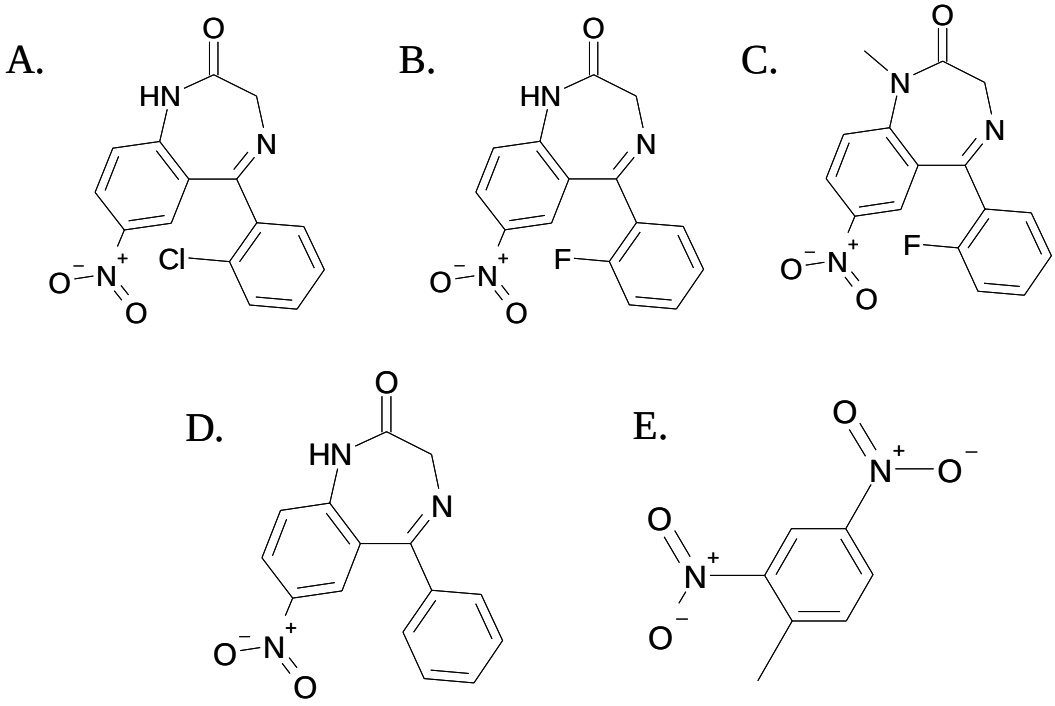


Figure 30. Nitrated Benzodiazepines used for the indirect chip method using Cy5 dye (A) clonazepam; (B) desmethylflunitrazepam; (C) flunitrazepam; (D) nitrazepam; and an explosive compound used for the internal standard (E) 2,4-dinitrotoluene.

A particular challenge faced in the separation of similarly charged drug compounds, is that even with the addition of SDS, hydrophobic interactions still might not be sufficient to yield baseline resolution. Organic modifiers can also be used in these devices to alter selectivity by affecting the partition coefficients and capacity factors of the analytes. These same principles were evaluated in microfluidic systems to achieve a separation of four nitrated benzodiazepines.

Experimental

Chemicals and Reagents

Cy5 monohydrazide dye (M.W. = 784.9 g/mol) was obtained from Amersham Biosciences (Piscataway, NJ). HPLC grade acetonitrile and methanol, as well as boric acid and sodium docecyl sulfate (SDS) was purchased from Fisher Scientific (Pittsburgh, PA). Sodium tetraborate were purchased from Acros (Morris Plains, NJ). The explosive compounds were purchased from Radian International (Austin, TX). Flunitrazepam was purchased from Sigma-Aldrich (St. Louis, MO), while desmethylflunitrazepam, clonazepam, and nitrazepam were purchased from Lipomed (Cambridge, MA).

The buffer stock for the detection of explosives was 100 mL of a 30 mM boric acid, 6 mM sodium borate, 30 mM sodium dodecyl sulfate (SDS) and 5.2 µm Cy5 dye at pH=8.5. A 50 µg/mL standard of nitrobenzene (NB), 2,4,6-trinitrotoluene (TNT), tetryl, 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT), 4-nitrotoluene (4-NT), 2-amino-4,6-dinitrotoluene (2-Am-4,6-DNT), and 4-amino-2,6-dinitrotoluene (4-Am-2,6-DNT) were dissolved in 47.5 µL of buffer and 2.5 µL of acetonitrile. The final concentration of benzodiazepine run buffer was 15 mM boric acid, 3 mM sodium borate, 15 mM SDS, and 2.6 µM Cy5 dye with percentages of the appropriate organic solvent. Standards ranging from 25 to 250 µg/mL flunitrazepam, desmethylflunitrazepam, clonazepam and sometimes nitrazepam were made from 1 mg/mL stock solutions and diluted in the appropriate amount of buffer.

Instrument and Conditions

All microfluidic experiments were performed on the Micralyne Micro Tool Kit (µ-TK) (Edmonton, AB, Canada), shown in Figure 31, with a 635 nm red diode laser module. The system contains two high voltage power boards with two +6 kV power supplies. Separations were performed on Micralyne's Low fluorescence Schott Borofloat™ glass Standard Microfluidic Chips. Both the channel plate and cover plate had a thickness of 1.1 mm yielding total dimensions of 16 × 95 × 2.2 mm. The device had a simple T-shaped design with an injection arm length of 9.64 mm and a separation arm length of 80.89 mm. The isotropically etched channels were 50 µm wide by 20 µm deep. Circular sample reservoirs 2.0 mm in diameter and capable of holding approximately 4 µL of buffer or sample were etched at the end of each channel arm. For these experiments the detector was aligned at 45 mm from the channel intersection. Peak area quantitation was performed by exporting the raw data from the Laboratory Virtual Instrument Engineering Workbench (LabView) 5.0 program into ChemSW ChromView software.

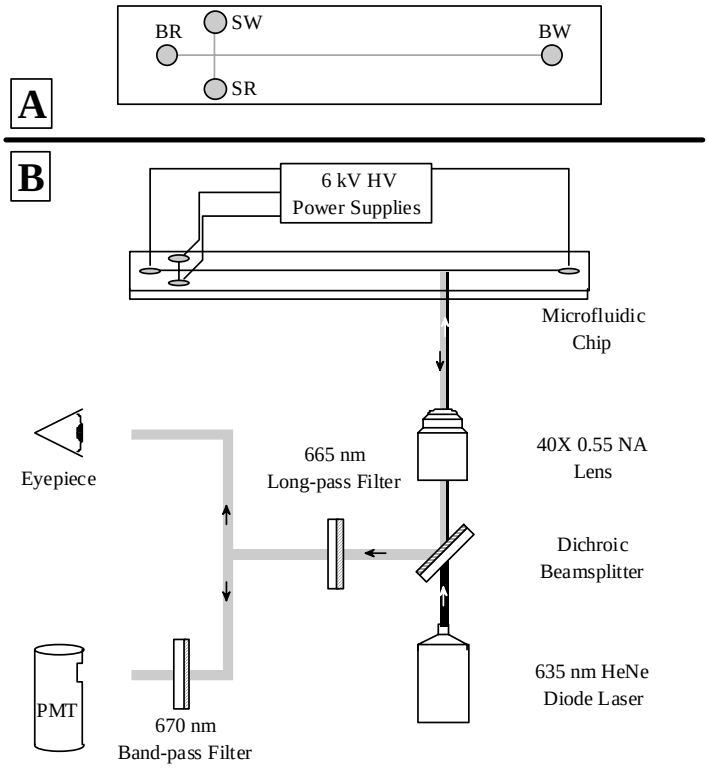


Figure 31. Schematic for the µ-Tool Kit configured with a red laser. Image Courtesy of John Crabtree at Micralyne.

The chips were preconditioned and cleaned using protocols outlined by Crabtree et al. (Crabtree, 2001) Rinsing was accomplished using a plastic syringe fitted with a micropipette tip cut to fit both the syringe and sample reservoir. The inlet, sample, and waste wells were filled with the appropriate solution; suction was then applied to the outlet well by pulling back on the syringe creating a pumping system. New chips were rinsed with 1.58 M HNO_3 followed by 1.00 M NaOH and then 18 MΩ water. Prior to each day, the devices were rinsed with NaOH for 15 minutes and run buffer for 1 minute.

Buffer Optimization

Optimization was performed by first repeating and then reoptimizing experiments done by Bailey et al. in order to calibrate the µ-TK. (Wallenborg, 2000) A comparable run-time was achieved for a standard of eight explosive compounds using the following conditions: a buffer consisting of 29.25 mM boric acid, 5.85 mM sodium tetraborate borate, 29.25 mM SDS, and 5 µM Cy5 dye at pH 8.5. The separation voltage was 2 kV and the effective length from the channel intersection to the detector was 55 mm. A variety of changes were made to accommodate the benzodiazepines including buffer concentration, separation voltage, and organic modifier concentration in order to achieve an optimal buffer of 15 mM SDS/15 mM boric acid/3 mM sodium tetraborate/2.6 µM Cy5 buffer with 20% methanol. The separation voltage was 4.0 kV and the effective length from the channel intersection to the detector was at 45 mm.

Results and Discussion

A separation of a series of explosives is shown and described in Figure 32. In previous studies different concentrations of borate were

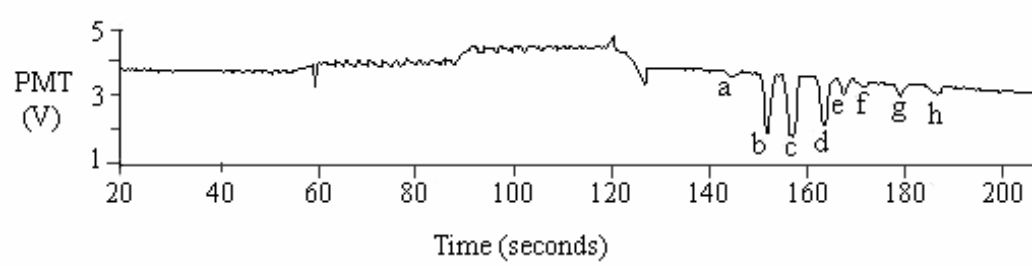


Figure 32. The separation of nitrated explosives using a 29.25 mM boric acid/5.85 mM sodium tetraborate borate/29.25 mM SDS/5 μM Cy5 dye at pH 8.5 buffer. The separation voltage was 2 kV and the effective length from the channel intersection to the detector was 55 mm. (a) nitrobenzene (NB); (b) Trinitrotoluene (TNT); (c) tetryl; (d) 2,4-dinitrotoluene (2,4-DNT); (e) 2,6-dinitrotoluene (2,6-DNT); (f) 4-nitrotoluene (4-NT); (g) 2-amino-4,6-dinitrotoluene (2-Am-4,6-DNT); (h) 4-amino-2,6-dinitrotoluene (4-AM-2,6-DNT).

used as the buffer and Cy5 or Cy7 were used as fluorescent probes. Ultimately, a buffer consisting of 29.25 mM boric acid/5.85 mM sodium tetraborate borate/29.25 mM SDS/5 μM Cy5 dye at pH 8.5 was employed in the method. Adjustments in effective channel length were necessary in order to provide baseline separation of all eight explosives with a runtime of 3 minutes and 10 seconds, due to differences in the chip dimensions.

It is important to note that the intensity differences of the different explosives can be explained by examining the mechanism by which fluorescence quenching occurs. Under non-quenching conditions, a fluorophore (F) absorbs a photon to reach an excited state (F*) and then return to the ground state by emitting a photon. In the instance of quenching, a charge transfer reaction will occur. In this interaction, the analyte serves as an electron acceptor (Q) and the fluorophore F* is the electron donor. Providing the free energy (ΔG) of the charge transfer reaction is less than zero, this interaction will result in a decrease in fluorescence due to the destruction of the excited state and the formation of the ions Q^- and F^+. This can be mathematically expressed in Equation 10 where E is the redox potential of either the fluorophore and ΔE_{F-F^*} is the lowest singlet-singlet excitation energy of the fluorophore.

$$\Delta G = E\left(\frac{F}{F^+}\right) - \Delta E_{F-F^*} - E\left(\frac{Q}{Q^-}\right) \qquad (10)$$

Experiments were next initiated to optimize the MECC buffer used for the explosives to permit analysis of the 3 benzodiazepines clonazepam, flunitrazepam and its

desmethyl metabolite. Trinitrotoluene (TNT) was chosen initially as the internal standard, however by using dinitrotoluene (2,4-DNT), the quenching was less intense and more comparable to the mono-nitro quenching exhibited by the benzodiazepines.

When using the method developed for explosives, the benzodiazepines eluted in a time of 147 seconds (using a 20 second injection period). Reducing the effective length of the separation by 10 cm only decreased the run time by 10 seconds. Therefore, the concentration of the buffer was reduced and the voltage was increased in order to further adjust the time parameter. The new buffer contained 15 mM SDS/15 mM boric acid/3 mM sodium tetraborate with a separation voltage of 4 kV and the run time was decreased to 80 seconds.

Based on initial experiments as well as findings in the MEKC study, an organic modifier was utilized to separate the closely related structures. As was seen in previous separations, flunitrazepam and clonazepam co-eluted because of their similarities in structure and polarity. The optimization involved varying the relative concentrations of organic modifier in order to find the most favorable buffer for benzodiazepine detection on a chip. Figure 33 shows the analysis of the benzodiazepine standard using the optimized buffer system.

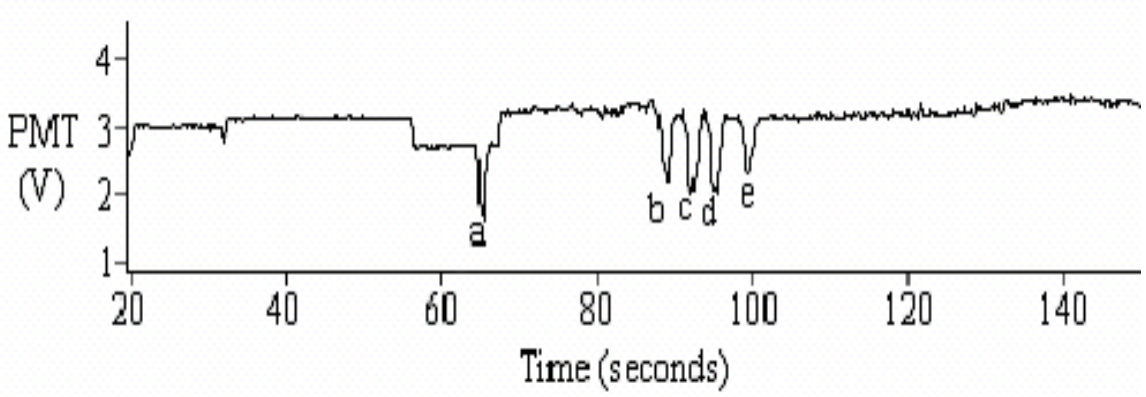


Figure 33. The optimized separation nitrated benzodiazepines using a 15 mM SDS/15 mM boric acid/3 mM sodium tetraborate/2.6 µM Cy5 buffer with 20% methanol. The separation voltage was 4.0 kV and the effective length from the channel intersection to the detector was at 45 mm. (a) 2,4-DNT (I.S.); (b) desmethylflunitrazepam; (c) flunitrazepam; (d) nitrazepam; (e) clonazepam.

Conclusions

In these experiments, an on-chip method for the detection of 4 nitrated benzodiazepines has been developed. A preexisting explosives method was optimized for the detection of benzodiazepines on Micralyne equipment with their standard microfluidic device. In order to accomplish this, an organic modifier, methanol, was added at 20% to achieve a baseline separation. While further studies will be necessary to detect benzodiazepines that do not contain this functional group, microfluidics has proven to be successful in carrying out indirect fluorescence detection methods.

Overall Conclusions

In a series of studies we have demonstrated the promising applications of capillary electrophoresis in the detection of drugs used in sexual assault. Our results show that exceptional separations of charged and neutral compounds are possible using the combination of capillary electrophoresis and chromatography. To achieve these results, we have used both pseudo stationary phases and imbedded stationary phases composed of polymer monoliths. The work with pseudo stationary phases involved the separation of various classes of drugs including GHB and benzodiazepines, amphetamines and benzyl piperazines and various opiates. Studies using monolithic stationary phases demonstrated their promise as tools for separation of drugs but also revealed that further work is needed to enhance separation efficiency. A variety of synthetic approaches were explored. Overall the best results were obtained using beta cyclodextrins combined with organic modifiers. These separations show exceptional selectivity and can be combined with various sensitivity enhancement tools to give detection capabilities as good or better than more commonly used gas chromatographic screening methods. In addition, when used with on-line extraction, the analytical procedure is easy to implement requiring no derivatization and is applicable to a variety of drugs in different matricies. We have also shown that these new separation techniques can be used in combination with disposable microfluidic devices, further increasing their versatility. Overall this work demonstrates an exceptional flexibility and range of applications for capillary electrophoresis when utilized for the detection of date rape drugs.

References

Bailey, C. G.; Wallenborg, S. R. Electrophoresis 2000, 21, 3081-3087.

Cahours, X.; Cherkaoui, S.; Rozing, G.; Veuthey, J. L. Electrophoresis 2002, 23, 2320-2326.

Ciolino, L. A.; Mesmer, M. Z.; Satzger, R. D.; Machal, A. C.; McCauley, H. A.; Mohrhaus, A. S. J. Forensic Sci. 2001, 46, 1315-1323.

Crabtree, H. J.; Cheong, E. C. S.; Tilroe, D. A.; Backhouse, C. J. Anal. Chem. 2001, 73, 4079-4086.

de Boer, D.; Bosman, I. J.; Hidvegi, E.; Manzoni, C.; Benko, A. A.; dos Reys, L.; Maes, R. A. A. Forensic Sci.Int. 2001, 121, 47-56.

de Boer, D.; Bosman, I. Pharm. World Sci. 2004, 26, 110-113.

Elliott, S. J. Anal. Toxicol. 2001, 25, 152-152.

Ericson, C.; Liao, J. L.; Nakazato, K.; Hjerten, S. J. Chromatogr. A 1997, 767, 33-41.

Euerby, M. R.; Gilligan, D.; Johnson, C. M.; Roulin, S. C. P.; Myers, P.; Bartle, K. D. J. Microcolumn Sep. 1997, 9, 373-387.

Euerby, M. R.; Johnson, C. M.; Smyth, S. F.; Gillott, N.; Barrett, D. A.; Shaw, P. N. J. Microcolumn Sep. 1999, 11, 305-311.

Fed. Regist. 2002; Vol. 67, p 58161-59162.

Forensic Drug Abuse Advisor Inc. Legal Ecstasy (MDMA)? Forensic Drug Abuse Advisor 2001, 13 (8), 60.

Hilder, E. F.; Svec, F.; Frechet, J. M. J. Chromatogr. A 2004, 1044, 3-22.

Itsuoda. 2nd experiment with Smoked BZP Freebase: BZP & GHB. http://www.erowid.org/experiences/exp.php?ID=5126. (accessed September 19, 2004).

Jiang, G. F.; Attiya, S.; Ocvirk, G.; Lee, W. E.; Harrison, D. J. Biosens. Bioelectron. 2000, 14, 861-869.

Johnson, C. M.; McKeown, A. P.; Euerby, M. R. Modes of CEC Separation In Capillary Electrochromatography; Deyl, Z., Svec, F., Eds.; Elsevier: Amsterdam, 2001; Vol. 62, pp 87-110.

Kerrigan, S.; Goldberger, B. A. In Principles of Forensic Toxicology, 2nd ed.; Levine, B., Ed.; American Association for Clinical Chemistry, Inc: Washington, DC, 2003, pp 187-205.

Kopp, M. U.; Crabtree, H. J.; Manz, A. Curr. Opin. Chem. Biol. 1997, 1, 410-419.

Lazar, I. M.; Li, L. J.; Yang, Y.; Karger, B. L. Electrophoresis 2003, 24, 3655-3662.

LeBeau, M. A.; Miller, M. L.; Levine, B. Forensic Sci.Int. 2001, 119, 161-167.

Lee, K. P.; Choi, S. H.; Ryu, E. N.; Ryoo, J. J.; Park, J. H.; Kim, Y.; Hyun, M. H. Anal. Sci. 2002, 18, 31-34.

Lurie, I. S.; Dal Cason, T. A.; LeBelle, M. J.; Brenneisen, R.; Weinberger, R. E. Anal. Chem. 1994, 66, 4019-4026.

Lurie, I. S.; Hays, P. A.; Casale, J. F.; Moore, J. M.; Castell, D. M.; Chan, K. C.; Issaq, H. J. Electrophoresis 1998, 191, 51-56.

Mank, A. J. G.; Yeung, E. S. J. Chromatogr. A 1995, 708, 309-321.

Marinetti, L. J. Gamma-Hydroxybutyric Acid and Its Analogs, Gamma-Butyrolactone and 1,4-Butanediol In Benzodiazepines and GHB: Detection and Pharmacology; Salamone, S. J., Ed.; Humana Press: Totowa, 2001, pp 95-126.

Mazzeo, J. R. Micellar Electrokinetic Chromatography In Handbook of Capillary Electrophoresis; 2nd ed.; Landers, J. P., Ed.; CRC Press: New York, 1997, pp 49-73.

McGuarnicle. GHB & BZP Near- Death Experience: GHB, BZP, Cannabis. http://www.erowid.org/experiences/exp.php?ID=3567. (accessed September 19, 2004).

Motzka, T. A.; Matiskella, J. D.; Partyka, R. A. Tetrahedron Lett. 1974, 14, 1325-1327.

Nagaraj, S.; Rahavendran, S. V.; Karnes, H. T. J. Pharm. Biomed. Anal. 1998, 18, 411-420.

Rice, K. C. J. Org. Chem. 1975, 40, 1850-1851.

Rogan, M. M.; Altria, K. D. Introduction to the Theory and Applications of Chiral Capillary Electrophoresis; Beckman Instruments: California, 1993.

Rossier, J.; Reymond, F.; Michel, P. E. Electrophoresis 2002, 23, 858-867.

Sanders, G. H. W.; Manz, A. Trac-Trends Anal. Chem. 2000, 19, 364-378.

Sentellas, S.; Puignou, L.; Galceran, M. T. J. Sep. Sci. 2002, 25, 975-987.

Smith, N. W.; Carter-Finch, A. S. J. Chromatogr. A 2000, 892, 219-255.

Sreenivasan, K. J. Appl. Polym. Sci. 1996, 60, 2245-2249.

Staack, R. F.; Fritschi, G.; Maurer, H. H. J. Chromatogr. B 2002, 773, 35-46.

Strausbauch, M. A.; Massen, B. J.; Wettstein, P. J.; Landers, J. P. Electrophoresis 1995, 16, 541-548.

Svec, F.; Peters, E. C.; Sykora, D.; Frechet, J. M. J. J. Chromatogr. A 2000, 887, 3-29.

Svec, F.; Peters, E. C.; Sykora, D.; Yu, G.; Frechet, J. M. J. HRC-J. High Resolut. Chromatogr. 2000, 23, 3-18.

Tagliaro, F.; Manetto, G.; Bellini, S.; Scarcella, D.; Smith, F. P.; Marigo, M. Electrophoresis 1998, 19, 42-50.

Telepchak, M. J.; August, T. F.; Chaney, G. Forensic and Clinical Applications of Solid Phase Extraction; Humana Press: New Jersey, 2004.

Terabe, S.; Otsuka, K.; Ichikawa, K.; Tsuchiya, A.; Ando, T. Anal. Chem. 1984, 56, 111-113.

Terabe, S.; Otsuka, K.; Ichikawa, K.; Tsuchuya, A.; T., A. Anal. Chem. 1984, 56, 111-113.

Thormann, W.; Lurie, I. S.; McCord, B.; Marti, U.; Cenni, B.; Malik, N. Electrophoresis 2001, 22, 4216-4243.

Tomlinson, A. J.; Benson, L. M.; Guzman, N. A.; Naylor, S. J. Chromatogr., A 1996, 744, 3-15.

U.S. DEA. Drug Intelligence Brief. BZP and TFMPP: Chemicals Used to Mimic MDMA's Effects. http://www.dea.gov/pubs/intel/02005/02005.html. (accessed September 19, 2004).

U.S. DEA. Intelligence Brief: Mixed MDMA/Piperazine Tablets in San Diego, California. Microgram Bull. 2003, 36 (7), 153.

U.S. DEA. Intelligence Brief: Tablets Containing Mixed Piperazines in Algona, Iowa. Microgram Bull. 2003, 36 (6), 118-119.

U.S. DEA. Piperazine Mixture Tablets in Chicago, Illinois. Microgram Bull. 2003, 36 (1), 6-10.

U.S. DEA. Selected Intelligence Brief: Ecstasy and Club Drug Trafficking in the Pacific Northwest. Microgram Bull. 2004, 37 (1), 10-20.

Varesio, E.; Veuthey, J. L. J. Chromatogr. A 1995, 717, 219-228.

Verpoorte, E. Electrophoresis 2002, 23, 677-712.

Wallenborg, S. R.; Bailey, C. G. Anal. Chem. 2000, 72, 1872-1878.

Wallenborg, S. R.; Lurie, I. S.; Arnold, D. W.; Bailey, C. G. Electrophoresis 2000, 21, 3257-3263.

Weinberger, R. Practical Capillary Electrophoresis; Academic Press: Boston, 1993.

Yeung, E. S. Acc. Chem. Res. 1989, 22, 125-130.

Yu, C.; Xu, M. C.; Svec, F.; Frechet, J. M. J. J. Polym. Sci. Pol. Chem. 2002, 40, 755-769.

Appendix

Papers Published and in Preparation Resulting from this Work

Bishop, S.C.; McCord, B.R. Gratz, S.R.,Loeliger, J. R.; Witkowski, M.R. Simultaneous Separation of Different Types of Amphetamine and Piperazine Designer Drugs by Capillary Electrophoresis with a Chiral Selector, *J. Forensic Sciences*, 2005, in press.

Al Najjar, A;Butcher, J; McCord, B. Determination of multiple drugs of abuse in human urine by capillary electrophoresis with fluorescence detection, *Electrophoresis*, 2004, *25 (10-11),* 1592-1600.

Bishop, S.; Lerch, M.; McCord, B. A Micellar Electrokinetic Chromatographic Screening Method for Common Sexual Assault Drugs, *Forensic Science International* 2004, *141(1),* 7-15.

All Najjar, A.; McCord, B. Determination of heroin metabolites in human urine using capillary zone electrophoresis with B-cyclodextrin and UV detection, *Journal of Pharmaceutical and Biomedical Analysis*, 2003, *33(3),* 463-473.

Bishop, S.C.; Lerch, M.; McCord, B.R. Microfluidic detection of benzodiazepines, in preparation.

Al Najjar, A. ; McCord, B. An automated method for the on-line extraction of drugs of abuse by capillary electrophoresis, in preparation.