ADVANCED CAPILLARY ELECTROPHORETIC TECHNIQUES FOR THE DETECTION OF DATE-RAPE AND CLUB DRUGS FOR A FORENSIC SETTING

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FOR A FORENSIC SETTING

by

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Capillary electrophoresis (CE) is a versatile tool for the isolation, separation, and
detection of date-rape and club drugs. Advanced applications of electrophoretic
techniques allow for the separation of small, similarly charged compounds by the
utilization of mobile phase additives and chromatographic stationary phases.

Two novel screening procedures were developed using mobile phase additives.
The first used sodium dodecyl sulfate (SDS) to detect gamma-hydroxybutyric acid
(GHB), gamma-butyrolactone, as well as eight classical and low-dose benzodiazepines.
Although SDS previously had been used for the separation of drugs, this was the first
simultaneous separation of benzodiazepines and GHB. The second method used the
additive hydroxypropyl-beta-cyclodextrin to detect 1-benzylpiperazine (BZP), 1-(3-
trifluoromethylphenyl)piperazine (TFMPP), and three piperazine analogs.

An additional aspect of this project was to develop new systems for the analysis
of the above drugs using capillary electrochromatography (CEC). A stationary phase in
the capillary permitted the analysis of neutral drugs without resorting to pseudostationary
phases. The buffers used in CEC were comparable to those used in high performance
liquid chromatography (HPLC), yet were more compatible with mass spectrometric
detection. While LC-like stationary phases are still among the most popular utilized for
CEC, monolithic polymer materials have the potential to provide an environment more
suited to the generation of electroosmotic flow, in combination with partitioning,
necessary for optimal analysis. Pore size and chromatographic evaluations of various
poly(butyl methacrylate-co-ethylene dimethacrylate) stationary phases were examined for their utility in small molecule separations.

Miniaturization of the techniques described above can result in devices that are small, portable, and disposable. The microfluidic analysis of four nitrated benzodiazepines was accomplished on a commercial device using SDS and a cyanine monofunctional hydrazide dye in indirect fluorescence mode. In addition, cyanine monoreactive N-hydroxysuccinimide (NHS) ester was used for direct derivatization of amine-containing compounds. Protocols for the fabrication of glass and poly(dimethylsiloxane) devices were provided as an alternative to the commercial devices, providing a cheaper and more flexible separation technique.

These chapters provide innovative ways to extract, separate, and detect drug-facilitated sexual assault drugs. Along with several new methods, novel technologies for CE drug analysis were explored such as polymeric stationary phases and microfluidic devices.

Approved:

Bruce R. McCord

Associate Professor of Chemistry
This dissertation is dedicated to all of the victims of drug-facilitated sexual assault. My heart goes out to anyone whose lives have been affected by this horrific criminal act.
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I guess I really am going to Disneyland!?! 😊
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Chapter 1: Forensic Toxicology: Date-Rape and Club Drugs

Introduction

Toxicology has been defined as the study of adverse effects of chemicals on living organisms.\(^1\) Although any substance can be considered a poison at a high enough dose, drugs are by far the most studied form of potentially poisonous compounds. In appropriate dose, drugs are designed to relieve the symptoms of or fight the cause of different disease states. When used improperly, drugs can be extremely harmful and even lethal. The history of substance use in the world is long, dating back to circa 3400 B.C., when the opium poppy was cultivated in lower Mesopotamia. It was referred to as *Hul Gil*, the ‘joy plant’.\(^2\) From the past to the present, the world has seen a cornucopia of different substances of abuse. Both natural compounds, such as opium and belladonna alkaloids and synthetic drugs, such as lysergic acid diethylamide (LSD) and phencyclidine (PCP), encompass the wide range of recreational drugs currently in use.

In the last few decades, nutrition and exercise have been a growing obsession in the United States. As a result, there has recently been an emergence of dietary supplements that provide anything from herbal remedies, to weight loss and/or muscle gain. Along with the traditional illegal drugs, new trends in rave party drugs like ecstasy, methamphetamine, and ketamine have swept the nation enticing people like never before.
While not as ancient as some of the substances themselves, the struggle to detect and quantitate compounds of abuse is as old as science. Pioneers in the field of toxicology managed to perform the first blood alcohol tests using steam distillation and relied mainly on colorimetric assays to identify certain compounds. Throughout the years, alcohol analysis became automated and identification took on the form of more complex immunoassay kits. Techniques for drug analyses are constantly being improved by the development of electronic equipment, high-speed computers, and miniaturization capabilities. Methods are not only becoming more accurate, but the results are obtained faster and utilize compact instrumentation that continues to get smaller and smaller.

When the problem of detecting an unknown drug either in bulk form or in vivo suddenly becomes a legal issue, precision and accuracy take on increased importance. The legal system in the United States follows very stringent codes and in order to convict criminals, the scientific methods used to provide evidence in court have to be validated, well-documented, and performed meticulously. Drug laboratory clinicians must not only be aware of new techniques to assist them with their demanding job, they must also be alert to new substances of abuse that may be present during an analysis. When a new drug emerges, methods must be adjusted or developed rapidly. However, to be accepted into current practice, the new methods must be valid, financially viable, time effective, and within the capabilities of the analysts.

The research described herein includes new methods of drug detection and awareness of new compounds of interest that will be useful for a forensic laboratory setting. These methods strive to include all of the desirable characteristics of clinical methods such as speed, sensitivity, and precision. Cutting edge discoveries regarding
clandestine synthetic products and detection of new designer drugs of abuse are also addressed. The goal of this research was to provide insight into new compounds and develop techniques to detect illicit drugs of the new millennium using electrophoresis technology. Particular attention was focused on compounds associated with drug-facilitated sexual assault.

**Drugs**

During the course of this research, a wide variety of different illegal and prescription drugs were analyzed to test different chromatographic procedures and novel stationary phases. Appendix 1 (drugs) and Appendix 2 (miscellaneous compounds) illustrate structures, molecular weights, and Chemical Abstracts Service (CAS) number information for all of the compounds of interest. In addition, a brief history and pharmacological data relevant to this work is provided for all compounds of interest.

Central nervous system (CNS) depressants, when used in moderation, produce a calming, drowsy state, sometimes resulting in sleep.\(^3\) In the cases of misuse or an overdose, results can include a coma and even death from respiratory and/or cardiac depression. Alcohol and opium alkaloids are among the oldest sedative hypnotics used, followed by bromide and chloral hydrate in the middle of the 19\(^{th}\) century. The first barbiturate drug was implemented in 1912, known as phenobarbital. While being used to treat anxiety and insomnia, barbiturates were linked to many suicides, fatal overdoses, and severe additive reactions when used with alcohol. This class of sedative-anxiolytic
substances was the most popular until the advent of benzodiazepines in 1961 with chlordiazepoxide, or Librium.\textsuperscript{3} Since then, there have been many variations on the structure as demonstrated by the variety of commercially available drugs shown in Table 1.\textsuperscript{4,5}

Therapeutically, benzodiazepines have since replaced barbiturates because they are less addictive substances that still produce sedative and tranquilizing sensations. Benzodiazepines are comprised of a 1,4-diazepine ring with a benzene ring fused to carbons 6 and 7 and typically a phenyl group attached to carbon 5. The lower dose benzodiazepines, which include alprazolam, lorazepam, midazolam and triazolam, have a shorter duration of action along with a faster clearance rate, when compared to the higher dose forms, such as diazepam, or Valium.\textsuperscript{5} Combined with their rapid metabolism that differs from classical benzodiazepines as shown in Figure 1,\textsuperscript{5-8} the smaller effective dose of these drugs make routine toxicological analysis challenging. The fast-acting, lethargic effects of these substances unfortunately make them ideal drugs for the use of sedation in the instance of rape.

Throughout history, the most famous benzodiazepine associated with date-rape is flunitrazepam, or Rohypnol. Because of overwhelming concerns this drug was rendered illegal to possess in the United States by the Drug-Induced Rape Prevention and Punishable Act of 1996.\textsuperscript{9} Referred to by the media as “roofies,” these drugs still remain in the illicit drug circuit in the U.S. The severity of the drugging allegations even prompted its distributor, F. Hoffmann-La Roche, to add blue dye the pills it markets for legal use in other countries.
**Table 1.** Classifications of various benzodiazepines.

<table>
<thead>
<tr>
<th>Classic 1,4-diazepines</th>
<th>7-Nitrobenzodiazepines</th>
<th>Triazolobenzodiazepines</th>
<th>Imidazolobenzodiazepines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lorazepam(^1)</td>
<td>Flunitrazepam</td>
<td>Alprazolam(^1)</td>
<td>Midazolam(^1)</td>
</tr>
<tr>
<td>Chlordiazepoxide</td>
<td>Desmethyflunitrazepam</td>
<td>Triazolam(^1)</td>
<td></td>
</tr>
<tr>
<td>Prazepam</td>
<td>Nitrazepam</td>
<td></td>
<td></td>
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<td>Clonazepam</td>
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<tr>
<td>Nordiazepam</td>
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<tr>
<td>Oxazepam</td>
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</tbody>
</table>

\(^1\)Selected low-dose benzodiazepines
Figure 1. Traditional commercial immunoassay kits are designed to detect the urinary metabolites of classic benzodiazepines, such as diazepam, and are insufficient for the detection of low-dose benzodiazepines such as triazolam. (A) Metabolism pathway for a classic benzodiazepine; (B) Metabolism pathway for a low-dose benzodiazepine. These biotransformations are the result of various cytochrome P-450s, drug-metabolizing enzymes responsible for the oxidative metabolism of xenobiotic and endobiotic compounds.
If the pills are illegally smuggled into the U.S., the dye will alarm unsuspecting victims of possible food or drink contamination.\(^5\)

Cocaine and amphetamine-like drugs are examples of psychostimulants, which are substances with antidepressant and mood-elevating properties.\(^3\) They have been known to increase alertness, decrease appetite, and improve task performance. Side effects often include anxiety and insomnia, which is why multi-drug users often use a benzodiazepine to “come down” from a stimulant high. Originally, Coca-Cola contained approximately 60 milligrams of cocaine per 8 oz serving, and until 1903, was a favorite among busy housewives.\(^3\) The 1914 Harrison Narcotic Act banned the drugs in medicines and beverages. Illegal use of cocaine was on the rise in the 1920s and subsequently decreased in the 1930s with the increased availability of amphetamines.\(^3\)

Amphetamine abuse has existed since its inception as a clinical drug during the 1930s in the form of Benzedrine inhalers.\(^10\) Throughout World War II, amphetamines were used by soldiers to stay alert. In the decades following the war, abuse of this stimulant was widely recognized.\(^11\) Once amphetamines were controlled and classified as a Schedule II substance, methamphetamine emerged as a popular substitute owing to its easy manufacturing. Clandestine methamphetamine laboratories still remain a major problem in the United States. Amphetamines and methamphetamines are the starting point for additional designer drugs such as, 3,4-methylenedioxyamphetamine (MDA) and 3,4-methylenedioxymethamphetamine (MDMA), otherwise known as ecstasy. MDMA is currently extremely popular among the club drug scene at raves, which are parties where drugs are openly distributed.\(^12\) Ecstasy use has steadily been on the rise since 1992. This can be illustrated, as seen in Table 2, by a Drug Enforcement
Administration’s (DEA) report showing a dramatic increase of MDMA tablet seizures over a seven year period. The 2002 National Survey on Drug Use and Health reported that over 10 million persons 12 or older admitted to using ecstasy at least once. These numbers are a dramatic increase from the 6.4 million in 2000.

Since the 1960s, gamma-hydroxybutyric acid (GHB) has been used as a clinical hypnotic agent. GHB is made up of a hydroxyl group, three straight chain carbons and a carboxyl group (Figure 2). After gaining popularity among bodybuilders in the 1980s as a way to increase muscle mass, GHB made its way into the club scene in the 1990s. Owing to its stupor-like properties, this colorless, tasteless drug has also been associated with date-rape. GHB was placed on the Drug Enforcement Administration’s Schedule I substance list in 2000 following the passing of the ‘Hillery J. Farias & Samantha Reid Date-Rape Drug Prohibition Act’; a bill named after two young ladies who were unknowingly administered lethal doses of the drug. Shortly thereafter, the drug scene experienced an emergence of structurally- and chemically-similar GHB analogs. Gamma-butyrolactone (GBL), and 1,4-butanediol were introduced as “legal” substitutes. These compounds can be transformed into GHB in vivo, as demonstrated in Figure 2. As each new analog of GHB appears on the street, legislation struggles to keep up with the growing list of drug alternatives. In 2000, the Maryland Poison Control Center fielded 61 calls concerning ingestion of GHB and its analogs with 32 resulting in hospital treatment. Of the percentage of urine testing positive for GHB, 71% of those results are suspected to be caused by analog abuse. The importance of clinical methods that incorporate the parent compound and potential analog substances is crucial in maintaining the control of these illegal drugs.
**Table 2.** Drug Enforcement Administration’s (DEA) report over a 7 year period of seizures involving MDMA tablet possession.\(^{13}\)

<table>
<thead>
<tr>
<th>Year</th>
<th>Seizures</th>
</tr>
</thead>
<tbody>
<tr>
<td>1993</td>
<td>196</td>
</tr>
<tr>
<td>1994</td>
<td>11,722</td>
</tr>
<tr>
<td>1995</td>
<td>27,759</td>
</tr>
<tr>
<td>1996</td>
<td>11,912</td>
</tr>
<tr>
<td>1997</td>
<td>79,599</td>
</tr>
<tr>
<td>1998</td>
<td>143,612</td>
</tr>
<tr>
<td>1999</td>
<td>954,878</td>
</tr>
</tbody>
</table>
Figure 2. Metabolism and elimination of GHB and its analogs.
On July 17, 2002 the FDA approved the drug Xyrem®, with an active ingredient of GHB (labeled on the container as sodium oxybate to avoid negative stigma), to treat cataplexy attacks. While this narcolepsy drug is a Schedule III controlled substance, illicit use of Xyrem® is still punishable by Schedule I penalties.17

Traditional Clinical Methods

For facilities geared towards illicit drug analysis, technicians rely on qualitative screening methods followed by a confirmatory technique. In postmortem screening, a toxicologist is often only concerned with the detection of drugs at levels that can contribute to the cause of death. This thinking may backfire in the instances of either multi-drug ingestion fatalities or in investigations of drug-facilitated sexual assault in which substances are rapidly broken down into low levels of parent and metabolite concentrations. Often, forensic postmortem laboratories are called upon to investigate these cases owing to their sophisticated instrumentation and the careful chain of custody already implemented in these facilities. In such instances, laboratories must have methods sensitive enough to qualitatively identify all compounds, as even trace indications of these drugs can be important in legal proceedings. Drug detection becomes especially important for the detection of GHB in biological fluids. Unlike compounds such as heroin that yield an active, detectable metabolite (in this example: morphine), GHB is rapidly metabolized into carbon dioxide and water through the Krebs cycle (Figure 2). Detectable quantities of GHB only exist for a few hours after ingestion as the
material is rapidly eliminated from the victim’s body. While there are several techniques used by clinical laboratories in the pursuit of date-rape and club drug detection, they are not without flaws.

A popular category of screening techniques for drugs of abuse is immunoassays. These include radioimmunoassays (RIA), enzyme-linked immunosorbent assays (ELISA), fluorescence polarization immunoassays (FPIA), and enzyme multiple immunoassay tests (EMIT), all which differ according to the detection method. Immunoassays in general, are constructed based upon the ability of antigen (Ag) and an antibody (Ab) to bind together according to the law of mass action:18

\[
Ab + Ag \leftrightarrow AbAg
\]  

Immunoassays focus on classes of drugs, not the specific drug in question, to screen samples based on competitive binding in an AbAg complex. Commercial kits are available for testing a wide variety of drug classes and are available from multiple distributors. Despite providing simple, rapid analysis, immunoassays have certain drawbacks such as cross-reactivity problems. These assays utilize a target metabolite common to the specific drug class as the binding factor for the determination of drugs that are present. For example, an opiate immunoassay is based on the metabolite morphine and will screen for analytes such as heroin, opium, and certain codeine containing products. Because of the use of a metabolite marker, false-positives and false-negatives are not uncommon. Incidentally, most private and government laboratories have increased cut-off positive levels for opiate immunoassays due to the fact that cross-
reactivity of these compounds with the opiates present in poppy seeds and over the counter (OTC) drugs can result in false positives, falsely implying drug use. Older benzodiazepine immunoassay kits used oxazepam as the target metabolite. Unfortunately, as shown in Figure 1, low dose benzodiazepines such as alprazolam, midazolam, and triazolam metabolize into $\alpha$-hydroxy and 4-hydroxy equivalents and therefore are not compatible with some kits. Furthermore, flunitrazepam and its metabolites have been reported to interact poorly with the EMIT antibody as well as exhibiting poor cross-reactivity in the FPIA systems.

Another problem with drug screening by immunoassays is that certain compounds may not currently have a relevant immunoassay. For example, there have been no reliable quick screening methods reported for the detection of GHB. Spot tests such as in several products by Drink Safe Technologies’ do exist (Figure 3). The manufacturer of this technology claimed that if a drop of drink was placed on the coasters, a color change would indicate the presence of a drug, alerting potential victims. However such procedures are not nearly sensitive enough for toxicological applications.

Gas chromatography, coupled to mass spectrometry (GC/MS), remains the gold standard for forensic drug analysis. Because of the structural fingerprint obtained from a mass spectrum, laboratories continue to rely on this procedure as an identifying technique. A sample extract is usually prepared, evaporated, and reconstituted in a small amount of organic solvent for injection onto the GC column. Unfortunately, a compound must possess certain properties to be GC-compatible.
Figure 3. Various products from Drink Safe Technology © that advertise the ability to detect date-rape drugs in beverages. (A) Drink Safe Advertisement; (B) Drink Safe Bar Coaster; (C) Drink Safe test strip cards.
Without being able to survive harsh injector port temperatures or volatility capabilities inherent to the instrumentation, detection becomes tedious. While this can be solved by reactions such as derivatizations or hydrolysis conversions, these steps can be time consuming.

HPLC may be a more versatile technique as far as the scope of compounds capable of analysis are concerned, but there is often a loss of peak capacity and number of theoretical plates when using this technique owing to a now laminar flow profile. Additionally, as with GC, an extract must be prepared in an organic solvent in order to be compatible with the reversed phase LC methods used for this technique.

**New Clinical Methods**

Capillary electrophoresis (CE) has certain advantages over the previously mentioned techniques as a tool for the forensic laboratory. Although CE is not a new analytical method, its application to clinical and forensic drug analysis in the United States has been limited.

CE has been widely embraced by the forensic DNA community due to its high chromatographic efficiency,\textsuperscript{23-25} its application in toxicological laboratories is just beginning. Confirmation of peak identity is an important issue in forensic laboratories, and there is currently no simple method to interface certain CE buffer systems to a mass spectrometer. Instead CE can be used as a screening technique. However, the sometimes complex training that may be involved in utilizing CE techniques is problematic in clinical settings which still rely on traditional methods. These complications can be
solved with time and increased research. For example, in Canada, CE is currently replacing GC/nitrogen-phosphorous detector (NPD) as a toxicological screening technique.\textsuperscript{26} Also, techniques employing CE have shown exceptional utility in separating and detecting chiral compounds in drug seizures.\textsuperscript{27-29} As a result, electrophoretic techniques should be considered for greater exploration for their use in forensic laboratories.

In the past, comparisons have been made between gas chromatography (GC) nitrogen/phosphorus detection and capillary zone electrophoresis (CZE) for forensic analysis.\textsuperscript{26,30} The advantages of using CE over traditional GC are that samples do not require extensive extraction or derivatization. CE is an aqueous-based technique that has been proven to be useful in both toxicology and in the analysis of seized drugs, and has been the subject of several review articles.\textsuperscript{31-33}

Capillary electrophoresis is an analytical separation technique based on a molecule’s ability to migrate in a charged environment.\textsuperscript{34} The separation is typically induced when a voltage is applied across a narrow fused silica polyimide coated capillary (50 to 100 $\mu$m) filled with an electrolyte. The capillary length can be described as having a total length, $L_{\text{total}}$, and a distance to the detector window encompassing the separation portion, called the effective length, $L_{\text{effective}}$. The velocity (cm/sec) of an ion is described by the following equations (2 to 4), where $E$ is the applied electric field (V/cm) and $\mu_e$ is the electrophoretic mobility with migration time of the ion, $t_m$ (sec):\textsuperscript{34}

$$E = \frac{\text{Volts}}{L_{\text{total}}}$$

(2)
\begin{align*}
\mu_e &= \frac{L_{\text{effective}}}{t_m \cdot E} \quad (3) \\
v &= \mu_e \cdot E \quad (4)
\end{align*}

If electrophoretic mobility were the only mechanism involved in electrophoresis, it would not be considered such a versatile analytical tool. However, a more universal means of flow exists within the capillary known as electroosmotic flow (EOF).\textsuperscript{34} In this fundamental example, the negative charges of the deprotonated Si-OH groups on the capillary wall attract positive charges from the buffer solution. This phenomena creates a surface charge is known as the zeta potential. Following a normal polarity scheme, where the inlet electrode is positive and outlet is negative, the so-called charged double layer, or Helmholtz layer, will migrate towards the outlet creating a bulk flow. The EOF will carry all positive and neutral species in the general direction of the on-column detector located just before the outlet electrode (traveling the distance $L_{\text{effective}}$). For other applications, the poles can be reversed for the detection of negatively charged analytes. The EOF can be expressed by equation 5, where $\varepsilon$ is the dielectric constant, $\zeta$ is the zeta potential and $\eta$ is the buffer viscosity.\textsuperscript{34}

\begin{equation}
\begin{align*}
\nu_{\text{EOF}} &= \frac{\varepsilon \cdot \zeta}{\eta \cdot E} 
\end{align*}
\end{equation}

It is crucial to recognize the important effect that the buffer pH has on the EOF. At high pH, the silanol groups are mostly deprotonated, causing a maximal EOF. Alternatively, the EOF can be suppressed when Si groups are protonated at a lower pH.
A major advantage of CE is the flat flow profile generated by this bulk flow. Unlike the hyperbolic, or laminar flow, characteristic of high performance liquid chromatography (HPLC), the flat profile does not contribute to peak broadening of the solute zone. Because of this negligible contribution to band broadening, a higher number of theoretical plates can be obtained. The solute zone can only be maintained however, if the surface tension is sufficiently large to uniformly carry the centermost bulk components.

The above scenario is the most simplified version of CE known as capillary zone electrophoresis, or CZE. It has been the goal of this research to explore the application of other more complex forms of this technique for the separation of similarly charged drug compounds. These techniques will be discussed in the following chapters. Chapters 2 and 3 outline two novel simultaneous separations of drugs of interest. Chapters 4, 5, and 6 explore innovative separation technologies including polymer stationary phases and microfluidic devices.
Chapter 2: Micellar Electrokinetic Chromatographic Screening Method for the Detection of Date-Rape Drugs

Background

Drug-Facilitated Sexual Assault

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.\textsuperscript{35} 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.\textsuperscript{35}

The media hyps the classic example of “mickey-finns” or “knock-out drops” type substances being surreptitiously spiked into a victim’s beverage as the primary modus operandi of date rapists. In reality, a study performed by ElSolhy and Salamone indicated that alcohol is still by far the most prevalent form of date-rape incapacitation.\textsuperscript{36} 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.\textsuperscript{37} Complicating matters further, investigations have revealed that multiple drug ingestion is common among victims reporting sexual assault. Table 3 displays a list of common DFSA drugs, whether taken willingly by the victim or administered by the perpetrator.\textsuperscript{38}
Table 3. List of potential DFSA drugs.\textsuperscript{3,38}

<table>
<thead>
<tr>
<th>Generic Name</th>
<th>Trade Name (if applicable)</th>
<th>Street Name (if applicable)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-(3-trifluoromethylphenyl)piperazine</td>
<td>TFMPP</td>
<td>1,4-butanediol diol 14B, thunder nectar, weight belt cleaner</td>
</tr>
<tr>
<td>Alprazolam</td>
<td>Xanax</td>
<td>Z bars</td>
</tr>
<tr>
<td>Amobarbital</td>
<td>Amytal</td>
<td>Speed, dex, crank</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>Adderall</td>
<td>BZP, piperazine, A2</td>
</tr>
<tr>
<td>Benzylpiperazine</td>
<td></td>
<td>Chlordiazepoxide</td>
</tr>
<tr>
<td>Chloral hydrate</td>
<td>Notec</td>
<td>Knockout drops, Mickey Finn (with alcohol)</td>
</tr>
<tr>
<td>Chlordiazepoxide</td>
<td>Librium</td>
<td></td>
</tr>
<tr>
<td>Clonazepam</td>
<td>Klonopin</td>
<td></td>
</tr>
<tr>
<td>Cocaine</td>
<td></td>
<td>Crack (freebase), coke, snow, nose candy, dust</td>
</tr>
<tr>
<td>Codeine</td>
<td>Nucofed</td>
<td></td>
</tr>
<tr>
<td>Dextromethorphan</td>
<td>Robitussin</td>
<td>DXM, robodrops</td>
</tr>
<tr>
<td>Diazepam</td>
<td>Valium</td>
<td></td>
</tr>
<tr>
<td>Diphenhydramine</td>
<td>Benadryl</td>
<td></td>
</tr>
<tr>
<td>Dronabinol, delta-9-THC</td>
<td>Marinol</td>
<td>THC, weed, grass, dope, mary jane alcohol</td>
</tr>
<tr>
<td>Ethanol</td>
<td></td>
<td>Roofies, roche, forget me pills</td>
</tr>
<tr>
<td>Flunitrazepam</td>
<td>Rohypnol</td>
<td></td>
</tr>
<tr>
<td>Gamma-butyrolactone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gamma-hydroxybutyric acid</td>
<td>Xyrem</td>
<td>Easy lay, grievous body harm, liquid ecstasy</td>
</tr>
<tr>
<td>Gamma-valerolactone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heroin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ketamine</td>
<td>Ketalar</td>
<td>K, special K, lady K, vitamin K</td>
</tr>
<tr>
<td>Lorazepam</td>
<td>Ativan</td>
<td></td>
</tr>
<tr>
<td>Lysergic acid diethylamide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methamphetamine</td>
<td>Methedrine</td>
<td>LSD, acid, blotter, tabs</td>
</tr>
<tr>
<td>Methylenedioxyamphetamine</td>
<td>Methamphetamine</td>
<td>Ice (freebase), meth, crystal</td>
</tr>
<tr>
<td>Methylenedioxymethamphetamine</td>
<td>Methylenedioxymethamphetamine</td>
<td>MDA</td>
</tr>
<tr>
<td>Midazolam</td>
<td>Versed</td>
<td>MDMA, ecstasy, XTC, X, E, adam</td>
</tr>
<tr>
<td>Morfine</td>
<td>Roxanol</td>
<td></td>
</tr>
<tr>
<td>Nefazodone</td>
<td>Serzone</td>
<td></td>
</tr>
<tr>
<td>Nitrazepam</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pentobarbital</td>
<td>Nembural</td>
<td></td>
</tr>
<tr>
<td>Phencyclidine</td>
<td>Sernyl</td>
<td>PCP, crystal, angel dust</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>Luminal</td>
<td></td>
</tr>
<tr>
<td>Prazepam</td>
<td>Centrax</td>
<td></td>
</tr>
<tr>
<td>Secobarbital</td>
<td>Seconal</td>
<td></td>
</tr>
<tr>
<td>Trazodone</td>
<td>Desyrel</td>
<td></td>
</tr>
<tr>
<td>Triazolam</td>
<td>Halcion</td>
<td></td>
</tr>
<tr>
<td>Zolpidem</td>
<td>Ambien</td>
<td></td>
</tr>
</tbody>
</table>
The one statistic that has been common among reports and papers is that many occurrences of sexual assault are overwhelmingly underreported.\textsuperscript{35}

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.\textsuperscript{39} Another compound, flunitrazepam, or “roofies”, is one of many benzodiazepine drugs that are used for their sedative properties to incapacitate victims.

\textit{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.

While flunitrazepam is of some concern, any low dose benzodiazepines 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. 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. Owing to the instability of GHB’s acid functionality, methods for its analysis involve derivatization steps or esterification to the lactone, GBL. Thus, new methods are needed which can provide a more rapid and efficient screening for these compounds.

**Micellar Electrokinetic Chromatography (MEKC)**

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 (described in the previous chapter) 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\textsuperscript{42,43} and GHB,\textsuperscript{44} 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.\textsuperscript{30} 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.\textsuperscript{41} 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. 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 $1 \times 10^{-4}$ 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) and a 1 mg (common doses 0.5 to 2 mg) dose of various benzodiazepines to simulate a prescription tablet. 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 °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 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.\(^{47}\) 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.\(^{48}\) 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 4. 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 Figure 4. 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.
Figure 4. 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.
The results of the mobility versus acetonitrile concentrations for both 20 mM and 30 mM SDS at pH=9.2 are shown in Figure 5. The plots comparing the resolution of three sets of closely eluting peaks to acetonitrile concentration are shown in Figure 6. Resolution was calculated for GHB and the internal standard, flunitrazepam and clonazepam, and alprazolam and lorazepam. Run-to-run and column-to-column reproducibility of five consecutive runs on two different columns are displayed in Table 4. Two major causes of the lack of reproducibility are diminishing migration times and loss of peak resolution for the benzodiazepines during these consecutive runs. This fluctuation can be attributed to the evaporation of the acetonitrile in the run buffer.

The benzodiazepines were calibrated in sets of two or three with concentrations ranging from 2.5 µg/mL to 100 µg/mL. GHB was run at 0.1 to 2.5 mg/mL, while the GBL concentrations were from 0.5 to 10 mg/mL (Table 5). The detection limits for all the analytes of interest are also listed in Table 5. Unfortunately, the MECC method was not sensitive enough to offer adequate quantitative data for 1,4-butanediol.

**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.\(^{41}\)
Figure 5. The mobility of benzodiazepines with respect to the percentage of acetonitrile organic modifier. Conditions were the same as in Figure 4. (A) 20 mM SDS in borate at pH=9.2; (B) 30 mM SDS in borate at pH=9.2.
Figure 6. Resolution of three sets of closely eluting peaks with changing acetonitrile concentrations: GHB and sulfanilic acid, flunitrazepam and clonazepam, alprazolam and lorazepam. Resolution was calculated by relating the distance between two peaks and the widths of those peaks measured at half the peak height. Conditions were the same as in Figure 4. (A) 20 mM SDS in borate at pH=9.2; (B) 30 mM SDS in borate at pH=9.2.
Table 4. Run-to-run and column-to-column reproducibility of three different capillaries run five times.a

<table>
<thead>
<tr>
<th>Compound</th>
<th>Column #1</th>
<th></th>
<th>Column #2</th>
<th></th>
<th>Column #3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mobility</td>
<td>Peak Area</td>
<td>Mobility</td>
<td>Peak Area</td>
<td>Mobility</td>
<td>Peak Area</td>
</tr>
<tr>
<td></td>
<td>% RSD</td>
<td>% RSD</td>
<td>% RSD</td>
<td>% RSD</td>
<td>% RSD</td>
<td>% RSD</td>
</tr>
<tr>
<td>GBL</td>
<td>0.81</td>
<td>5.1</td>
<td>0.39</td>
<td>2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GHB</td>
<td>0.48</td>
<td>7.9</td>
<td>0.20</td>
<td>5.6</td>
<td>0.74</td>
<td>17</td>
</tr>
<tr>
<td>Internal Standard (IS)</td>
<td>0.48</td>
<td>0.19</td>
<td></td>
<td></td>
<td>0.75</td>
<td></td>
</tr>
<tr>
<td>Flunitrazepam</td>
<td>0.64</td>
<td>2.1</td>
<td>0.25</td>
<td>1.4</td>
<td>0.83</td>
<td>7.4</td>
</tr>
<tr>
<td>Clonazepam</td>
<td>0.62</td>
<td>1.9</td>
<td>0.32</td>
<td>3.4</td>
<td>0.79</td>
<td>4.9</td>
</tr>
<tr>
<td>Chlordiazepoxide</td>
<td>0.60</td>
<td>2.1</td>
<td>0.37</td>
<td>2.5</td>
<td>0.86</td>
<td>3.4</td>
</tr>
<tr>
<td>Alprazolam</td>
<td>0.45</td>
<td>2.0</td>
<td>0.10</td>
<td>5.8</td>
<td>0.91</td>
<td>0.7</td>
</tr>
<tr>
<td>Lorazepam</td>
<td>0.46</td>
<td>3.4</td>
<td>0.23</td>
<td>6.3</td>
<td>0.92</td>
<td>0.8</td>
</tr>
<tr>
<td>Triazolam</td>
<td>0.36</td>
<td>5.8</td>
<td>0.28</td>
<td>2.8</td>
<td>0.91</td>
<td>2.9</td>
</tr>
<tr>
<td>Midazolam</td>
<td>0.49</td>
<td>4.4</td>
<td>0.37</td>
<td>3.8</td>
<td>0.93</td>
<td>1.7</td>
</tr>
<tr>
<td>Prazepam</td>
<td>0.66</td>
<td>3.0</td>
<td>0.52</td>
<td>4.8</td>
<td>1.1</td>
<td>2.0</td>
</tr>
</tbody>
</table>

aThe runs were performed in a 50 µm ID capillary with a total length 80 cm and an effective length of 72 cm. The electrokinetic injection was for 4 seconds at 30 mbar. The electrophoretic buffer was 20 mM SDS in borate pH=9.2 with 7% acetonitrile.
Table 5. Calibration curve data for GHB, GBL, and the benzodiazepines.ª

<table>
<thead>
<tr>
<th>Compound</th>
<th>Slope(^b) (mAU s mL mg(^{-1}))</th>
<th>(R^2)</th>
<th>Detection Limit(^c) (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GHB</td>
<td>0.00170 ± 0.0001</td>
<td>0.9902</td>
<td>31.8</td>
</tr>
<tr>
<td>GBL</td>
<td>0.178 ± 0.009</td>
<td>0.9916</td>
<td>152</td>
</tr>
<tr>
<td>Flunitrazepam</td>
<td>0.055 ± 0.001</td>
<td>0.9988</td>
<td>1.17</td>
</tr>
<tr>
<td>Lorazepam</td>
<td>0.0660 ± 0.0006</td>
<td>0.9997</td>
<td>0.979</td>
</tr>
<tr>
<td>Prazepam</td>
<td>0.0630 ± 0.0008</td>
<td>0.9993</td>
<td>1.97</td>
</tr>
<tr>
<td>Clonazepam</td>
<td>0.057 ± 0.001</td>
<td>0.9975</td>
<td>3.33</td>
</tr>
<tr>
<td>Alprazolam</td>
<td>0.078 ± 0.002</td>
<td>0.9976</td>
<td>3.14</td>
</tr>
<tr>
<td>Triazolam</td>
<td>0.043 ± 0.001</td>
<td>0.9979</td>
<td>4.37</td>
</tr>
<tr>
<td>Chlordiazepoxide</td>
<td>0.0264 ± 0.0004</td>
<td>0.9991</td>
<td>1.58</td>
</tr>
<tr>
<td>Midazolam</td>
<td>0.0643 ± 0.0004</td>
<td>0.9999</td>
<td>0.722</td>
</tr>
</tbody>
</table>

ªGHB was run at concentrations ranging from 100 to 2500 mg/mL, GBL from 0.5 to 10 mg/mL, and benzodiazepines from 2.5 to 100 mg/mL. The electrokinetic injection was for 8 seconds at 30 mbar. The electrophoretic buffer was 20 mM SDS in borate pH=9.2 with 7% acetonitrile. Column length was the same as Figure 4.

\(^b\)Slope and standard deviation were calculated using Microsoft Excel.
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 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. A list of drinks and the major CE artifacts caused by their components are shown in Table 6. 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. 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.

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. The 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 trends learned from the optimization study, the interfering peak is easily moved out from beneath the GHB peak (as seen in Figure 7) when the buffer is adjusted to a concentration of 30 mM SDS with 20% acetonitrile.
Table 6. Beverages monitored for the GHB kinetic study.\(^a\)

<table>
<thead>
<tr>
<th>Beverage</th>
<th>pH</th>
<th>Artifact Mobility (Vcm(^2)/sec)</th>
<th>Peak Area (mAU s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bottled Water</td>
<td>6.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gatorade</td>
<td>3.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apple/Cranberry Juice</td>
<td>3.0</td>
<td>0.00043</td>
<td>13.7</td>
</tr>
<tr>
<td>Orange Juice</td>
<td>4.0</td>
<td>0.00046</td>
<td>2.44</td>
</tr>
<tr>
<td>Sprite</td>
<td>3.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coca-Cola</td>
<td>2.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beer</td>
<td>4.4</td>
<td>0.00037</td>
<td>2.07</td>
</tr>
<tr>
<td>White Wine</td>
<td>3.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red Wine</td>
<td>3.7</td>
<td>0.00032</td>
<td>2.56</td>
</tr>
<tr>
<td>Vodka</td>
<td>7.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bailey's Irish Cream</td>
<td>6.8</td>
<td>0.00030</td>
<td>123</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.00029</td>
<td>104</td>
</tr>
</tbody>
</table>

\(^a\)The samples were prepared by a 1:10 dilution in deionized water and internal standard. Samples were analyzed at \(t = 0\), 24, and 48 hours after sitting uncovered at an ambient temperature. Conditions were the same as in Table 5.
Figure 7. Separation of GHB from an interfering peak in beer utilizing changes in both SDS and acetonitrile concentrations: a) Blank beer sample diluted 1:10 with a run buffer of 20 mM SDS with 7% acetonitrile; b) Beer spiked with GHB using a run buffer of 30 mM SDS with 10% acetonitrile; c) Beer spiked with GHB using a run buffer of 30 mM SDS with 15% acetonitrile; d) Beer spiked with GHB using a run buffer of 30 mM SDS with 20% acetonitrile.
Unknown Beverage Samples

For samples containing benzodiazepines, an ethyl acetate extraction was necessary prior to analysis. Simple dilution of these beverages was not possible because the standard dose of these drugs is 0.25 to 2 mg. The percent recovery for the one step ethyl acetate procedure is shown in Table 7. Therefore, when encountering an unknown sample, the recommended procedure is to dilute an aliquot 1:10 as previously described for GHB analysis. A second aliquot should be prepared for extraction to detect the drugs, which may be present at lower concentration.

An interference study was performed to determine other potential drugs that might be present in beverages. By using both optimized buffers, we were able to prove that 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 8. 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 $3.4 \times 10^{-4}$ Vcm$^2$/sec, which was identical to the mobility of clonazepam. When the analyzed with the 30 mM SDS buffer, ketamine’s mobility became greater than clonazepam by a difference of $4 \times 10^{-3}$ Vcm$^2$/sec. By exploiting these mobility differences along with the spectral data, the MECC method becomes valuable for date-rape drug detection.
**Table 7.** Extraction efficiencies for the ethyl acetate extraction.\(^a\)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Percent Recovery (%)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flunitrazepam</td>
<td>90 ± 3</td>
</tr>
<tr>
<td>Lorazepam</td>
<td>87 ± 8</td>
</tr>
<tr>
<td>Prazepam</td>
<td>71 ± 4</td>
</tr>
<tr>
<td>Clonazepam</td>
<td>97 ± 3</td>
</tr>
<tr>
<td>Alprazolam</td>
<td>95 ± 3</td>
</tr>
<tr>
<td>Triazolam</td>
<td>90 ± 7</td>
</tr>
<tr>
<td>Chlordiazepoxide</td>
<td>92 ± 8</td>
</tr>
<tr>
<td>Midazolam</td>
<td>95 ± 6</td>
</tr>
</tbody>
</table>

\(^{a}\)To all standard solutions 100 mL of buffer phosphate was added. Each group was extracted twice with 0.5 mL of solvent. Conditions were the same as in Table 5.

\(^{b}\)Error given in terms of standard deviation calculated from Microsoft Excel.
<table>
<thead>
<tr>
<th>Analyte</th>
<th>(a) Mobility $\times 10^{-4}$ (Vcm$^2$/sec)</th>
<th>Analyte</th>
<th>(b) Mobility $\times 10^{-4}$ (Vcm$^2$/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GHB</td>
<td>3.9</td>
<td>GHB</td>
<td>3.3</td>
</tr>
<tr>
<td>Sulfanilic Acid</td>
<td>3.7</td>
<td>Sulfanilic Acid</td>
<td>3.2</td>
</tr>
<tr>
<td>Salicylic Acid</td>
<td>3.6</td>
<td>Ketamine</td>
<td>3.1</td>
</tr>
<tr>
<td>Flunitrazepam</td>
<td>3.5</td>
<td>Salicylic Acid</td>
<td>3.1</td>
</tr>
<tr>
<td>Clonazepam</td>
<td>3.4</td>
<td>Flunitrazepam</td>
<td>2.8</td>
</tr>
<tr>
<td>Ketamine</td>
<td>3.4</td>
<td>Heroin</td>
<td>2.8</td>
</tr>
<tr>
<td>Chlordiazepoxide</td>
<td>3.3</td>
<td>Clonazepam</td>
<td>2.7</td>
</tr>
<tr>
<td>Heroin</td>
<td>3.1</td>
<td>Chlordiazepoxide</td>
<td>2.6</td>
</tr>
<tr>
<td>Benzylpiperazine</td>
<td>3.0</td>
<td>Benzylpiperazine</td>
<td>2.6</td>
</tr>
<tr>
<td>Alprazolam</td>
<td>2.8</td>
<td>Alprazolam</td>
<td>2.2</td>
</tr>
<tr>
<td>Lorazepam</td>
<td>2.8</td>
<td>Cocaine</td>
<td>2.2</td>
</tr>
<tr>
<td>Triazolam</td>
<td>2.7</td>
<td>Lorazepam</td>
<td>2.2</td>
</tr>
<tr>
<td>Midazolam</td>
<td>2.6</td>
<td>Methamphetamine</td>
<td>2.1</td>
</tr>
<tr>
<td>Methamphetamine</td>
<td>2.6</td>
<td>Triazolam</td>
<td>2.1</td>
</tr>
<tr>
<td>MDMA</td>
<td>2.5</td>
<td>MDMA</td>
<td>2.1</td>
</tr>
<tr>
<td>Cocaine</td>
<td>2.5</td>
<td>Midazolam</td>
<td>2.0</td>
</tr>
<tr>
<td>Prazepam</td>
<td>2.4</td>
<td>Prazepam</td>
<td>1.9</td>
</tr>
<tr>
<td>Dextramethorphan</td>
<td>2.4</td>
<td>Dextramethorphan</td>
<td>1.7</td>
</tr>
</tbody>
</table>

\(^a^\text{Samples were run in borate buffer at pH=9.2 with (a) 20 mM SDS with 7\% acetonitrile and (b) 30 mM SDS with 10\% acetonitrile. All other conditions were the same as in Table 5.}\)
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.
Chapter 3: Simultaneous Separation of Amphetamine-like and Piperazine-like Compounds by Chiral-mediated Capillary Electrophoresis

Background

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. 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. 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 (pMeOPP), 1-[2-methoxyphenyl]-piperazine (oMeOPP), and 1-[3-chlorophenyl]-piperazine (mCPP), which are shown in Appendix 1.

In 1944, 1-benzylpiperazine was first used as a potential anti-parasitic agent. Since then, BZP has not been used for any other legitimate medical purpose, and it is for this reason that BZP and TFMPP are on the DEA’s schedule I list. Ingestion of BZP results in euphoria and an increase in heart rate, pulse rate, and systolic blood pressure. TFMPP, which produces hallucinogen-like effects similar to d-amphetamine and mCPP, is also a serotonin-releasing agent. The drug mCPP is an active metabolite of the
non-tricyclic antidepressant drug Trazodone® (Desyrel, Trazon, Trialodine). Many neuropharmacological studies have been performed on both rats and humans to determine the extent of mCPP’s anxiogenic effects as a 5-HT$_2$ receptor agonist. Studies were performed in 2001 and 2003 by Tancer and Johanson in order to compare the effects of MDMA with mCPP and d-amphetamines. Their latest study involved administering mCPP and d-amphetamine to young individuals with a history of stimulant and MDMA abuse in order to examine drug induced behavior. Although mCPP did not exhibit reinforcing effects characteristic of MDMA and D-amphetamine, the response to the drug was generally positive with euphoric feelings and increased drug likeability. The subjective effects of MDMA were similar to that of the mCPP profile.

Reports of piperazine tablet seizures have appeared frequently in the Drug Enforcement Administration’s 2003 and 2004 Microgram Bulletins. There have also been accounts of piperazine tablets being used in combination with gamma-hydroxybutyric acid (GHB) and gamma-butyrolactone (GBL). Recently, the owner of Genapharm, Inc. in Austin, Texas pleaded guilty to charges of possessing controlled drugs with the intent to distribute. Over 5,000 tablets of BZP, made to look like ecstasy, were seized by authorities. Figure 8 illustrates the U.S. states where BZP and TFMPP encounters have occurred.
Figure 8. BZP and TFMPP Encounters by Law Enforcement Map (as of 10/9/03) prepared by the U.S. Department of Justice Drug Enforcement Administration, Office of Diversion Control (Chemical Operations Section). Adapted from Ref. 68.
Clinical Analysis of Piperazines

With the emergency scheduling and its subsequent illicit use, BZP samples encountered in forensic analysis generally originate from clandestine manufacturing sites. As with any clandestinely manufactured drug, the purity of the substance may be in question since the synthetic route used to manufacture the drug can vary. Reactions could contain low levels of the drug substance under investigation and may also contain concentration(s) of by products and/or starting materials. These unwanted compounds can interfere with the analysis of the substance. Techniques for the analysis of these drugs must also be able to detect the synthetic analogs as well as the illegal product. Drug analogs are substituted for the illicit compound in an attempt by a user to avoid detection and arrest.

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.\textsuperscript{69,70} De Boer et al. suggested a variety of analytical strategies for detection of BZP in capsules,\textsuperscript{51} 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.\textsuperscript{29,71-75} 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.

**Chiral Mediated Capillary Electrophoresis**

Chiral-mediated CE involves the separation of enantiomers by electrophoretic means. Cyclodextrins are uncharged and have negligible UV-absorbance in their natural states above 200 nm; for these reasons they have become very popular as chiral pseudophases for CE. Chemically, cyclodextrins are comprised of a ring of glucose molecules in which multiple interactions, such as hydrophobic, electrostatic, and hydrogen bonding, can orient molecules in solution and lead to chiral separations. The resultant cyclodextrin/solute inclusion complex alters the electrophoretic mobility of the solute resulting in a separation. The size of the cyclodextrin’s cavity depends upon the number of glucose units in the molecule. The most effective cavity size for small molecules is the β form which contains 7 glucose units.

Cyclodextrins can be used in their native form or as chemically modified derivatives that can enhance enantioselectivity. 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 solid phase extraction eluting solvent was prepared daily and consisted of a ratio of 78/20/2 (v/v/v) methylene chloride/isopropyl alcohol (IPA)/ammonium hydroxide. The chiral CE buffer was prepared by a modification of the literature procedure. Sodium phosphate and 85% phosphoric acid were combined to yield 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 °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.

The liquid chromatography with mass spectral detection (LC-MS) instrument used was an Agilent 1100 Series LC-MSD operated in the positive ion mode with a fragmentor voltage of 100 Volts. Nitrogen was used as the drying gas at 300 °C with a flow rate of 10 L/min. The nebulizer pressure was maintained at 20 psig. The chromatographic method for the MS analysis of piperazines utilized a Zorbax SB-C18 column (150 mm x 2.1 mm) with a mobile phase consisting of acetonitrile and a volatile ion-pairing agent (10 mM tridecafluoroheptanoic acid). The mobile phase was delivered
at 0.30 mL/min and was run as a 20 minute gradient from 37% acetonitrile to 90% acetonitrile.

**Liquid-Liquid Extraction**

A modified version of Kinberger’s procedure was used for the liquid-liquid extraction of standard mixtures and spiked urine. In a 4 mL conical screw cap vial, 1.0 mL of standard sample and 200 µL of 1.0 M sodium hydroxide were added. The specimen was then shaken and extracted 3 times with 1.0 mL solvent. The aqueous layer was drawn out with a stainless steel syringe and the organic layers were combined in a clean conical vial. To the organics, 100 µL of 0.10 M hydrochloric acid was added. The layers were mixed and the aqueous portion was transferred to a 100 µL sample vial.

**Solid Phase Extraction (SPE)**

The extraction was performed using an altered version of the Varian Bond Elut Certify Amphetamines in Urine procedure. The SPE columns (1210-2051) were conditioned using 2.0 mL of methanol and then 2.0 mL of 100 mM pH 6.0 phosphate buffer drawn through with a low vacuum (≤ 3 inches Hg). To 1.0 mL of standard or sample, 1.0 mL of the phosphate buffer was added to adjust the pH. After the specimen was slowly loaded onto the column, it was rinsed with 1.0 mL of acetic acid, dried for 5 minutes under vacuum, rinsed with 3.0 mL of methanol, and then dried for 2 minutes (≥10 inches Hg). The analytes were eluted using 2.0 mL of fresh CH₂Cl₂/IPA/NH₄OH,
evaporated to dryness under a steady stream of nitrogen, and then reconstituted using 100 µL of 1:1 methanol to deionized water.

_Synthesis of Benzylpiperazine and Its Analog Compounds_

Owing to the increased legal control of BZP and TFMPP, we anticipate that new methods for the clandestine synthesis of piperazine analogs will develop over time. Because of this potential problem, greater focus needs to be placed on the detection of those piperazine analogs that are not yet scheduled such as DBZP, oMeOPP, pMeOPP, and mCPP.

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 (^1H-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^1NMR), gas chromatography with mass spectral detection (GC-MS), and liquid chromatography with mass spectral detection (LC-MS). The purities of each synthetic product were determined using LC-MS. The resulting products from two
The syntheses shown in Figure 9 were then analyzed using the capillary electrophoresis method described in this chapter. For the sake of brevity, only LC-MS ratio comparisons are provided. Data from characterization methods described above are not shown.

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.

**Results and 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 9. 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. Figure 10 illustrates the result of increased HP-β-CD on migration time and order of the 10 component standard mixture of amphetamines and piperazines. As the amount of cyclodextrin increased, the migration time of the drugs also increased. While there was no major difference in the resolution and order of elution, 20 mM HP-β-CD offered the best selectivity for all of the analytes of interest. Figure 11 shows that the migration time also increases when the concentration of phosphate in the run buffer was increased. 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.

The run-to-run, day-to-day, and column-to-column reproducibility can be found in Table 9. Calibration curves were generated to verify the linearity of detector response to the piperazine-type compounds. The curve data and limits of detection are displayed in Table 10.
Figure 10. Mobility of piperazines and amphetamines with respect to the concentration of the chiral selector hydroxypropyl-β-cyclodextrin (HP-β-CD). The capillary had total length of 64.5 cm, an effective length of 56 cm, and an internal diameter of 50 µm. Injections were 40 mbar for 4 seconds, the temperature was kept at 25 °C, and the run voltage was 25 kV.
Figure 11. Mobility of piperazines and amphetamines with respect to the concentration of sodium phosphate in the buffer. Conditions are the same as in Figure 4.
Figure 12. The optimized chiral separation of amphetamine and piperazine compounds using the standard drug mixture 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) mCPP.
Figure 13. UV-spectral data for the piperazine-like compounds taken from the standard mixture.
Table 9. Run-to-run reproducibility data from four different runs performed on two different days and column-to-column data for a comparison with a second column. The “n” for these experiments is equal to 4.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Compound</th>
<th>Day-to-Day Migration (min.)</th>
<th>Std. dev.</th>
<th>RSD</th>
<th>Column-to-Column Migration (min.)</th>
<th>Std. dev.</th>
<th>RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>BZP</td>
<td>7.55</td>
<td>0.1</td>
<td>1.1</td>
<td>7.33</td>
<td>0.2</td>
<td>3.0</td>
</tr>
<tr>
<td>Phenethylamine</td>
<td>10.5</td>
<td>0.4</td>
<td>4.0</td>
<td>10.1</td>
<td>0.3</td>
<td>2.6</td>
</tr>
<tr>
<td>DBZP</td>
<td>10.6</td>
<td>0.0</td>
<td>0.2</td>
<td>11.5</td>
<td>0.4</td>
<td>3.5</td>
</tr>
<tr>
<td>1,2-MeOPP</td>
<td>11.8</td>
<td>0.1</td>
<td>1.1</td>
<td>11.5</td>
<td>0.4</td>
<td>3.5</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>13.4</td>
<td>0.1</td>
<td>0.4</td>
<td>13.1</td>
<td>0.3</td>
<td>2.6</td>
</tr>
<tr>
<td>Methamphetamine</td>
<td>13.6</td>
<td>0.1</td>
<td>0.4</td>
<td>13.3</td>
<td>0.3</td>
<td>2.6</td>
</tr>
<tr>
<td>1,4-MeOPP</td>
<td>14.1</td>
<td>0.1</td>
<td>0.4</td>
<td>13.8</td>
<td>0.4</td>
<td>2.6</td>
</tr>
<tr>
<td>MDA</td>
<td>14.4</td>
<td>0.1</td>
<td>0.4</td>
<td>14.1</td>
<td>0.4</td>
<td>2.7</td>
</tr>
<tr>
<td>MDMA</td>
<td>17.0</td>
<td>0.2</td>
<td>1.3</td>
<td>16.3</td>
<td>0.7</td>
<td>4.3</td>
</tr>
<tr>
<td>MDMA</td>
<td>18.0</td>
<td>0.1</td>
<td>0.5</td>
<td>17.6</td>
<td>0.5</td>
<td>3.1</td>
</tr>
<tr>
<td>MDMA</td>
<td>18.4</td>
<td>0.1</td>
<td>0.5</td>
<td>18.0</td>
<td>0.6</td>
<td>3.1</td>
</tr>
<tr>
<td>MDMA</td>
<td>18.9</td>
<td>0.1</td>
<td>0.5</td>
<td>18.4</td>
<td>0.6</td>
<td>3.1</td>
</tr>
<tr>
<td>TFMPP</td>
<td>19.6</td>
<td>0.1</td>
<td>0.5</td>
<td>19.1</td>
<td>0.6</td>
<td>3.3</td>
</tr>
<tr>
<td>mCPP</td>
<td>20.3</td>
<td>0.1</td>
<td>0.5</td>
<td>19.7</td>
<td>0.6</td>
<td>3.2</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Conditions are the same as in Figure 12.
Table 10. Calibration curve data for 6 piperazine-like compounds.\(^a\)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Slope(^b) (mAU s mL mg(^{-1}))</th>
<th>(R^2)</th>
<th>Detection Limit(^c) (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BZP</td>
<td>0.26 ± 0.02</td>
<td>0.9868</td>
<td>0.577</td>
</tr>
<tr>
<td>DBZP</td>
<td>0.46 ± 0.03</td>
<td>0.9859</td>
<td>5.19</td>
</tr>
<tr>
<td>1,2-MeOPP</td>
<td>1.50 ± 0.09</td>
<td>0.9895</td>
<td>3.46</td>
</tr>
<tr>
<td>1,4-MeOPP</td>
<td>0.95 ± 0.07</td>
<td>0.9838</td>
<td>0.0374</td>
</tr>
<tr>
<td>TFMPP</td>
<td>1.19 ± 0.09</td>
<td>0.9845</td>
<td>1.28</td>
</tr>
<tr>
<td>m CPP</td>
<td>0.71 ± 0.04</td>
<td>0.9885</td>
<td>4.27</td>
</tr>
</tbody>
</table>

\(^a\)Conditions were the same as in Figure 12.
\(^b\)Slope and standard deviation were calculated using Microsoft Excel.
\(^c\)Detection limits were calculated using Microsoft Excel linear estimation.
Spiked Urine Samples

Since previous work on the extraction and analysis of piperazines from forensic samples was limited, both liquid-liquid and solid phase extractions were investigated for their usefulness. Extraction methods for piperazine samples were developed using existing methods published for amphetamine samples.\textsuperscript{78} Owing to the polar nature of these compounds, liquid-liquid extractions are often difficult. This problem was overcome by performing a back extraction into 0.10 M hydrochloric acid (HCl). The percent recovery for diethyl ether and ethyl acetate are shown in Table 11. Diethyl ether gave the highest percent recovery. The use of phosphoric acid in place of HCl did not improve upon the extraction. Table 12 contains percent recovery data for the solid phase extraction for 20 µg/mL and 50 µg/mL standard water samples. Urine samples spiked at 50 µg/mL were also analyzed to demonstrate similar extraction efficiencies.

Synthesized Samples

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.
**Table 11.** Liquid-liquid extraction percent recoveries for 50 µg/mL samples extracted with two different solvents.\(^{a,b}\)

<table>
<thead>
<tr>
<th>Solvent</th>
<th>BZP</th>
<th>DBZP</th>
<th>1,2-MeOPP</th>
<th>1,4-MeOPP</th>
<th>TFMPP</th>
<th>m CPP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diethyl Ether</td>
<td>88%</td>
<td>60%</td>
<td>82%</td>
<td>79%</td>
<td>99%</td>
<td>96%</td>
</tr>
<tr>
<td>Ethyl Acetate</td>
<td>71%</td>
<td>65%</td>
<td>78%</td>
<td>85%</td>
<td>67%</td>
<td>45%</td>
</tr>
</tbody>
</table>

\(^{a}\)In a conical vial, 1-mL of 50 µg/mL water standard and 200 µL of 1.0 M sodium hydroxide was added. The specimen was extracted with 3 × 1.0 mL solvent. The organic layer was back-extracted with 100 µL of 0.1 M hydrochloric acid. The aqueous portion was transferred to a sample vial and injected. Extractions were performed two times for each solvent.

\(^{b}\)Conditions were the same as in Figure 12.
Table 12. Solid phase extraction (SPE) of water and urine samples.\textsuperscript{a,b} (A) Spiked water standards at 50 µg/mL; (B) Spiked water standards at 20 µg/mL; (C) Two different urine samples spiked at 50 µg/mL. The “n” for these experiments is equal to 3.

\begin{tabular}{lcccccc}
A. & BZP & DBZP & 1,2-MeOPP & d-Amphetamine & 1,4-MeOPP & TFMPP & m CPP \\
\hline
50 ug/mL & Average Percent Recovery & 85.3 & 77.4 & 93.4 & 64.1 & 90.1 & 84.7 & 78.9 \\
& Standard Deviation & 2.5 & 8.6 & 6.9 & 16.5 & 6.1 & 10.7 & 6.6 \\
\hline
\end{tabular}

\begin{tabular}{lcccccc}
B. & BZP & DBZP & 1,2-MeOPP & d-Amphetamine & 1,4-MeOPP & TFMPP & m CPP \\
\hline
20 ug/mL & Average Percent Recovery & 68.4 & 62.8 & 81.1 & 34.5 & 74.2 & 79.8 & 66.4 \\
& Standard Deviation & 1.5 & 5.2 & 10.4 & 15.5 & 5.1 & 11.2 & 8.0 \\
\hline
\end{tabular}

\begin{tabular}{lcccccc}
C. & BZP & DBZP & 1,2-MeOPP & d-Amphetamine & 1,4-MeOPP & TFMPP & m CPP \\
\hline
Urine 1 & Percent Recovery & 90.3 & 87.1 & 94.6 & 88.3 & 91.1 & 87.9 & 84.7 \\
Urine 2 & Percent Recovery & 91.4 & 96.0 & 96.1 & 88.9 & 89.9 & 88.9 & 87.7 \\
\hline
\end{tabular}

\textsuperscript{a}In a conical vial, 1-mL of 50 µg/mL water standard and 200 µL of 1.0 M sodium hydroxide was added. The specimen was extracted times with 1.0 mL solvent. The organic layer was back-extracted with 100 µL of 0.1 M hydrochloric acid. The aqueous portion was transferred to a sample vial and injected. Extractions were performed two times for each solvent.

\textsuperscript{b}Conditions are the same as in Figure 12.
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.
The overall peak ratios for each synthesis that was determined by the CE method were the same as obtained by LC-MS, demonstrated in Figure 15 and Figure 16.

**Conclusions**

A novel CE method for the simultaneous detection of piperazine and amphetamines compounds has been developed for the analysis of these drugs in spiked urine and synthesized samples. The method utilizes hydroxypropyl-β-cyclodextrin for the separation of enantiomers of amphetamine-type drugs. Both a liquid-liquid extraction, using diethyl ether, and a solid phase extraction, using a commercial procedure, were offered for sample pretreatment options. Consistent with the reaction scheme, synthesis #1 yielded BZP as the primary product, while synthesis #2 favored 1,4-DBZP.

Despite the new scheduling of BZP and TFMPP, it is clear by the analysis of the synthetic products that other non-scheduled piperazines can be present when clandestine procedures are used. Future studies are needed to characterize other reactions and develop improved techniques to quantitate reaction products. Additionally, tailoring the CE method to provide lower limits of detection would be helpful for the detection of low-level toxicological samples. This can be done using field amplified sample stacking and other preconcentration techniques.
Figure 15. LC-MS data of BZP diHCl (white crystals) from BZP synthesis #1. The chromatographic method for the MS analysis of piperazines utilized a Zorbax SB-C18 column (150 mm x 2.1 mm) with a mobile phase consisting of acetonitrile and a volatile ion pairing agent (10 mM tridecafluoroheptanoic acid). The mobile phase was delivered at 0.30 mL/min and was run as a 20 min gradient from 37% acetonitrile to 90% acetonitrile.
Figure 16. LC-MS data of DBZP (white crystals) from BZP synthesis #2. Conditions were the same as in Figure 15.
Chapter 4: Capillary Electrochromatography for Small Molecules

Background

Capillary electrochromatography (CEC) is an analytical technique that is a hybrid between capillary electrophoresis (CE) and high-performance liquid chromatography (HPLC). CEC uses the same types of stationary phases utilized in HPLC, but requires no pump for the analysis. Instead the column flow is generated using electro-driven pumping created by high voltage electroosmosis. The capillary contains stationary phase from the tip of the inlet up to or before the detector window. Apart from the modified columns, CEC employs standard capillary electrophoresis instrumentation consisting of a capillary column, buffers, a high-voltage power supply, and a detector with suitable electronics.

Equation 4 in Chapter 1 details the parameters responsible for EOF of an open capillary, as utilized with CE. In packed capillary techniques, such as CEC, with an induced electric field, linear velocity ($v$) can be described by the following equation where $\varepsilon_r$ is the vacuum permittivity, $\varepsilon_0$ is the dielectric constant of the solution, $E$ is the applied field, $\zeta$ is the zeta potential, and $\eta$ the viscosity of the solvent.\(^{80}\)

$$v = \frac{(\varepsilon_0 \varepsilon_r E \zeta)}{\eta} \quad (6)$$
When EOF is described in terms of the stationary phase, the migration takes the form of the following equation where $1/\kappa$ is the thickness of the double layer and $\sigma$ is the charge density.\textsuperscript{80}

$$\mu = \left( \frac{1}{\kappa} \right) \left( \frac{\sigma E}{\eta} \right)$$  \hspace{1cm} (7)

It is important to note that for silica phases EOF will be dependent on the size, abundance, and ionization of silanol groups packed into the capillary.

The combination of chromatography and electrophoresis offers several advantages. In CEC, a flat electroosmotic flow profile is produced, which provides improved efficiencies when compared to the hyperbolic, or laminar flow, profile generated with pressurized liquid chromatography. The addition of a stationary phase not only provides molecular partitioning, but also creates additional surface charge sites inside the capillary. Therefore, as shown by Equation (7), EOF is dependent on the surface charge of both the free silanol groups on the capillary wall and, most importantly, the stationary phase. It is worthy to note that the linear velocity, $v$, in CEC is independent on particle size, as shown by the Equation (6). Because of this independence, electrically driven flow separations are free from the problems associated with backpressure, which often plague HPLC. Therefore, particles used in CEC can be much smaller and columns can be much longer than those used in micro-HPLC. The amalgamation of flat, low-pressure flow profiles and long columns results in a method
that produces highly efficient separations that are more commonly seen with capillary GC.80,81

A major advantage of CEC in forensic analysis is 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 CEC, these mobile-phase additives are replaced by stationary phases similar to those used in LC and LC-MS.

Despite CEC’s advantages over its two parent instruments, HPLC and CE, there are several reasons why the technique has been slow to develop.82 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.83 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 chapter, 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.\(^{84}\) 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.\(^{80,85}\) 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.\textsuperscript{80}

In order to achieve a better understanding of the complex interactions involved in modified silica CEC, literature procedures were evaluated for their usefulness for clinical drug screenings pertinent to date-rape and club drugs. While these methods were not optimized in detail, they were included for their relevance towards the separation of date-rape and club drugs.

*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.\textsuperscript{86,87} but because of their limited chromatographic properties, alternative methods have been developed. 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.88

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. Examples of monomers, porogens, and initiators used in this chapter’s experiments are shown in Appendix III.

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.

**Polymer Pore Size Studies**

The porous properties of the monolithic-polymer stationary phases were studied using mercury intrusion and gas sorption. While mercury intrusion was used to determine the larger-pore sizes, its complementary technique, nitrogen adsorption/desorption, was employed to measure the smaller-size range of the polymers. In order to interpret the collected pore size data, a detailed review of both measuring techniques is provided below.

Mercury intrusion porosimetry (MIP) is an analytical technique used to describe the density and morphology of porous substances. Among its many applications, it has been widely used in the areas of geology to study rocks, engineering to study cement, and
chemistry to study porous materials. It is most often employed for pore volumes, or pore size distributions,\textsuperscript{89} as it will be here, used to assess the pore size of the resultant monolithic polymers.

Basic fluid dynamics are used to describe the theory behind intrusion/extrusion studies.\textsuperscript{90} Interfacial tensions are the basis behind the capillary action of a wetting liquid, which has a surface contact angle of less than 90°, and will spontaneously enter the capillary. A non-wetting liquid with a contact angle greater than 90° however, needs to be forced into a capillary. Because the amount of pressure used to push the non-wetting liquid is a function of pore size, interstitial or porous spaces can be easily measured.\textsuperscript{91}

Present day porosimetry measurements are almost always made using mercury owing to its non-wetting properties. Equation 8 is a rearrangement of the Washburn equation derived in 1921,\textsuperscript{90} which states that the pressure required to force a non-wetting liquid into a porous space, $P$, is inversely proportional to the size, or diameter, of that space (assuming the pore is cylindrical), $D$, and directly proportional to the surface tension of the liquid, $\gamma$, and angle of contact with the solid, $\theta$.\textsuperscript{91}

$$D = -4\gamma \cos \theta P$$  \hspace{1cm} (8)

This equation was applied to intrusion measurements in 1945 by Ritter and Drake to define the concept of the intrusion of a minimum pore size under an applied pressure.\textsuperscript{89} Therefore the size of the pore is inversely proportional to the applied pressure.

A modern mercury intrusion porosimeter contains a sample cup with a capillary stem, referred to as a penetrometer, which also serves as a co-axial capacitor. All gases
and water vapors are removed from the container, leaving only the solid sample, the mercury, and the mercury vapor. The capillary tube and cup are filled with mercury prior to the application of a series of pressures to measure the intrusion of the mercury. This is demonstrated by the rise and fall of mercury in the capillary and detected by pressure transducers that produce an electrical signal proportional to the amplitude of the applied pressure. A comparison of the applied pressure and mercury volume will take the form of an intrusion curve, which shows the pore diameter (µm) versus the log differential pore volume (cm³/g). The information can be gathered and reported to show the median pore volume that will describe the approximate size of the pores. It is important to recognize that MIP measures the pores inside the polymers in the dry state. Chromatography stationary phases will be saturated, which causes polymers to be slightly swollen. Regardless of this fact, mercury intrusion still remains to be the best and most accurate approximation of the monoliths pore size.  

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.

The 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 Unimicro. 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):H₂O.

*Methacrylate Polymers*

*Methacrylate Monoliths*

The polymerization procedure for the methacrylate polymers was adapted from protocols originally developed by Svec and coworkers. 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 lengths, leaving a few centimeters of excess capillary for trimming purposes.

For the polymerization step, the desired stationary phase monomer solution was prepared in disposable black top glass vials as per Table 13, Table 14, and Table 15. All components were added drop wise by a Pasteur pipette while being measured on an analytical balance. 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.
Table 13. Poly(butyl methacrylate-co-ethylene dimethacrylate) monoliths with acrylamido-2-methylpropane-sulfonic acid (AMPS) surface functionality prepared for UV initiation with azobisisobutyronitrile (AIBN) initiator and polymerized for 4 hours. For these particular experiments the AMPS concentration was set at 0.12% w/w,\(^1\) however variations on AMPS can be made by altering the water solution.\(^2\)

<table>
<thead>
<tr>
<th></th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>70</td>
</tr>
<tr>
<td>Monomers (40%)</td>
<td></td>
</tr>
<tr>
<td>BMA (g)</td>
<td>0.60</td>
</tr>
<tr>
<td>EDM (g)</td>
<td>0.40</td>
</tr>
<tr>
<td>Porogens (60%)</td>
<td></td>
</tr>
<tr>
<td>1-Propanol (g)</td>
<td>1.05</td>
</tr>
<tr>
<td>1,4-Butanediol (g)</td>
<td>0.30</td>
</tr>
<tr>
<td>Water(^1) (g)</td>
<td>0.15</td>
</tr>
<tr>
<td>Percent 1-Propanol in Porogens</td>
<td>70%</td>
</tr>
<tr>
<td>Median Pore Volume (nm)</td>
<td>4440</td>
</tr>
</tbody>
</table>

\(^1\)The source of the water comes from a solution of AMPS (0.078 g) in water (3.8 g) for a 0.12% concentration.

\(^2\)Variations on this solution are made to increase, or decrease, the concentration of AMPS [e.g. AMPS (0.2 g) in water (3.8 g) for a 0.3% concentration.]
Table 14. Poly(butyl methacrylate-co-ethylene dimethacrylate) monoliths with acrylamido-2-methylpropane-sulfonic acid (AMPS) surface functionality prepared for UV-initiation with 2,2-dimethoxy-2-phenyl-acetophenone (DMPA) initiator and polymerized for 4 hours. Octyl methacrylate (OMA) was added to sample “75B” in order to determine the effect on median pore volume.

<table>
<thead>
<tr>
<th>Samples</th>
<th>75A</th>
<th>75B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomers (40%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMA (g)</td>
<td>0.60</td>
<td>0.40</td>
</tr>
<tr>
<td>OMA (g)</td>
<td>0.00</td>
<td>0.20</td>
</tr>
<tr>
<td>EDMA (g)</td>
<td>0.40</td>
<td>0.40</td>
</tr>
<tr>
<td>Porogens (60%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-Propanol (g)</td>
<td>1.13</td>
<td>1.13</td>
</tr>
<tr>
<td>1,4-Butanediol (g)</td>
<td>0.23</td>
<td>0.23</td>
</tr>
<tr>
<td>Water¹ (g)</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>Percent 1-Propanol in Porogens</td>
<td>75%</td>
<td>75%</td>
</tr>
<tr>
<td>Median Pore Volume (nm)</td>
<td>2550</td>
<td>8460</td>
</tr>
</tbody>
</table>

¹The source of the water comes from a solution of AMPS (0.2 g) in water (3.8 g).
Table 15. Poly(butyl methacrylate-co-ethylene dimethacrylate) monoliths with 2-acrylamido-2-methylpropane-sulfonic acid (AMPS) surface functionality prepared for UV-initiation with 2,2-dimethoxy-2-phenyl-acetophenone (DMPA) initiator and polymerized for 4 hours.

$$\text{Table 15. Poly(butyl methacrylate-co-ethylene dimethacrylate) monoliths with 2-acrylamido-2-methylpropane-sulfonic acid (AMPS) surface functionality prepared for UV-initiation with 2,2-dimethoxy-2-phenyl-acetophenone (DMPA) initiator and polymerized for 4 hours.}$$

<table>
<thead>
<tr>
<th></th>
<th>Samples</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Monomers (40%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMA (g)</td>
<td>0.60</td>
<td>0.60</td>
<td>0.60</td>
<td></td>
</tr>
<tr>
<td>EDMA (g)</td>
<td>0.40</td>
<td>0.40</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>Porogens (60%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-Decanol (g)</td>
<td>0.90</td>
<td>1.00</td>
<td>1.10</td>
<td></td>
</tr>
<tr>
<td>Cyclohexanol (g)</td>
<td>0.45</td>
<td>0.35</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>Cyclohexanol(^1) (g)</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>Percent 1-Decanol in Porogens</td>
<td>60%</td>
<td>67%</td>
<td>73%</td>
<td></td>
</tr>
<tr>
<td>Median Pore Volume (nm)</td>
<td>122</td>
<td>104</td>
<td>94.9</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)The source of the cyclohexanol comes from a solution of AMPS (0.2 g) in Cyclohexanol (3.8 g).
The capillaries were then subjected to either a hot water bath at 70°C or the UV-light box, as shown in Figure 17.

The vial of monomer solution was secured with parafilm and kept in the refrigerator for up to a week. A portion could be polymerized alongside the capillary and subjected to microscopy and pore studies. The polymers used for mercury intrusion porosimetry (MIP) and nitrogen adsorption/desorption studies were polymerized in 2 mm internal diameter pyrex rods to help simulate the capillary on a larger scale. The solutions were exposed to UV-light for approximately 1.5 hours or longer if needed. Due to the large volume of monomer solution and the make-up of the glass, the black-capped vials (product) will need to be exposed for a longer period of time. After polymerization, the glass vials were broken and the polymer was washed with methanol (Soxhlet extraction) and dried prior to intrusion studies.

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.
Figure 17. Temperature controlled UV-light box utilized for all UV-initiated monolithic materials polymerizations.
VBC/DVB Monoliths

For the preparation of the monolith, the vinylbenzyl chloride (VBC) to divinylbenzene (DVB) ratio was kept at 1:1 (v:v). As with the methacrylate monoliths, the ratio of monomer to porogens was 4:6 (v:v) with the porogen being a combination of one or two small, solvent molecules. Table 16 shows the preparations of the columns made for our study. For these experiments AIBN was added in a ratio of 0.1% (w/v) of the total solution and the polymerization was then thermally induced. The final mixture was then purged with nitrogen through the desired capillary and when the gas was turned off, capillary action filled the column with monomer solution. When the desired length was achieved, the ends were sealed with septa and placed in a water bath at varying times and temperatures. After polymerization, the column was purged with nitrogen, to attempt to remove any volatiles, rinsed with ACN, and then rinsed with the desired buffer. Some of the columns were surface treated with N,N-dimethyloctylamine in an attempt to functionalize the chloromethyl groups on the polymer. Similarly, a bulk solution of the monomers was reacted in black vials and characterized using mercury intrusion porosimetry.
Table 16. Vinylbenzyl chloride (VBC)/divinylbenzene (DVB) monoliths (polymerized at 65°C for 3 hours and 75°C for 24 hours) prepared by thermal initiation with azobisisobutyronitrile (AIBN) initiator. Attempts were made to treat columns with N,N-dimethyloctylamine as a functionalization agent to generate EOF.

<table>
<thead>
<tr>
<th>Samples</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Monomers</strong> (40%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VBC (mL)</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>DVB (mL)</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><strong>Porogens</strong> (60%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-Propanol (mL)</td>
<td>5.7</td>
<td>5.4</td>
<td>4.5</td>
</tr>
<tr>
<td>Formamide (mL)</td>
<td>0.3</td>
<td>0.6</td>
<td>1.5</td>
</tr>
<tr>
<td>Percent 1-Propanol in Porogens</td>
<td>95%</td>
<td>90%</td>
<td>75%</td>
</tr>
<tr>
<td>Median Pore Volume (nm)</td>
<td>5240</td>
<td>5000</td>
<td>2890</td>
</tr>
</tbody>
</table>
Results and Discussion

Silica Columns

CEC Hypersil C18

Open capillary 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. Examples of improper and proper electropherogram results from using the conditioning method are provided in Figure 18. 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.98 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:H₂O. When studied using a C18 column, the best results were achieved with a 4:3:3 (v:v:v) ACN:MES:H₂O buffer.
Figure 18. Demonstration of (A) improper and (B) proper generation of a stable current and EOF using the column conditioning program necessary prior to performing CEC.
Using these conditions, the method was capable of separating 4 barbiturates along with an additional 3 benzodiazepines, as shown in Figure 19. 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. Although not the particular focus of the current research, further studies with a gradient and more drugs of the same classes might be interesting to use with this buffer system. 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. Also, additional experiments should be performed using interfering compounds similar to the runs performed during the date-rape drug study as described in Chapter 2.

**Monolithic Polymer Columns**

**Column Preparation**

Column preparation procedures were adapted from Svec et al., Horvath et al. used a more complex conditioning step regarding the 3-(trimethoxysilyl)propyl methacrylate by noting the optimal condition under which the trimethoxysilyl group reacted with the silanols at high temperatures. Unfortunately, at such optimial temperatures, polymerization of the vinyl groups occured. An inhibitor, 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH), was added at the amount of 0.01% (w/v) to a 50% (v/v) solution of the silanizing agent in dimethylformamide (DMF).
Figure 19. 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. After this research group reviewed scanning electron micrograph (SEM) images of columns made with and without reactions containing DPPH, gaps between the capillary wall and the polymer clearly appeared in non-inhibited columns. Even though work in this chapter was continued using the original surface modification procedure, further examination of this alternative method is necessary. To find the best method, a comparison should be made between the 20% in acetone silanizing procedure currently being used with the 50% in DMF inhibited procedure outline above.

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. 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. A SEM image of the 75% 1-propanol poly(butyl methacrylate-co-ethylene dimethacrylate) monolith sample “75A” preparation outlined in Table 2 is shown in Figure 20.

Chromatographic Results

The initial monolith experiments were performed with materials, as described in Table 13. Separations of aryl benzene compounds were generated in order to test the column performance, shown in Figure 21, using a 25 cm effective length poly(butyl methacrylate-co-ethylene dimethacrylate) (BMA/EDMA) monoliths with 0.3% AMPS and 65% 1-propanol 25% 1,4-butanediol, 10% water porogens. The separation was performed using an 8:2 acetonitrile to 5 mM sodium phosphate pH=7 buffer. While the compounds were completely resolved, sample overloading was the cause of such broad peaks.
Figure 20. SEM image of poly(butyl methacrylate-co-ethylene dimethacrylate) monolith sample “75A” prepared as described in Table 14.
Figure 21. Separation of aryl benzenes on a 25 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 using a ratio of 8:2 (v:v) acetonitrile to 5 mM sodium phosphate buffer at pH=7. The samples were injected for 5 seconds at 5 kV and separated using 20 kV with a 2 bar pressure on both the inlet and outlet electrodes. (A) Pyridine; (B) Benzene; (C) Chlorobenzene.
Little selectivity was demonstrated by the methcrylate column for the separation of 3 benzodiazepines, as shown in Figure 22. 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 pH=7. The extreme change in run time in run Figure 22 (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, demonstrating the lack of column selectivity under these conditions.

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 owing to its properties it might be useful as solid phase extraction media. A graph of the pore size studies, data given in Table 13 with 0.12\% AMPS, is shown in Figure 23. The pore size was not linear with percent 1-propanol; however this could be used as a rough estimate regarding what sizes to expect for future preparations.
Figure 22. 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 next series of monomer mixtures were made in response to the lack of sensitivity exhibited on the previous column. While reproducible chromatographic results were not obtained, pore size measurements were taken to assess the potential of the phases and provide useful size data. Table 14 outlines a second methacrylate mixture that was made with the addition of octyl methacrylate (OMA). The preparation was made concurrently with an identical sample omitting the OMA to determine the affect on pore size. OMA was chosen because of the long chain of carbons that might hopefully demonstrate chromatographic properties similar to C18 silica material. Results showed an extreme difference in the pore sizes of these materials with the replacement of 0.2% BMA with OMA. The 75% 1-propanol columns without the octyl compound resulted in a median pore volume of 2550 nm and while the OMA containing polymer had a pore size of 8460 nm.

Table 15 outlines the preparation for a UV-initiated methacrylate polymer that only makes use of two porogens, cyclohexanol and 1-decanol. In this mixture, the AMPS functionality was induced in order to dissolve the polymer in cyclohexanol using additional heat, instead of dissolving it in water at room temperature as in previous experiments. These new changes altered the morphology of the methacrylates and created an improved stationary matrix. Test separations with thiourea (neutral marker) and toluene yielded migration times of 0.9 and 1.7 minutes, respectively, employing polymers prepared as in Table 15. The migration time of thiourea increased to 7.3 minutes while toluene’s migration time was 21.5 minutes using the new porogen system at 65% 1-decanol.
Figure 23. Mercury intrusion studies performed on poly (butyl methacrylate-co-ethylene dimethacrylate) with polymers 0.12 wt. % 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) prepared as per Table 13. This graph was prepared in Microsoft Excel and demonstrates the effects of varying the percent of 1-propanol in the porogen mixture on the pore size.
These improvements were reflected in the pore size data where the median pore volume was reduced to 95 nm at 73% 1-decanol. The new retention may be the result of increased interactions with analytes of interest because of these smaller pore sizes.

**VBC/DVB**

Horváth et al. characterized monoliths made of polystyrene (PS) with either divinylbenzene (DVB) or vinylbenzyl chloride (VBC). A variety of porogens were used, however in order to make a column suitable to generate sufficient EOF, the surface was functionalized with N,N-dimethyloctylamine. While the functionalization resulted in no apparent EOF, the results from the pore size measurements are provided along with the preparation in Table 16.

**Conclusions**

One of the severe drawbacks to the above monolithic procedures was the lack of uniformity of the resulting polymers. Not only was there variability in pore sizes of the same preparations, there were differences in column reproducibility. Despite the ease of the procedure, column-making was both materials and time-consuming when the finished column was not be porous enough for proper flow, or functionality rich enough for suitable EOF. At times, the pore size distribution was often too large to accommodate the rinsing pressures needed for a working column. Moreover, when attempting to troubleshoot column preparations with new functional groups such as OMA, reproducibility was a necessity, so the high-rate of column failing was disappointing.

An increase in silica stationary phase CEC research has resulted in some favorable results. For example Bailey et al. performed a direct comparison of an indirect
laser induced fluorescence detection of explosives by both MECC and CEC.\textsuperscript{102} While both techniques exhibited fluctuations in the background caused by thermal instabilities, the problem was much greater in MECC. This was the result of the larger separation currents in MECC that resulted from the, highly conductive run buffers necessary to achieve separation. Another useful comparison was the chromatographic differences in open-tubular versus packed capillary CEC. A study on benzodiazepines was performed by Jinno et al. using a modified cholesteryl bonded silica column.\textsuperscript{103} Faster run times and increased resolution was obtained for the packed column when the drugs were neutral.

The separation of barbiturates and benzodiazepines on C18 silica yielded interesting results and would be worth pursuing. While the chromatographic results of the CEC monolithic columns were slightly disappointing due to the lack of selectivity, gaining knowledge of the materials through pore size measurements and highly structured experiments will prove useful for future experiments.

**Future Work**

One way to minimize the variations in pore-size is to create a polymer with pores that will always offer the same properties, similar to the properties of silica when tightly packed, yet still offer the advantage of in situ preparation. Chirica and Remcho prepared a series of monolithic phases using dissolved silica to create pores.\textsuperscript{104} However, with the silica beads still requiring the same type of packing protocols as traditional columns, not much is gained in the way of preparation simplicity in comparison to traditional packing procedures. Additionally, with no regulation of how the end polymer is distributed
throughout the mixture, pore uniformity might not always be better than the above described monoliths.

One solution may be in finding a mixture composed entirely of monomers that can be polymerized and then treated. A solid polymer can generate a porous polymer if the temperature is raised above the glass transition temperature (Tg) of one of the monomers, yet below the Tg of the second monomer. This ultimately causes the polymer with the lower Tg to melt and be rinsed away. Work performed by Hillmyer and coworkers demonstrates this principle.\textsuperscript{105,106} This laboratory is constructing polystyrene (PS)-polylactide (PLA) block copolymers. After formation of the monomer, the material is subjected to mild degradation conditions to chemically etch the PLA out of the matrix. This product is then subjected to a bulk shear in order to align the polymer structures into rod like structures.
Chapter 5: Microfluidic Drug Detection Based on Commercial Chips and Cy-5 Dye Applications

Background

Capillary Electrophoresis on a Chip

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. While the field of DNA analysis has seen respectable progress, advances in small molecule drug detection have been limited.

Even though micromachining has been applied to gas and liquid chromatography instrumentation, electrophoretic separations are much more suited to downsizing, owing to the lack of carrier gases and the ease of moving solutions using electroosmotic driven pumping. 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 electrotrodes. While commercial markets have been focused in other areas such as proteomics and genomics, the potential of CE for forensic analysis is an attractive idea. 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.
There are a number of important issues to be solved in developing microfluidic approaches to chemical analysis. This chapter will present ways to detect small drug molecules on a chip, while Chapter 6 will provide information on lithography techniques and protocols for in-house development of glass and poly(dimethylsiloxane) (PDMS) devices.

Detection Methods

Basic and acidic drug methods that were developed for traditional CE instrumentation,\textsuperscript{33} 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.\textsuperscript{114-119} Another advantage of optical on-chip detection is its increase in sensitivity and selectivity when compared to UV detection.\textsuperscript{116} 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. 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. 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.

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. 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.

$$C_{LOD} = \frac{C_m}{(TR \times DR)}$$
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.\textsuperscript{102,115,119,123} 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 fluorescence. 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.\textsuperscript{102,124} Since several of the more potent benzodiazepines involved in DFSA (e.g. flunitrazepam and clonazepam, as shown in Figure 24) 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. The micelle used in this method, SDS, was the same as that used in Chapter 2 for the benzodiazepine beverage study. 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.\textsuperscript{125}

In this study, Cy5 N-hydroxysuccinimide ester was employed as the visualizing fluorophore (Figure 25). Because the quantum yield of Cy dyes are lower inside the micelle,\textsuperscript{102} 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.\textsuperscript{102,123,126}
Figure 24. Nitrated Benzodiazepines used for the indirect chip method using Cy5 dye (A) clonazepam; (B) desmethyflunitrazepam; (C) flunitrazepam; (D) nitrazepam; and an explosive compound used for the internal standard (E) 2,4-dinitrotoluene.
Figure 25. Structure of Cy5 Mono NHS Ester (M.W. = 792 g/mol).
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. As described in Chapter 2, an organic modifier was used to aid in the micellar separation of common DFSA drugs. Organic modifiers 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 dodecyl 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 26, 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 26. 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. 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 HNO₃ 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. 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.
Liquid-liquid Extraction

A liquid-liquid extraction method developed the date-rape drug study from Chapter 2 was utilized.127 To 1 mL of a water standard containing 10 µg/mL of each of the 4 benzodiazepines, 100 µL of buffered phosphate was added followed by extracting the sample two times with 0.5 mL ethyl acetate. The organic layer was dried under a stream of nitrogen and then reconstituted in 100.0 µL of buffer containing 2,4-DNT as an internal standard.

Solid Phase Extraction (SPE)

The solid phase extraction method was an altered version of the Benzodiazepines in Serum or Plasma method.129 Columns 1210-2051 were prepared by drawing portions of methanol, deionized water and 100 mM phosphate buffer pH 6.0 followed by loading the buffer adjusted sample at 1 mL/min. The column was rinsed with 3 mL of water, 1 mL of 1.0 M acetic acid, 3 mL of methanol and dried for 5 minutes at a pressure of greater than 10 inches Hg. The benzodiazepines were then eluted with 2 mL CH₂Cl₂/IPA/NH₄OH (78/20/2) (v/v/v) at 1 mL/min. The eluent was then evaporated to dryness with N₂ and reconstituted in the appropriate amount of buffer.

Results and Discussion

A separation of a series of explosives is shown and described in Figure 27. In previous studies different concentrations of borate were (continued on page 133)
Figure 27. The optimized 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 (∆G) 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 ∆E_{F−F^+} is the lowest singlet-singlet excitation energy of the fluorophore.

\[
\Delta G = E\left(\frac{F}{F^{+}}\right) - \Delta E_{F−F^{+}} - E\left(\frac{Q}{Q^{-}}\right)
\] (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, as shown in Figure 28, in order to find the most favorable buffer for benzodiazepine detection on a chip. In Figure 29, the resolution of flunitrazepam and clonazepam was determined for each of the different solvent combinations in order to confirm the best conditions for the two drugs.
Figure 28. The mobility of benzodiazepines with respect to percent organic modifier. The buffer consisted of 15 mM SDS/15 mM boric acid/3 mM sodium tetraborate/2.6 μM Cy5. The effective length from the channel intersection to the detector was set at 45 mm while the voltage was varied for these three sets of modifier studies. (A) 370.9 V/cm; (B) 370.9 V/cm; (C) 494.5 V/cm.
Figure 29. Resolution of flunitrazepam and clonazepam with adjusted concentrations of organic modifier. The conditions are the same as in Figure 28.
A standard containing 250 µg/mL of the four benzodiazepines and 25 µg/mL of 2,4-DNT was utilized in the optimization studies and the resultant mobilities are shown in Figure 30. While both acetonitrile and methanol exhibited similar profiles at 20%, methanol was chosen as the organic modifier because it yielded sharper analyte peaks and a smaller system peak.

Calibration curves were generated for the four benzodiazepines ranging from 50 µg/mL to 250 µg/mL using the buffer containing a methanol concentration of 20%. The calibration data and detection limits are shown in Table 17. This method was most sensitive for flunitrazepam at 7.86 µg/mL. Run to run variations in the results were also compared for three days in Table 18.

In order to simulate toxicological samples, extractions were performed on water standards to optimize pre-treatment techniques and establish the usefulness of this method. The liquid-liquid procedure used in our previous work yielded recoveries ranging between 79% for flunitrazepam to 88% for clonazepam as shown in Table 19. While a solid phase procedure was attempted as well, recoveries were highly variable ranging from 32-104% for clonazepam and flunitrazepam, respectively (Table 20). While SPE is a cleaner extraction procedure, more reliable results were obtained with the simple one-step ethyl acetate extraction.
Figure 30. 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) desmethyflunitrazepam; (c) flunitrazepam; (d) nitrazepam; (e) clonazepam.
Table 17. Calibration data for 4 benzodiazepines.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Compound</th>
<th>Slope\textsuperscript{b} (mAU s mL mg\textsuperscript{-1})</th>
<th>R\textsuperscript{2}</th>
<th>Detection Limit\textsuperscript{c} (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desmethylflunitrazepam</td>
<td>0.0044 ± 0.0005</td>
<td>0.9620</td>
<td>66.7</td>
</tr>
<tr>
<td>Flunitrazepam</td>
<td>0.00391 ± 0.00005</td>
<td>0.9995</td>
<td>7.86</td>
</tr>
<tr>
<td>Nitrazepam</td>
<td>0.0037 ± 0.0002</td>
<td>0.9901</td>
<td>33.5</td>
</tr>
<tr>
<td>Clonazepam</td>
<td>0.0028 ± 0.0004</td>
<td>0.9490</td>
<td>77.7</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Conditions were the same as in Figure 30.
\textsuperscript{b}Slope and standard deviation were calculated using Microsoft Excel.
\textsuperscript{c}Detection limits were calculated using Microsoft Excel linear estimation.
Table 18. Day to day reproducibility of 4 benzodiazepines and the Internal Standard 2,4-DNT.\(^a\)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Day 1 Mobility (% RSD)</th>
<th>Day 2 Mobility (% RSD)</th>
<th>Day 3 Mobility (% RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Internal Standard (IS)</td>
<td>0.64</td>
<td>0.60</td>
<td>1.3</td>
</tr>
<tr>
<td>Desmethylflunitrazepam</td>
<td>0.89</td>
<td>0.82</td>
<td>1.4</td>
</tr>
<tr>
<td>Flunitrazepam</td>
<td>0.96</td>
<td>0.85</td>
<td>1.4</td>
</tr>
<tr>
<td>Nitrazepam</td>
<td>1.1</td>
<td>0.86</td>
<td>1.1</td>
</tr>
<tr>
<td>Clonazepam</td>
<td>1.2</td>
<td>0.92</td>
<td>1.4</td>
</tr>
</tbody>
</table>

\(^a\)The conditions were the same as in Figure 30.
\(^b\)Percent relative standard deviation based on \(n=4\).
Table 19. Percent recoveries for the liquid-liquid extraction.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Compound</th>
<th>Percent Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desmethylflunitrazepam</td>
<td>81 ± 4</td>
</tr>
<tr>
<td>Flunitrazepam</td>
<td>79 ± 4</td>
</tr>
<tr>
<td>Nitrazepam</td>
<td>86 ± 4</td>
</tr>
<tr>
<td>Clonazepam</td>
<td>88 ± 4</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Conditions were the same as in Figure 30.
Table 20. Percent recovery for the solid phase extraction.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Compound</th>
<th>Percent Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desmethyflunitrazepam</td>
<td>50 ± 10</td>
</tr>
<tr>
<td>Flunitrazepam</td>
<td>104 ± 1</td>
</tr>
<tr>
<td>Nitrazepam</td>
<td>44 ± 4</td>
</tr>
<tr>
<td>Clonazepam</td>
<td>32 ± 9</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Conditions were the same as in Figure 30.
Conclusion

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.

Towards Direct Detection of Amines via microfluidic devices

After the initial optimization work was completed using Cy5 dye with the Micralyne µ-TK for the indirect detection of benzodiazepines, further applications were investigated. Because of the N-hydroxysuccinimide (NHS) group’s ability to directly derivatize amines, a method using a Cy5 mono-NHS ester dye to label primary and secondary amines was developed on traditional instrumentation to be evaluated for its usefulness for microfluidics.

To date, there has only been one forensic example of piperazines separation by capillary electrophoresis. The chiral capillary electrophoresis method developed by our laboratory separates 14 piperazine and amphetamine compounds in 23 minutes. The motivation behind creating a Cy5 derivatization for these amine containing compounds would be to ultimately adapt this separation to the µ-TK for a faster analysis. While this
Cy5 study was focused upon a group of designer drugs, with further buffer optimization this technique could be applied to a wide variety of amine containing compounds.

Experimental

Chemicals and Reagents

Cy5 monoreactive N-hydroxysuccinimide (NHS) ester dye (M.W. = 792 g/mol) was obtained from Amersham Biosciences (Piscataway, NJ). Benzodiazepines were obtained from sources discussed previously with 7-amino- flunitrazepam and 7-amino-clonazepam also purchased from Lipomed (Cambridge, MA). All piperazines and amphetamines were obtained and prepared as stated in Chapter 3. Dimethylsulfoxide (DMSO) (99.9% purity) was obtained from Sigma (St. Louis, MO). Sodium dodecyl sulfate was obtained from Fisher Scientific (Pittsburgh, PA) and native β-cyclodextrin was obtained from TCI America (Portland, OR).

Instrumentation and Conditions

The Beckman P/ACE System 2050 (Fullerton, CA) with the Beckman P/ACE LIF Detector and Beckman 635 Laser Module was used to analyze all experiments. The laser excitation was set at 635 nm and the emission of the Cy5 labeled drug was 670 nm. Samples were injected for 5 sec at low pressure with a separation voltage of 20 kV. The total capillary length (Polymicro Technologies, Phoenix, AZ) was 47 cm for the buffer studies and 77 cm for the derivatization studies, both using 50 µm ID capillary.
Buffer and Sample Preparation

All blank samples and derivatized drugs were analyzed using a micellar electrokinetic capillary electrophoretic (MECC) buffer with laser induced fluorescence detection (LIF). The borate-SDS buffer was established using a stock buffer previous used for an indirect Cy5 containing buffer for the detection of nitrated benzodiazepines developed in our laboratory. The stock buffer for this study was prepared omitting the Cy5 dye buffer additive and diluted 1:4 for these experiments. The optimized buffer consisted of 7.5 mM SDS, 7.5 mM boric acid and 1.5 mM sodium borate buffer.

The amine-type drugs used for this study were 1-benzylpiperazine (BZP), 1-(3-trifluoromethylphenyl)-piperazine (TFMPP), 1-(3-chlorophenyl)-piperazine (mCPP), amphetamine, methamphetamine, ephedrine, and phenylpropanolamine (PPA). Additionally, because of their potential as DFSA drugs, a variety of benzodiazepines were also analyzed. These included flunitrazepam, 7-aminoflunitrazepam, 7-aminoclonazepam, lorazepam, and chlordiazepoxide. Other miscellaneous drugs utilized in this study were phenobarbital, theophylline, metoprolol and morphine. Both amine and non-amine containing compounds were studied to not only to evaluate the success of the derivatization, but to also rule out the appearance of any false positive peaks.

Derivatization reaction

The amine derivatization procedure was provided by Amersham Biosciences. This derivatization occurs when the carboxylic functional group on the Cy5 dye undergoes nucleophilic attack by the amino group of the drug as shown in Figure 31. In these experiments, 1.0 mg Cy5 monoreactive NHS ester was dissolved in 1.26 mL of
DMSO to produce a 1 mM dye solution. This stock solution was stored in the dark at –15 °C in order to avoid photolytic and thermal decomposition.

A 1 mg/mL MeOH stock solution of drug was diluted to a concentration of 1 µg/mL with DMSO. In order to achieve a 1:1 starting ratio of dye to drug, to 100 µL drug solution, an equivalent molar amount of dye solution was added for a final concentration of 10 µM (Table 21). Triethylamine was diluted 1:100 with DMSO and added to the solution and the reaction was agitated over-night in the dark at ambient temperature. Prior to MECC analysis, the samples were diluted 1:5 with DMSO.

**Buffer optimization**

The buffer was initially optimized using derivatized amphetamine, BZP, and ephedrine as the analytes in a borate buffer with different concentrations of either β-cyclodextrin (β-CD) or sodium dodecylsulfate (SDS) to provide the secondary mode of separation. A stock buffer of 30 mM boric acid and 6 mM sodium borate was prepared and varied as shown in Table 22 for different concentrations of SDS and for different concentration of β-CD.
Figure 31. Derivatization of Cy5 dye with an amine drug to give the labeled drug.
Table 21. Amount of dye added to each drug solution.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Added dye solution (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-Amino-clonazepam</td>
<td>35</td>
</tr>
<tr>
<td>7-Amino-flunitrazepam</td>
<td>35</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>74</td>
</tr>
<tr>
<td>BZP</td>
<td>57</td>
</tr>
<tr>
<td>Chlordiazepoxide</td>
<td>33</td>
</tr>
<tr>
<td>Ephedrine</td>
<td>61</td>
</tr>
<tr>
<td>Flunitrazepam</td>
<td>32</td>
</tr>
<tr>
<td>Lorazepam</td>
<td>31</td>
</tr>
<tr>
<td>mCPP</td>
<td>51</td>
</tr>
<tr>
<td>Methamphetamine</td>
<td>67</td>
</tr>
<tr>
<td>Metoprolol</td>
<td>37</td>
</tr>
<tr>
<td>Morphine</td>
<td>35</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>43</td>
</tr>
<tr>
<td>Phenylpropanolamine</td>
<td>66</td>
</tr>
<tr>
<td>TFMPP</td>
<td>43</td>
</tr>
<tr>
<td>Theophylline</td>
<td>56</td>
</tr>
</tbody>
</table>
Table 22. Buffer concentrations for the optimization study using a standard of amphetamine, benypiperazine and ephedrine.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Boric Acid (mM)</th>
<th>Sodium Borate (mM)</th>
<th>SDS (mM)</th>
<th>β-CD (mM)</th>
<th>Current (µA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>1.5</td>
<td>7.5</td>
<td>0</td>
<td>9.0</td>
</tr>
<tr>
<td>7.5</td>
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<td>0</td>
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<td>5.9</td>
</tr>
<tr>
<td>7.5</td>
<td>1.5</td>
<td>5.0</td>
<td>0</td>
<td>8.7</td>
</tr>
<tr>
<td>7.5</td>
<td>1.5</td>
<td>7.5</td>
<td>0</td>
<td>9.0</td>
</tr>
<tr>
<td>7.5</td>
<td>1.5</td>
<td>10.0</td>
<td>0</td>
<td>9.7</td>
</tr>
<tr>
<td>7.5</td>
<td>1.5</td>
<td>15.0</td>
<td>0</td>
<td>10.8</td>
</tr>
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<td>1.5</td>
<td>0</td>
<td>7.5</td>
<td>5.6</td>
</tr>
<tr>
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<td>1.5</td>
<td>0</td>
<td>7.5</td>
<td>8.3</td>
</tr>
<tr>
<td>10.0</td>
<td>1.5</td>
<td>7.5</td>
<td>0</td>
<td>9.2</td>
</tr>
<tr>
<td>15.0</td>
<td>3.0</td>
<td>15.0</td>
<td>0</td>
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<td>9.8</td>
</tr>
<tr>
<td>15.0</td>
<td>3.0</td>
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<tr>
<td>30.0</td>
<td>6.0</td>
<td>30.0</td>
<td>0</td>
<td>26.7</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Sample injections 5 sec at low pressure with a separation voltage of 20 kV. The total capillary length was 47 cm with a 50 µm internal diameter.
Discussion

Cy5 monoreactive NHS ester

The Cy5 monoreactive NHS ester dissolved in DMSO was analyzed with the optimized buffer on a 47 cm capillary using a 5 second pressure injection, and a separation voltage of 20 kV. Shown in Figure 32, the Cy5 monoreactive NHS dye peak was determined to have eluted at 5.9 min. By using the Beckman Cy5 test dye, which was reconstituted in water, the peak at 4.8 min was determined to be the result of a non-reactive Cy5 hydrolysis product, thus explaining the location of the NHS dye peak. Cy5 dye is easily decomposed to its hydrolysis product after reconstitution and is subject to further hydrolysis as depicted in Figure 33. This is observed throughout these experiments by an increase in the peak intensity at 4.8 min. over time. Since the hydrolysis reaction appeared to occur rapidly in DMSO, future studies investigating more suitable solvents, such as dimethylformamide or acetonitrile, which reduce the occurrence of Cy5-hydrolysis would be beneficial.
Figure 32. Electropherogram of Cy5 NHS ester. Conditions are the same as in Table 22.
Figure 33. Hydrolysis of Cy5 NHS ester.
Buffer optimization

The same SDS/borate buffer that was used for the indirect detection of benzodiazepines was chosen for these experiments because Cy5 has proven to be stable under those conditions.\(^{133}\) Cyclodextrins were also evaluated due to their utility in previous CE separations of piperazines. Figure 34 demonstrates the migration results when varying the concentration of SDS while detecting amphetamine. Separation from the Cy5 peaks is not achieved until a concentration of 7.5 mM SDS or greater.

Derivatization of Amine-containing Drugs

The derivatization reaction was optimized by performing temperature and time variations to the original procedure.\(^{132}\) In order to achieve baseline separation of closely eluting compounds, a 77 cm capillary was used to analyze the derivatized amine drugs. The Cy5 labeled compounds were diluted 1:5 and analyzed using a 7.5 mM boric acid/1.5 mM sodium borate/7.5 mM SDS buffer that resulted in a current of approximately 5.7 \(\mu\)A. The amount of dye added to each drug solution is described in Table 21. The optimized separation of amphetamine, BZP, TFMPP, chlordiazepoxide, and mCPP is shown in Figure 35, which was performed with a 5 second pressure injection and a separation voltage of 20 kV. Further optimization will be needed for a more resolved separation of the closely eluting piperazine compounds.
Figure 34. Electropherograms of amphetamine in different buffer concentrations. (A) 0 mM SDS; (B) 3 mM SDS; (C) 7.5 mM SDS; (D) 10 mM SDS; (E) 15 mM SDS.
Figure 35. Electropherogram of amphetamine, BZP, TFMPP, chlordiazepoxide, and mCPP. Conditions are the same as in Table 22 with a 77 cm capillary.
To increase the rate of the reaction, derivatization was attempted at 50 °C and 60 °C. The reactions were agitated for 1, 2, 4, and 5 hours. An extended agitation (35 hours) was also performed to ensure that most of the dye reacted with the drug. After agitating the samples for 1 h or 2 h at 60 °C, amphetamine and BZP will give small peaks. If the sample is heated for a longer period of time, the BZP peak will disappear. This can be attributed to thermal decomposition of the drug, the dye, or the drug-dye-complex could be occurring. An additional consequence of heat was an increase in the amount of the Cy5 hydrolysis product, as compared to the Cy5 monoreactive NHS ester. If the reaction was allowed to run overnight at ambient temperature, the Cy5 hydrolysis peak is not as pronounced as in the higher temperature reactions.

When the samples are allowed to agitate for more than 20 hours at room temperature, drug peaks increased owing to the fact that more dye will react with the drugs. Peaks for secondary amines such as ephedrine, methamphetamine, metoprolol, theophylline, and phenobarbital could not be identified using this buffer system. The proton at the secondary nitrogen of ephedrine, methamphetamine, metoprolol, lorazepam, and theophylline is not sufficiently acidic to react with the NHS group. Heating the reaction mixture was not successful in improving reactivity. In addition to the previously listed secondary amines, primary amines, such as 7-amino-flunitrazepam, 7-amino-clonazepam, and PPA, do not show any signal. An additional factor that may contribute to the lack of signal for these types of compounds is a steric one. Since 7-amino-flunitrazepam and 7-amino-clonazepam are large molecules, a steric interaction may occur preventing reaction with the dye. Thus, no signal is observed. The negative
controls, morphine and flunitrazepam, did not react, as expected, because they are tertiary amines.

It was ultimately determined that the optimized drug derivatization did in fact involve agitating the samples overnight at ambient temperature. Even though all of the dye may not be able to react with the drug, speed of the overall analysis is always a concern. Since some of the drugs are detectable, speed was chosen over increase sensitivity to analysis time.

Buffer and capillary studies

The buffer used in the analysis of the Cy5 labeled drugs, such as amphetamine, TFMPP, BZP, chlordiazepoxide, and mCPP, was a 7.5 mM SDS/7.5 mM boric acid/1.5 mM sodium borate buffer. When using β-CD instead of SDS, no peaks could be identified. The peaks were broad and no separation was created. Figure 34 illustrate the effect of SDS on the separation of amphetamine. If the concentration of SDS was lower than 7.5 mM, the retention time was decreased and no separation was observed. With increased SDS concentration, up to 15 mM SDS, elution time increased while resolution decreased. The variation of boric acid showed little effect on the electropherogram except for a broadening of peak shape.

It was possible to separate amphetamine from BZP or TFMPP with the 7.5 mM SDS buffer. However, complete separation of all compounds was not achieved due to co-eluting BZP and TFMPP. In order to improve separation, the capillary length was varied. The tested total lengths were 47 cm, 57 cm, and 77 cm. Though BZP and
TFMPP could not be resolved using these capillary lengths, separation of amphetamine from BZP/TFMPP was improved using the 77 cm capillary. Figures 11 to 15 show the electropherograms of amphetamine, TFMPP, BZP, chlordiazepoxide, and 1-(3-chlorophenyl)-piperazine using a 77 cm capillary with 7.5 mM SDS buffer (No.3), 5s pressure injection, and 20 kV voltage.

Due to the structural similarities between BZP, TFMPP, and mCPP, separation of these compounds was not achieved. When injected separately, all three piperazines resulted in peaks with similar retention time. Likewise, when two piperazines, such as BZP and TFMPP, are injected, both co-elute as one signal.

Conclusions

The established method using a 7.5 mM SDS/7.5 mM boric acid/1.5 mM sodium borate buffer was found suitable to separate several Cy5 dye labeled drugs of different structure types, including amphetamines, benzodiazepines, and piperazines. It was shown that the derivatization reaction works with primary amines, for instance amphetamine, and secondary amines, such as BZP, TFMPP, mCPP, and chlordiazepoxide. With the above buffer system, no signals were observed for other tested drugs, methamphetamine, ephedrine, cathine, lorazepam, 7-amino-clonazepam, 7-amino-flunitrazepam, phenobarbital, theophylline and metoprolol. Since they are primary amines, it was expected that PPA, 7-aminoflunitrazepam, and 7-amino-clonazepan would react with the Cy5. A rationale based on steric considerations may explain the reason for this phenomenon. Flunitrazepam and morphine were not detected since Cy5 does not react
with tertiary amines. In order to complete the separation of the drugs listed above, future work need to focus on the development of a new buffer system.
Chapter 6: Microfluidic Device Fabrication

Background

The first well-known commercially marketed microelectromechanical system (MEMS) was the sensor used to detect rapid automobile deceleration in order to trigger the deployment of the airbag. Since then, miniaturized devices have found applications in many scientific disciplines. Implantable systems for in vivo disease monitoring, for DNA genotyping and for combinatorial synthesis, have all been produced using miniaturization techniques. A number of systems for chemical analysis have also been developed including miniaturized gas chromatography equipment and electrochemical sensors.

Manz and Becker first introduced the concept of the micro total analytical system (µ-TAS) in 1989, which was also referred to as a lab-on-a-chip. These devices commonly involved liquid-based separations developed on miniaturized instruments fabricated from glass and polymer wafers. The perfect candidate for such separations was argued by many to be capillary electrophoresis, a concept that was initially described in 1992. Electrophoretic channels on the substrate were produced by lithographic techniques. Liquid samples in these devices were then manipulated by altering the voltage in the channels that were etched into the chip. Mechanical pumps were not required; instead electroosmotic pumping was utilized to inject and mix samples.

There are a number of ways the forensic community may benefit from the application of microfluidic separation devices. First and foremost, inexpensive,
disposable devices would help limit sample carry-over and contamination. Disposable devices also improve throughput and permit easy tracking of evidence through techniques, such as bar coding. Many quick-screen colorimetric and immunoassay based kits have already moved in that direction; however these techniques are only used for qualitative analysis. These kits can also fail to provide a sufficient limit of detection or lead to false positives when cross-reactivity occurs. A semi-portable, inexpensive, single-use “mini-instrument” that can extract, separate, and identify compounds of interest from a complex matrix to offer quantitative data would be in great demand. This is the future of CE and CEC on a chip.

Fabrication Procedures

The trend towards micromachining CE devices resulted in the development of separation-based microfluidic fabrication technology. The same techniques that are first developed to construct semiconductor chips can be used to create micro-total analytical systems (µ-TAS). Two popular mediums for the fabrication of devices are glass and plastic. Glass devices provide the most uniform surface and the most similar environment when compared to traditionally capillaries, though their production is quite tedious.

The fabrication principle is known as lithography, which is defined as the transfer of a pattern from a mask to a substrate. A photolithographic mask must first be made, which is essentially a stencil of the desired pattern for the device. The mask itself is made out of a chrome-clad glass plate coated with an electron-beam sensitive resist.
Electron beam lithography (EBL) involves the use of direct writing to expose one pixel of the mask at a time to an electron beam, thus generating high-resolution patterns. Unlike optical lithography, EBL is not limited by the wavelength of the exposure light, which results in its greater resolution. The chrome resist utilized on the device then becomes soluble after treatment with the beam. It can then be washed away in the development process to generate patterns that are used in the image transfer process.

Before beginning the image transfer, the glass substrate must first be coated in a similar manner to the chrome mask using a photoresist, which is a chemical that becomes altered upon exposure to UV-light. Depending on its chemical composition, the resist can become soluble or insoluble in the development solution, after UV treatment. For glass devices, a positive photoresist is used such that exposed areas become soluble. The mask is then vacuum-sealed to the glass substrate and exposed using a contact printer. Following post-image development, a relief is imparted on the surface of the substrate. In this case, portions of the substrate still covered in resist are protected against wet chemical etchants that ultimately carve the desired pattern into the glass. Often, prior to the application of resists, thin deposits of metal are used to enhance the durability of the protected surface. Addition of metal may be necessary to shield the glass for deep pattern etching with strongly acidic variations of a buffered oxide etch, as in the case of CE channel generation.

A top plate containing reservoir holes is then bonded to the etched substrate. These holes permit the buffers, samples, and electrodes to access the etched channels in the completed chip. Two bonding procedures, high temperature furnace bonding and
room temperature bonding, have been implemented to achieve the most secure sandwiched device.\textsuperscript{140,141}

An alternative procedure, called soft lithography, which is also known as rapid prototyping or replica molding, is used to fabricate poly(dimethylsiloxane) (PDMS) chips.\textsuperscript{139,142-145} This technique makes use of a so-called “master” positive relief design that is used to cast a negative PDMS device. The master is made using a glass substrate coated with a negative photoresist that remains affixed to the substrate in the desired pattern (this technique is opposite in concept to what was described above). Because this technique uses a positive relief master, some researchers have utilized overhead transparency masks, as opposed to the drastically more expensive chrome masks. This is usually the case, because of the decreased amount of resolution needed to achieve the finished pattern. Regardless of the technique used to create the channels, a cover plate of glass containing predrilled access holes must be bonded to the PDMS substrate.

The advantage of the replica molding procedure is that numerous devices can be poured onto and then peeled away from one master copy. Therefore, after the master is produced, time consuming lithography is only necessary if the mold needs to be replaced. This greatly reduces the overall time required to fabricate a device. However, sufficient electroosmotic flow in PDMS channels still needs to be generated, in comparison to the silanol-rich glass substrates.

Protocols have been developed for the production of both glass and PDMS devices. The following sections outline the specific procedures for the fabrication, as well as modifications made to improve the lithography techniques. The devices were
developed for use with the methods described in the previous chapter for the detection of
drugs derivatized with Cy5 dye.

**Experimental**

**Glass Devices**

**Supplies and Reagents**

A 5 × 5-inch photolithographic mask was designed in-house and then fabricated at the Rochester Institute of Technology (RIT) Mask-Shop in the School of Microelectronic Engineering. All glass substrates were 3 inch × 1 mm borosilicate glass obtained from Bullen Ultrasonics, INC. (Eaton, OH). Photoresist AZ5206E and commercial Developer AZ351 were both obtained from Clariant Corp. (Somerville, MA). Acetone, methanol, hydrofluoric acid, and nitric acid were obtained from Fisher Scientific (Fair Lawn, NJ). Trichloroethylene (TCE) was purchased from Acros. Hydrochloric acid was purchased from EM science (Gibbstown, NJ).

The gold aqua regia etch (3:1 HNO₃:HCl) was prepared fresh. The chromium base etch was prepared by adding 33 g of K₃(Fe(CN)₆) in 100 mL of deionized water to 17 g of NaOH in 33 mL of deionized water. The buffered oxide etch (BOE) was prepared according to the ratio 20:14:66 (v:v:v) HF:HNO₃:H₂O and obtained from Dr. J.J. Heremans in the Ohio University Department of Physics and Astronomy. The sodium silicate solution was obtained from Sigma-Aldrich and diluted 1:5 (v:v) in
doubly-deionized water. The room temperature bonded glass was hydrolyzed in NH₄/H₂O₂/H₂O (2:1:3) (v:v:v).

**Equipment**

Metal deposition was performed using a Cooke Products Vacuum System Metal Evaporator with Thickness Monitor (TM-100). A Specialty Coating Systems, Inc. P-6000 Spin Coater was employed to deposit the photoresist onto the substrate and a Karl Suss MJB3 contact printer was used for the lithography. Viewing of the photolithography for the metal etching was performed on a 110 V 25 W 50/60 Hz microscope. The electrode holes were drilled using a Dumore Hi-Speed Sensitive Drill with a 1/16 inch drill bit while submerging the substrate in a pan of water. High temperature bonding and annealing was performed in a Sybron Thermolyne 10500 Furnace.

**Lithography Procedure**

The first photolithography mask pattern was modeled using a simple “T” design with a slight offset at the channel intersection to increase the injection size. The schematic for the in-house pattern is shown in Figure 36. The lithography procedure, wet chemical etching, and thermal bonding techniques were adapted from a procedure developed from Fan and Harrison.¹⁴⁰
Figure 36. Schematic of the lithographic mask used for all in-house devices.
All lithography procedures herein were performed by Dr. J.J. Heremans from the Ohio University Department of Physics and Astronomy while being assisted by the author. To prepare the bottom substrate, it was first necessary to anneal the glass by placing it in the Sybron furnace for 6 hours at 600 °C. The oven temperature was increased by 200 °C every thirty minutes and then held at 600 °C for the remaining time. At the conclusion of the annealing step, the oven was turned off and the glass was allowed to slowly cool to ambient temperature overnight. The glass substrates were then submerged in boiling trichloroethanol (TCE) for 5 minutes, boiling acetone for 5 minutes, and then boiling methanol for 5 minutes. Finally, the substrates were rinsed with doubly-deionized water and then dried in an oven at 95 °C for 20-30 minutes.

Two hundred Å of chromium and 1000 Å of gold were deposited on the clean substrate. The spin-coating of AZ5206E photoresist was performed at a minimum of 2500 revolutions per minute (rpm) to ensure sufficient dispersion. The glass substrate was then coupled to a photolithographic mask and exposed to UV-light for 8 seconds in order to crosslink the photoresist. It was then baked for 20 minutes at 100 °C to cure the substrate. After cooling, the substrate was submerged in developer solution AZ321 for 30 seconds and the pattern was inspected to locate any defects or underdeveloped areas.

Wet Chemical Etching Procedure

The gold was etched for approximately 3 to 8 seconds using a drop-wise addition of aqua regia. The substrate was then rinsed with copious amounts of doubly-deionized water. Chromium etching was performed using a chromium base etch for approximately 30 seconds followed by a water rinse. The substrate was inspected under a microscope to
ensure metals in all desired areas were removed. To create the channels, the substrate
was submerged in a buffered oxide etch (BOE) from 0.5 hour to 1.5 hours depending on
desired channel depth. The etched substrate was rinsed with copious amounts of fresh,
doubly-deionized water and then dried with N₂ to remove any water droplets from inside
the channels. The substrate was rinsed with acetone to remove any residual photoresist.
Finally, the gold and chromium etch was repeated to remove all remaining metal.

For the top of the microfluidics device, a blank borosilicate substrate was aligned
over the etched substrate and the well positions were marked with a permanent marker.
The electrode holes were drilled at those positions in the top substrate. The substrates
were matched together to ensure that the holes were aligned with the well. The substrates
were then permanently bonded together using either the thermal or room temperature
bonding procedure.

For the thermal bonding, the top substrate was cleaned with TCE, isopropanol,
and methanol. The aligned substrates were placed in a stainless steel holder. The cover
was held in place by a stainless steel weight. The entire apparatus was put in the furnace
and subjected to the bonding process (Table 23).
Table 23. Temperature program for thermal bonding of glass microfluidic devices.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
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<tbody>
<tr>
<td>200 ºC</td>
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</tr>
<tr>
<td>400 ºC</td>
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</tr>
<tr>
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</tr>
<tr>
<td>650 ºC</td>
<td>6 hours</td>
</tr>
<tr>
<td>Ambient</td>
<td>Overnight</td>
</tr>
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</table>
An alternative method involving a sodium silicate room-temperature bonding procedure was adapted from a procedure developed by Wang et al.\textsuperscript{141} The substrates were placed in a sonicating bath, with acetone, for 5 minutes, hydrolyzed at 70 °C for at least 20 minutes, rinsed, and then dried. Dilute sodium silicate was spin-coated on the top substrate at approximately 300 rpm for 10 seconds. The bottom substrate was immediately brought in contact with the top and clamped with alligator clips. The device was annealed at 90 °C for 1 hour.

\textit{PDMS Devices}

\textbf{Supplies and Reagents}

The photolithographic mask, glass cover-plates, and identical supplies were the same as those used above. Isopropanol (IPA) was obtained from Fisher Scientific (Fair Lawn, NJ). Nano SU-8 (50) negative UV resist in 69\% gamma butyrolactone was obtained from MicroChem Corp. (Newton, MA) and removed using 1,2-propanediol monomethyl ether acetate (PMEA) obtained from Shipley (Marlborough, MA). Sylagard Elastomer 184 PDMS and the curing agent was made by Dow Corning but ordered through Fisher Scientific (Fair Lawn, NJ). (Tridecafluoro-1,1,2,2,-tetrahydrooctyl)-1-trichlorosilane was obtained from United Chemical Technologies, Inc. (Bristol, PA). All equipment used for the lithography procedures was the same as that described previously for the glass chips.
Lithography Procedure

All beakers were cleaned with methanol, isopropanol, and then wiped with a lint-free cloth prior to coming in contact with solutions and the glass substrate. The substrate was submerged in TCE, acetone, and then methanol. After spinning drying, SU-8 (50) photoresist was coated onto the glass at 2500 rpm for 40 seconds. The substrate was subjected to a pre-exposure bake temperature at 65 °C and then ramped to 95 °C for 20 minutes. The contact printer was set at 12 mW for 25 seconds of exposure followed by a post-exposure bake of 65 °C for 1 minute and then 95 °C for 5 minutes. After brief cooling, the substrate was developed in PMEA for 6 minutes and quickly rinsed with IPA. The master was then placed in a desiccator for 1 hour with silane to increase its longevity.

Replica Molding Procedure

Sylgard 184 was prepared in a 10:1 ratio with the curing agent. It was then degassed in a solid phase extraction chamber under 10 mmHg with vacuum. After removing the viscous liquid from the chamber, it was poured over the master in a round plastic container similar in size to the substrate until the pattern was covered completely. The container was then placed in an oven overnight at 40 °C to facilitate curing. The next morning, the PDMS layer was peeled free of the master and the buffer reservoirs were created using tiny punches at the end of the separation channels. Reversible sealing of the PDMS layer was completed by pressing the flexible polymer onto a glass cover-plate identical to the glass device.
Results and Discussion

Glass Devices

The annealing step served to settle the surface hydrogen bonds on the glass to produce a suitable surface for etching. Since glass was isotropically etched in all directions, the channel depth could be approximated kinetically. Figure 37 shows representatives channels constructed on glass sides that were either (A) not annealed, or (B) annealed.

Before the metal step was added to the fabrication procedure, the masked photoresist alone was unable to withstand the strength of the BOE. Once the pattern was protected with metals, the etching was allowed to proceed as desired. The individual metal removal was performed with ease if the color changes from gold to chromium and then chromium to bare glass were carefully noted. Surface cleanliness also played a major role in the quality of the deposited layers. Figure 38 (A) shows a substrate with a pinhole on the surface of the deposited metal. The pinhole was most likely caused by dust on the substrate. Figure 38 (B) shows a channel defect caused by an imperfection that resulted in the metal being inadvertently etched away. A surface scratch of unknown origin is shown in Figure 38 (C).
Figure 37. Demonstration of the importance of annealing. (A.) Substrate cleaned with MeOH and unannealed. (B.) Substrate annealed at 650 °C for 6 hours.
Figure 38. Demonstration of inconsistent etching that occurred in the development of glass microfluidic devices. (A) Pinholes; (B) defects; and (C) surface scratches are all imperfections which can most likely be avoided by the exclusion of dust and foreign particles, rigorous cleaning procedures, and careful substrate handling.
With correct surface preparation procedures, such defects could be avoided. Intensive cleaning was necessary in order to remove all foreign particles from the surface of the glass. The attempt was first made to clean the substrates by only wiping them with TCE, IPA, and then methanol. However, cleaning the substrates by boiling them in the respective solvents appeared to remove the greatest number of impurities. For the initial substrates, Windex® was also used as a cleaning solution; however it did not seem to have any noticeable effect on the cleanliness of the glass.

Figure 39 illustrates the importance of spin-coating speed and proper development of the substrate. As can be seen in Figure 39 (A), poor photoresist layers result when spin-coating was performed at less than 2500 rpm and with an inadequate development time. Images of the wet-chemical etching from start to finish are shown in Figure 40.

Other cosmetic scratches and depressions were attributed to the stainless steel holder used in the thermal annealing process. There were advantages and disadvantages to both bonding methods. Because of the harsh temperatures applied during the thermal bonding procedure, the glass substrates would occasionally crack. This could be alleviated using a better holding device. The room temperature bonding, though a more mild method, resulted in an adhesion weaker than that of thermal bonding when equal pressures were applied. Different concentrations of dilute sodium silicate would vary the degree of bonding; therefore this procedure should be evaluated more thoroughly.
Figure 39. Demonstration of the importance of proper development. (A) Substrate in which the photoresist layer was spin-coated at < 2500 rpm and not allowed to fully develop. (B) Substrate in which the photoresist layer was spin-coated at 2500 rpm and allowed to develop (exposed to UV-light for 12 seconds and developed for 30 seconds) in solution.
Figure 40. The fabrication of a glass microfluidic device. Digital image taken After (A) photoresist development; (B) aqua regia etch; (C) chromium base etch; (D) HF:HNO₃:H₂O etch.
PDMS Devices

Several groups have noted the successful use of high resolution printers in lieu of the chrome mask. Not only is this technique simple, but it is a fraction of the cost of traditional chrome masks. A laboratory procedure outlining this practice is provided in Appendix 4.146 While this inexpensive procedure is an extremely valuable introduction to the principles of lithography, the more expensive chrome mask is needed for the purpose of producing a reliable and reproducible device. What can be referred to as “fuzzy edges” often result from insecure contact of the mask to the substrate at the time of exposure. Additionally, if the printer resolution is not high enough, UV-light will bleed through the black portions of the transparency causing exposure in areas where it was not desired.

The reversible sealing employed in the creation of the so-called “fourth wall” of the electrophoretic channel (the first three being that of the PDMS itself) was performed using contact pressure. This sort of bonding creates van der Waals contact with a flat surface, in this case the glass cover-plate, with minor imperfections.139 While this creates a water-tight seal, it can not hold up to pressures greater than 5 psi. Alternatively, irreversible bonding can be accomplished by exposing the PDMS surface to air plasma by the formation of Si-O-Si bonds between PDMS and glass after a loss of water. The creation of now covalent bonds can withstand pressures of 30 to 50 psi.

Another important factor in producing high-quality PDMS masters involves the chemical preparation of the glass surface for bonding. Typically, thermal heating can result in a relaxation of the Si bonds in the glass to create a surface that is more capable
of accepting the SU-8 (50) resist. Figure 41 shows an image of a PDMS master without the use of glass preparation. The arrow in the figure points to stresses on the SU 8 (50) resist, caused by insufficient bonding. These stresses will cause the photoresist pattern to shift from the desired straight channel. In this case, it occurs when drying the substrate with nitrogen after development. The master mold can be strengthened by adding a few drops of (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane into a vial, which is then placed into the desiccator with the glass slide. The silane will “silanize” the glass, thereby preparing a sufficient surface for bonding.

An important factor in the preparation of the PDMS replica substrate is the removal gas from the elastomer/curing agent mixture. The degassing of this 10:1 (elastomer:curing agent) mixture is crucial to the success of the PDMS portion of the device. Prior to evacuation, a large number of bubbles were visible. Once the bubbles are no longer visible, after evacuation, immediate use of the mixture is recommended.

Once the device has been fabricated, one must now be concerned with generating the proper amount of EOF for successful analyte detection. While this is determined on a procedure-to-procedure basis, several points must be addressed when PDMS devices are employed. Unlike glass, which has a surface much like traditional capillaries used for electrophoresis applications, unmodified PDMS possesses a hydrophobic surface. Exposure to oxygen or air plasma will create a hydrophilic surface in place of the methyl groups. The new silanol groups welcome the presence of aqueous buffers and will be less prone to analyte adsorption on the walls of the channels. After subjecting the surface to air plasma it is important to note that the channels must be filled with a polar liquid to maintain the desired charge.
Figure 41. Master mold channel intersection. Illustration of stressed placed on SU-8 (50) resist. This can be corrected with surface preparation procedures.
Conclusions

Both the glass and the PDMS fabrication procedure produced channeled substrates. Cleanliness appeared to be a major issue when etching both devices and would ultimately affect the reproducibility of mass produced devices. Attempts were made to combat substrate surface cleaning and suggestions were made for the future to assure a contamination-free environment. With proper technique, it was possible to produce high-quality microfluidic devices, as shown in Figure 40.

The next step would be to adapt the commercial chip for the nitratated benzodiazepines, as described in Chapter 5, for one of the in-house devices. While PDMS is a rapid prototyping procedure that can be performed with greater ease and less lithography per device, there seems to be more drawbacks at this point. The devices are less uniform and channel fouling is easier to promote upon handling of the molded substrates. Strong EOF generation, a characteristic very important to small neutral molecule migration, seems unlikely without the use of an oxygen plasma.

In order for microfluidic devices to make a successful entrance into the forensic community, the procedures should not only be faster and more reliable, which are the method goals offered in the last chapter, but the equipment must be cost effective. While some sort of replica prototyping, or soft lithography, seems to be the future answer, much more must be learned about the chemistry and physics involved in the microfluidics before speculations can be made about usefulness in a clinical laboratory environment.
Chapter 7: The Future of Drug Analysis by Advanced Capillary Electrophoretic Techniques

While CZE is a fairly simple form of electrophoretics, the development of techniques such as MECC and chiral analysis illustrate that complex chemical interactions can be used to achieve impressive separation efficiency. With the increasing application of CEC and microfluidics in the analytical laboratory, it can be anticipated that CE applications in toxicological analysis will continue to develop. As with any method, further improvement and increasing the scope of current studies are always necessary.

In date-rape drug screening, as described in Chapter 2, the use of a flow-cell or z-cell design will help lower the limit of detection for that analysis. In order to become useful for biological samples for urine and blood screening, the sensitivity must be increased for benzodiazepines in order to accommodate the dosage levels common in surreptitious drug-induced sexual assault. The proper urinary metabolites of low-dose benzodiazepines must also be taken into consideration when examining toxicological specimens.

For the detection of toxicological levels of GHB, more extensive extraction techniques are needed to prevent biological interferences and capillary fouling. For these measurements to be valid, however, analysts must be careful to consider the effect of endogenous concentrations of GHB in data analysis. While low-level GHB detection been a controversial subject in the past, there have been several papers published that outline ante and postmortem levels. Another emerging GHB analog only recently
discovered has been gamma-valerolactone, or GVL. New methods should be adjusted to
detect this and other analogs of GHB.

It has been evident from the casework at advanced forensic laboratories like the
FDA Forensic Center in Cincinnati, OH and through Internet drug user sources, such as
Erowid,\textsuperscript{46,64-66} that piperazines have already made their way into the illicit drug scene. As
with most new substances of abuse, now it is the job of all forensic laboratories to
provide valid screening protocols for qualifying samples. While the method described in
Chapter 3 outlines the first attempt at piperazine detection by CE, improvements still
need to be made in sensitivity. In order for these techniques to be useful, solid
pharmacological data is needed to establish proper cut-off concentrations and limits of
detection for these compounds. Once this research is complete, CE methods can be
tailored to detect this compound at lower levels and accommodate additional drugs of
interest. In addition, further clandestine synthetic procedures need to be evaluated for the
resultant products. Additional work has already been completed in the analysis of
clandestine syntheses of methyl-piperazine compounds.\textsuperscript{51} It is also inevitable that
various BZP analogs will shortly appear once the scheduled parent compounds become
scarce in the same way that GBL, 1,4-butanediol and recently GVL became common
once GHB was scheduled.

In capillary electrochromatography, the most important aspects that need to be
given further study are the complex mechanisms involved in small molecule separations.
Even though modified silica stationary phases are not specifically geared towards EOF
generation, researchers are finding ways around this problem and better methods are
constantly emerging. Cahours et al. compared MECC to CEC-MS for flunitrazepam and
its analytes on a Hypersil C18 bonded silica CEC column (packed 25 cm, with a 55 cm total length, 100 µm ID). While a slight increase in retention time was observed, the successful coupling to a mass spectrometer outweighs the additional time.

As mentioned previously, CEC techniques can offer important advantages in separation and detection of small molecules. Because of the inherent advantages in preparation, future work in CEC will involve monoliths. The in-situ preparation techniques for these new stationary phases outlined in Table 13, Table 14, and Table 15 for methacrylates and Table 16 for divinyl benzene-vinylbenzyl chloride, provide a starting point to advance toward more complex mixtures that can provide improved selectivity. While these polymer phases rely mostly on pore sizes and limited surface functionalities to achieve analyte-phase interactions, more complex phases could prove to be very useful. This is especially true in the instance of small molecule separations where thus far the polymers described in Chapter 4 have shown poor efficiency. Cyclodextrin-modified silica stationary phases have seen great success in HPLC separations. There have already been examples of cyclodextrin phases for both OT-CEC and modified packed silica.

A novel procedure in its initial stages of development was the synthesis of a methacrylate-cyclodextrin complex using a combination of literature procedures. The creation of this complex using isocyanatoethyl methacrylate will result in active monomers modified with native cyclodextrins. While this procedure shown in Figure 42 is only in its initial phase, promising results have been obtained by IR analysis.

Once sufficient development of stationary phases for CEC have taken place, it should be possible to integrate these techniques into a microfluidic device. This research
has provided some of the groundwork for the development of an electrophoretic device that enables pre-concentration, separation, and detection of small drug molecules. Initial research is already in progress for protein analysis via this technique.\textsuperscript{157}

Every year there are more and more drug facilitated sexual assault cases that go on unreported, unsolved, or not prosecuted due to the illusiveness of these incidents. In the club scene there has been an entire series of new trytamine compounds (2-CB, 2C-T-2, 2C-T-7), that just like the piperazines are not included in screening tests.\textsuperscript{158} It is only a matter of time before these and other newly designed drugs become prevalent. Advanced capillary electrokinetic techniques will need to continue to be developed to address the forensic problems involved in detecting these new substances.
Figure 42. Reaction to yield a reactive β-cyclodextrin with a methacrylate group using isocyanatoethyl methacrylate (IEM). This reaction will yield a methacrylate monomer functionalized with a cyclodextrin for polymer stationary phases.
References


(39) Wilson, T. Luster is Hit with a Third Civil Lawsuit; A Placer County woman joins two others seeking millions from the rapist, the great-grandson of cosmetics magnate Max Factor. *Los Angeles Times*, June 26, 2003, p B1.


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


1 Website no longer active.


(132) Amersham Biosciences N-Terminal Labelling of (D-ser2)-leucine-enkephalin with Cy5™ Mono NHS ester In CyDye™ mono-reactive NHS Esters: Reagents for the labelling of biological compounds with Cy™ monofunctional dyes; Amersham Biosciences Corp: Piskataway, 2003, pp 14-16.


Appendix I. Structures of Drugs

- **Alprazolam**
  - M.W.: 308.77
  - CAS: [28981-97-7]

- **7-Amino-clonazepam**
  - M.W.: 285.74
  - CAS: [4959-17-5]

- **7-Amino-flunitrazepam**
  - M.W.: 283.30
  - CAS: [34084-50-9]

- **Amobarbital**
  - M.W.: 226.28
  - CAS: [57-43-2]

- **Amphetamine**
  - M.W.: 135.21
  - CAS: [300-62-9]

- **1-Benzylpiperazine**
  - M.W.: 176.26
  - CAS: [2759-28-6]

- **1,4-Butanediol**
  - M.W.: 90.12
  - CAS: [110-63-4]

- **Chlordiazepoxide**
  - M.W.: 299.76
  - CAS: [58-25-3]

- **1-(3-Chlorophenyl)-piperazine**
  - mCPP
  - M.W.: 196.68
  - CAS: [6640-24-0]

- **Chloral hydrate**
  - M.W.: 165.40
  - CAS: [302-17-0]
Cocaine
M.W.: 303.56
CAS: [50-36-2]

Codeine
M.W.: 299.37
CAS: [76-57-3]

Dextromethorphan
M.W.: 271.40
CAS: [125-71-3]

1,4-Dibenzylpiperazine
DBZP
M.W.: 266.39
CAS: N/A

Diphenhydramine
M.W.: 255.36
CAS: [58-73-1]

Ephedrine
M.W.: 165.23
CAS: [299-42-3]

Flunitrazepam
M.W.: 313.29
CAS: [1622-62-4]

Gamma-butyrolactone
GBL
M.W.: 86.09
CAS: [96-48-0]

Gamma-hydroxybutyric Acid
GHB
M.W.: 104.11
CAS: [591-81-1]

Gamma-valerolactone
GVL
M.W.: 100.12
CAS: [108-29-2]
Heroin
M.W.: 369.42
CAS: [561-27-3]

Ketamine
M.W.: 237.73
CAS: [674-88-1]

Lorazepam
M.W.: 321.16
CAS: [846-49-1]

Lysergic acid diethylamide
LSD
M.W.: 323.44
CAS: [50-37-3]

Methamphetamine
MDMA
M.W.: 193.25
CAS: N/A

1-(2-Methoxyphenyl)piperazine
M.W.: 192.26
CAS: [35386-24-4]

1-(4-Methoxyphenyl)piperazine
M.W.: 192.26
CAS: [38212-30-5]
Secobarbital
M.W.: 238.29
CAS: [76-73-3]

Tetrahydrocannabinol
THC
M.W.: 314.47
CAS: [1972-08-3]

Theophylline
M.W.: 180.17
CAS: [58-55-9]

Trazodone
M.W.: 373.89
CAS: [19794-93-5]

Triazolam
M.W.: 343.21
CAS: [28911-01-5]
Appendix II. Miscellaneous Compounds

4-Amino-2,6-dinitrotoluene
M.W.: 197.15
CAS: [19406-51-0]

2-Amino-4,6-dinitrotoluene
M.W.: 197.15
CAS: [35572-78-2]

3-Chloroaniline
M.W.: 127.57
CAS: [108-42-9]

Dimethylsulfoxide
DMSO
M.W.: 78.13
CAS: [67-68-5]

2,4-Dinitrotoluene
2,4-DNT
M.W.: 182.14
CAS: [121-14-2]

2,6-Dinitrotoluene
2,6-DNT
M.W.: 182.14
CAS: [606-20-2]

2-(4-Morpholino)ethanesulfonic acid
MES
M.W.: 195.233
CAS: [4432-31-9]

Nitrobenzene
NB
M.W.: 123.11
CAS: [98-95-3]

4-Nitrotoluene
PNT
M.W.: 137.14
CAS: [99-99-0]

Sulfanilic acid
M.W.: 173.19
CAS: [121-57-3]

Thiourea
M.W.: 76.116
CAS: [62-56-6]
2,4,6-Trinitrophenylmethylnitramine
Tetryl
M.W.: 287.15
CAS: [479-45-8]

2,4,6-Trinitrotoluene
TNT
M.W.: 227.13
CAS: [118-96-7]
Appendix III. List of Monomer Material

2-Acrylamido-2-methylpropanesulfonic acid
AMPS
M.W.: 207.24
CAS: [15214-89-8]

2,2'-Azobis(2-methylpropionitrile)
AIBN
M.W.: 164.21
CAS: [78-67-1]

1,2-Butanediol
M.W.: 90.12
CAS: [26171-83-5]

1,4-Butanediol
M.W.: 90.12
CAS: [110-63-4]

Butyl methacrylate
BMA
M.W.: 142.20
CAS: [97-88-1]

Cyclohexanol
M.W.: 100.16
CAS: [108-93-0]

1-Decanol
M.W.: 158.28
CAS: [112-30-1]

2,2-Dimethoxy-2-phenylacetophenone
DMPA
M.W.: 256.30
CAS: [24650-42-8]
Appendix IV. Photolithography

The following procedure was adapted from: Collard, D.; Week, M. Introduction to Photolithography In Polymer Chemistry Experiments; Georgia Institute of Technology: Georgia, 2004, pp 2-4.

Preparation of the Photomask Template

1. Create a 3 x 3-inch black box in a computer program, such as Microsoft PowerPoint, Adobe Photoshop, Adobe Illustrator, etc.

2. Create a text box or design and position it in the center of the box. Any feature to be polymerized must be in white-colored font. Leave at least 1.5 inches of space around the 3 x 3-inch black box. Objects and text with less detail produce better results.

3. Print the photomasks on transparency film using a laser printer.

4. Cut out the black box, an example of which is shown below.
Polymer Patterning

Key steps for the polymer patterning are numbered in the figure below.

1. Cut out four-equal parts from a sheet of blank transparency film.
2. Align two thin-glass coverslips on the top and bottom of the transparency film.
3. Add 25-30 drops of liquid photoresist to the center of the film, leaving about 2.5 inches between the cover-slip and photoresist.
4. Cover the photoresist with a thick-glass slide (75 x 50 x 1 mm), allowing its top and bottom to rest on top of the thin cover-slips. The space between the thick-glass slide and the transparency film should be completely filled with photoresist. Minor bubbles are acceptable, but large spaces are not.
5. Place the photomask, containing the design, as prepared above, on top of the glass slide. Ensure that the photoresist is visible through the designs made on the transparency film.

6. Place another thick glass slide on top of the photomask.

7. Polymerize the exposed photoresist for 20-25 seconds with 365 nm UV light held ~0.5 inches above the resist.

8. Remove the mask and carefully peel the transparency from the thick, glass slide.

9. Wash any unpolymerized photoresist from the slide and pat dry with a towel.
Fin