FINAL REPORT

The development of microfluidic devices for the rapid isolation and detection of drugs of abuse,

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1. Abstract

The goal of this project was to develop technologies for the rapid analysis of drugs of abuse using microfluidic systems. Specifically we set out to examine and test microchip based devices to separate and analyze drug compounds which have been implicated in drug facilitated sexual assault, including phenethyl amines, GHB, and benzodiazepines,. In developing this methodology we had three main objectives: (1) the development of highly sensitive screening methods for specific classes of drugs using fluorescent detection, (2) the development of universal detection techniques for drugs of abuse using microfluidic systems, and (3) the confirmation of the presence of these drugs using CEC/MS (capillary electrochromatography mass spectrometry) techniques which could eventually be used in microchips as well. The analytical procedures were initially developed using standard mixtures and tested on spiked urine samples. As a result of this proposal, a number of procedures have been developed for publication in the forensic literature. These include a microfludic procedure for the indirect detection of nitrobenzodiazepines, a procedure for the trace detection of phenethyl amines using chip detection, an online preconcentration technique for the detection of a wide variety drugs in urine using CE, a technique for the ultra trace detection of benzodiazepines by CEC/MS and the development of chip based designs for improved resolution of samples by fluorescent and electrochemical detection.

The development of microfluidic devices for the rapid isolation and detection of drugs of abuse,

2. Executive Summary

The goal of this project was to explore currently available microfluidic systems for the detection of illicit drugs, particularly date rape drugs, and to examine various approaches for their analysis. In the development of these procedures, initial testing was performed using off-line techniques coupled with capillary electrophoresis. Systems which were proven useful were then further modified to an on-chip platform. This report is divided into three specific sections, relating to the three main projects: direct fluorescence detection of phenethyl amines, indirect fluorescence detection of nitrobenzodiazepines, and CEC/MS-TOF for the preconcentration and analysis of multiple benzodiazepines.

A. Direct Detection of Phenethyl Amines on Microfluidic Devices

Introduction

In this work, a microfluidic system was tested for use as a portable screening device for detection of drug facilitated sexual assault drugs. Fluorescent derivatizing agents were reacted with each drug used to provide high sensitivity detection. Four basic drugs which contain either primary (amphetamine) or secondary Amines (methamphetamine, ephedrine, and ketamine) were initially selected for derivatization experiments. Other compounds examined included amine metabolites of nitrobenzodiazepines, ketamine and methcathanone. Demethylated forms of tertiary amines such as morphine and cocaine were also examined.

A Micralyne µToolKit or µTK was used to perform the electrophoretic separations. It contained a high voltage power supply and an electrode stage which was controlled by LabView software from National Instruments (Austin, TX, USA). A 532 nm υ-doubled green Nd:YAG laser was coupled to the µTK for LIF detection in a confocal orientation. A PMT with a 568.2 nm bandpass filter collected the fluorescence emission which was recorded as an electropherogram in LabView. Low fluorescence Schott Borofloat™ glass chips from Micralyne (Edmonton, Alberta, Canada) were used with a simple cross intersection (Figure 1)

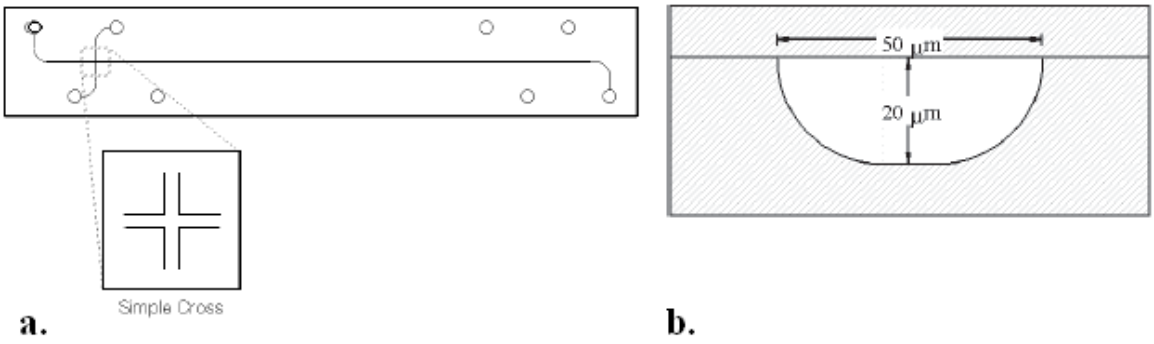


Figure 1. Micralyne's low fluorescence Schott Borofloat™ glass chip (Micralyne).

To perform fluorescent derivatization of a sample, 100 µL of a drug solution, 200 µL of the dye solution, and 100 µl of reaction buffer were placed in a 600 µL PCR tube. Tubes were capped, shaken, and then stored in a thermocycler with an amber lid. Reactions were heated to the specified temperature using an ABI 480 thermocycler.

Results

Eosin isothiocyanate (EITC) was selected as a derivatizing agent for our experiments. Eosin, like fluorescein, lacks the cationic charge that caused adherence to channel walls and decomposition problems at elevated temperatures. In addition isothiocyanates with

both fluorescein and rhodamine dyes have been proven to be reactive with primary and secondary amines [Alnajjar].

Amphetamine, methamphetamine, and ephedrine were successfully derivatized with EITC with no degradation effects of the dye. The detection limit for amphetamine with this probe was 20 ppb. Completion of the derivatization reactions required about 24 hours at room temperature. This can be reduced through increasing the temperature. [Alnajjar] No derivatization of ketamine was observed even when heat was applied and the reaction was allowed to proceed for 72 hours. It is probable that this is due to the steric hindrance of the amine group as compared to that on the phenethyl amines.

Two buffers, one slightly acidic, and one basic, were analyzed to determine which would yield the best separation of the basic drugs. A 20mM aqueous solution of 2-(N-Morpholino)ethanesulfonic acid (MES) had a pH of 6.1. The second buffer was an adaptation of Alnajjar's buffer for separating FITC derivatives of opiates [Alnajjar]. This basic buffer was composed of 20 mM $Na_2B_4O_7$, and the pH was adjusted to 9.8 with NaOH. While neither buffer yielded complete separation of the three phenethyl amines, resolution was best with the borate buffer. (Figure 2)

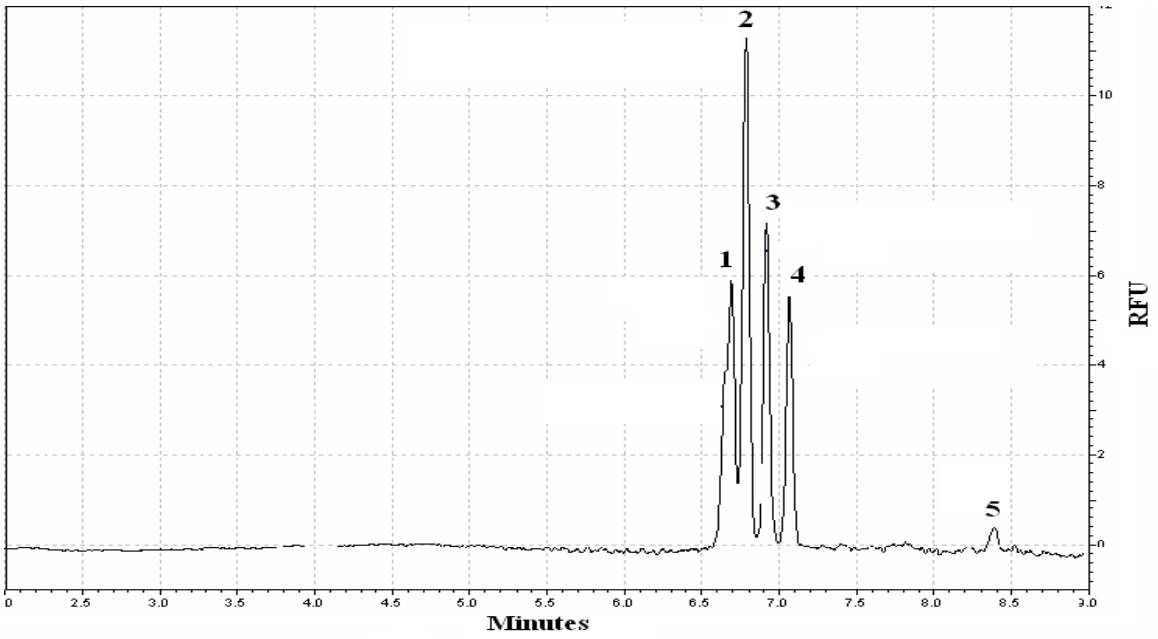


Figure 2. Separation of four phenethyl amines in a run buffer of 20 mM sodium borate, 20 mM β-cyclodextrin, 10% isopropanol, 10% acetonitrile, pH = 9.8. The peaks are 1. MDMA, 2. methamphetamine, 3. amphetamine, 4. ephedrine, and 5. EITC.

The effects of SDS and β-cyclodextrin on the resolution of amphetamine, methamphetamine, and ephedrine were compared utilizing two buffers which were previously optimized for separation of FITC derivatives [Alnajjar 2004]. β-cyclodextrins also altered the selectivity of the run buffer so that the analytes eluted earlier, prior to the excess dye, and in a different order as compared to SDS. Because it yielded at separation of all three analytes, β-cyclodextrin was selected as the pseudo-stationary phase for our buffer. Small amounts of organic solvents were also added to the run buffer to increase selectivity. Optimal results were obtained with 20% isopropanol.

This final buffer with 20% isopropanol was used to analyze a mixture of phenethyl amines including MDA, methamphetamine, amphetamine, ephedrine, and methcathinone (Figure 3). Other compounds observed to produce derivatives included amine metabolites of

nitobenzodiazepines such as 7-aminoclonazepam and 7-aminoflunitrazepam, and demethylated tertiary amines such as normorphine and norcocaine.

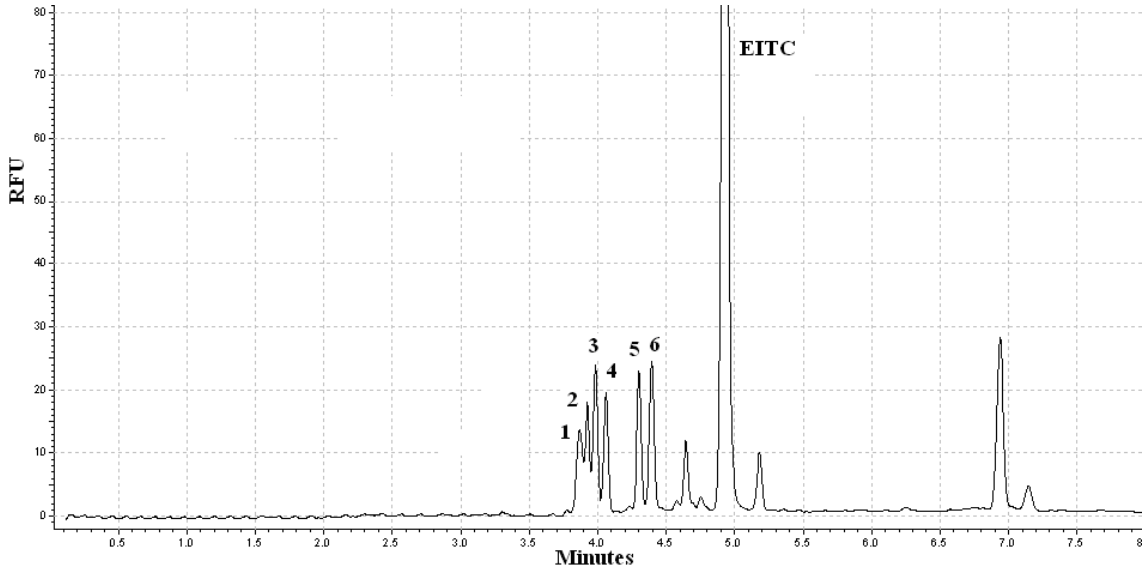


Figure 3. Separation of six drugs in a run buffer of 20 mM sodium borate, 20 mM β-cyclodextrin, 20 % isopropanol, pH = 9.8. The peaks are: 1. MDMA, 2. methamphetamine, 3. amphetamine, 4. ephedrine, 5. methcathinone, and 6. normorphine.

Microfluidic Separation

Once the buffer system was optimized for standard CE systems, the separation system was set up on the microfluidic device. Methamphetamine, amphetamine, and ephedrine could be distinguished, but additional analytes in the mixture such as MDMA coeluted with these peaks. While this is less of a problem when the system is used as a general screening technique, further experiments focused on improving resolution of the microchip systems.

One particular improvement involved adding external reservoirs to the chips to minimize evaporation and permit longer run times. These reservoirs (nanoports) were glued with epoxy resin to the surface of the chips to create wells for holding sample and buffer. The wells

eliminate problems with evaporation and siphoning effects. The overall results are shown in Figure 4 which provides a separation of the three drugs in less than a minute and a half.

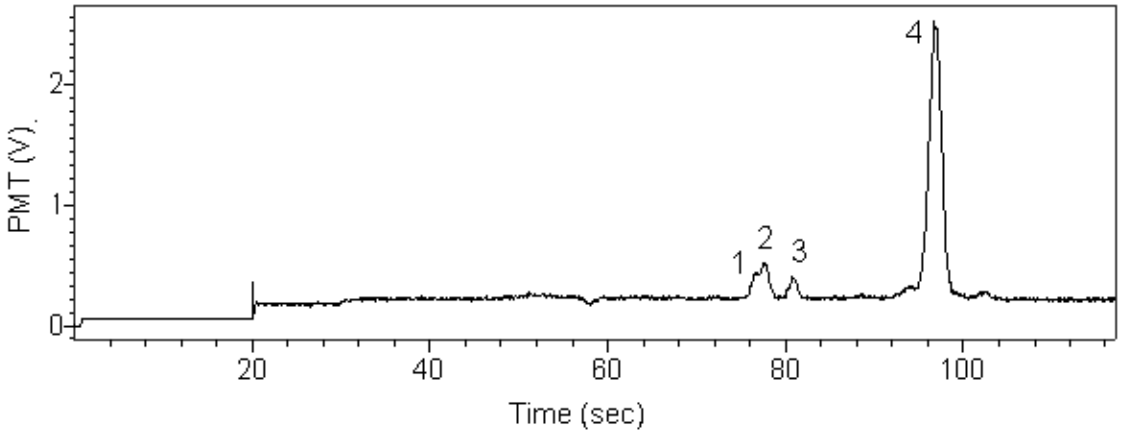


Figure 4. Separation on the μTK with the optimized run buffer of 20 mM sodium borate, 20 mM β-cyclodextrin, and 20% isopropanol, pH = 9.8, E = 250 V/cm. The peaks are 1. methamphetamine, 2. amphetamine, and 3. ephedrine.

Conclusions

A derivatization scheme for drugs containing primary and secondary amines was developed utilizing eosin isothiocyanate. The technique involves mixing the drug and reactive dye in a 20 mM sodium bicarbonate solution at a pH of 8.5 and permits the detection of low levels of phenethyl amines (20 ng/mL on chip.) The reaction is suitable for trace detection of phenethyl amines in urine and other body fluids.

B. Indirect Detection of Benzodiazepines and GHB on Microfluidic Devices

Introduction

While direct fluorescence is the most sensitive detection method for drugs of abuse, not all compounds produce fluorescence. Derivatization is required prior to analysis. Alternatively, indirect laser induced fluorescence is a method where a fluorophore is added to the electrophoretic buffer so that the baseline is constantly elevated [Kuhr]. The presence of analytes will result in a decreased baseline, giving a negative peak as a signal.

The mechanism here is thought to be dynamic quenching where an excited state charge transfer complex is formed between the fluorophore and the quencher. Nitrated compounds are strongly electron withdrawing and will form strong complexes with large quenching constants producing a loss of signal. Thus flunitrazepam and its metabolite desmethylflunitrazepam, as well as clonazepam, and nitrazepam, should also be amenable to this approach and we have examined their separation and indirect detection on microfluidic devices.

All separations were conducted on a Micralyne (Edmonton, Alberta, Canada) μTK system with a 750 nm diode laser and photo multiplier tube (PMT) for detection. Simple cross microfluidic chips composed of borofloat glass from Micralyne were used.

A buffer containing 15 mM SDS, 15 mM boric acid, and 3 mM sodium tetraborate was determined to be optimal for benzodiazepine separation using a maximum voltage of 4 kV. All benzodiazepines eluted within 80 seconds using this buffer system.

Results

The separation of the four benzodiazepines with this buffer is shown in Figure 5.

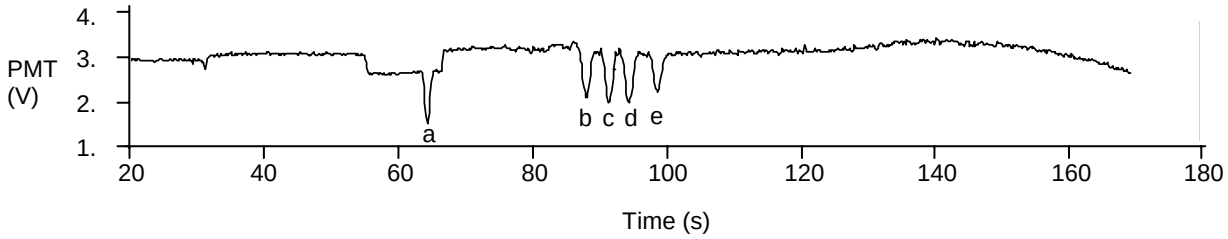


Figure 5. The separation of four benzodiazepines: a) 2,4-DNT, b) desmethylflunitrazepam, c) flunitrazepam, d) nitrazepam, e) clonazepam. The run buffer consisted of 15 mM SDS, 15 mM boric acid, 3 mM sodium tetraborate, 2.6 μM Cy5 with 20% methanol. The maximum voltage was set at 4.0 kV and the detector was at 45 mm.

Using the 20 % methanol buffer, a calibration curve was constructed for four benzodiazepines using peak areas relative to that of the internal standard. The benzodiazepines were run at concentrations ranging from 25 to 250 μg/mL. As an estimate of reproducibility, and mobilities of the four compounds were determined for four consecutive runs on three different days. The percent relative standard deviations for each drug were under 1.5%.

Conclusions

The use of microfluidics in the detection of nitro benzodiazepines by laser-induced indirect fluorescence has been shown. The reproducibility of the analysis is 1.4 % RSD or less. While the detection limits of the method are not sufficient for it to be used as a toxicological examinations, the procedure should prove useful a screening method for spiked beverages and other forensic samples. Overall the work demonstrates the feasibility of using microfluidic devices as a rapid screening tool for the detection of benzodiazepines used in beverages during drug facilitated sexual assault (DFSA).

C. CE/MS and Monolithic CEC/MS-TOF for Ultratrace Detection of Benzodiazepines

Introduction

As the results above show, fluorescent techniques coupled with microfluidic detection can provide rapid and highly specific detection for screening drugs such as benzodiazepines and phenethyl amines. However for use in court, more definitive techniques are required. CEC/MS provides this capability in a microfluidic format.

CEC is an analytical separation technique which combines the high peak efficiency of CE with the stationary phase selectivity of LC [Eeltink]. This technique has already proven to be suitable for the separation of benzodiazepines, but there has been no work done using CEC/MS for these types of samples [Taylor, Kiyokatsu]. The hyphenation of CEC to MS combines the speed and efficiency of CEC with the selectivity and sensitivity inherent to MS.

In this study, we have developed a **CEC/MS-TOF** separation of benzodiazepines *via* optimization of the *in situ* synthesis and application of porous acrylate-based monoliths.

A monolith is a continuous porous bed which contains two pore size distributions, mesopores and macropores. Mesopores give the structure morphology with high surface area. Macropores provide structural support and help to minimize back pressure. Figure 6 shows the hexyl acrylate monolith composed of microglobules, about 1 μm in diameter linked together. These pictures demonstrate that (i) the monolith is well linked to the capillary wall and (ii) the morphology of the monolith is similar to those usually presented in the literature [Barrioulet. These results demonstrate that the morphology of the hexyl acrylate monolith synthesized in our group is able to generate high separation efficiency with good retention reproducibility. The monolith was then evaluated for benzodiazepine separation and detection using a TOF-MS.

A

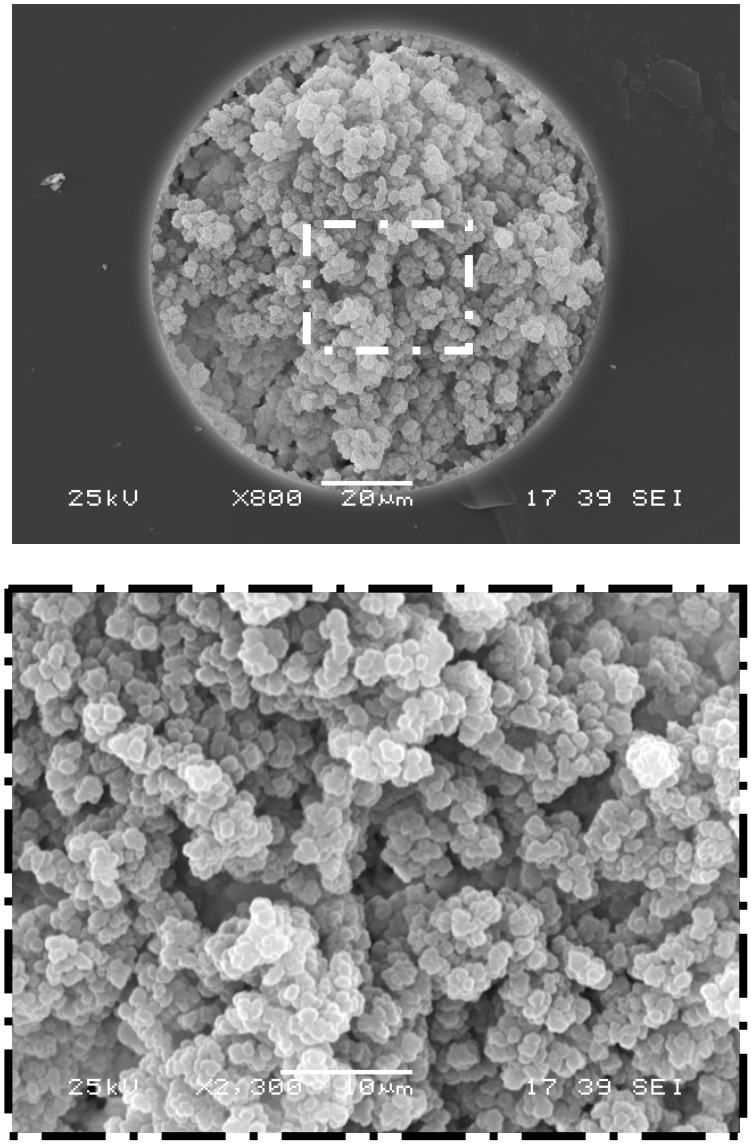


Figure 6: Scanning electron micrographs of hexyl acrylate monolithic column. A-entire capillary and, B-enlargement of the dotted zone.

CEC/MS-TOF of Benzodiazepines

An example of a CEC separation of the selected benzodiazepines with a 50 cm capillary length is presented Figure 7A. This electrochromatogram consists of the isolation and superposition of each protoned molecular ion. Figure 7B provides extracted ion chromatograms

from the standard which correspond to the nominal masses of nitrazepam *m/z*=282, clorazepate ($[M+H-CO_2]^+$) m/z=271 and diazepam, m/z=285.

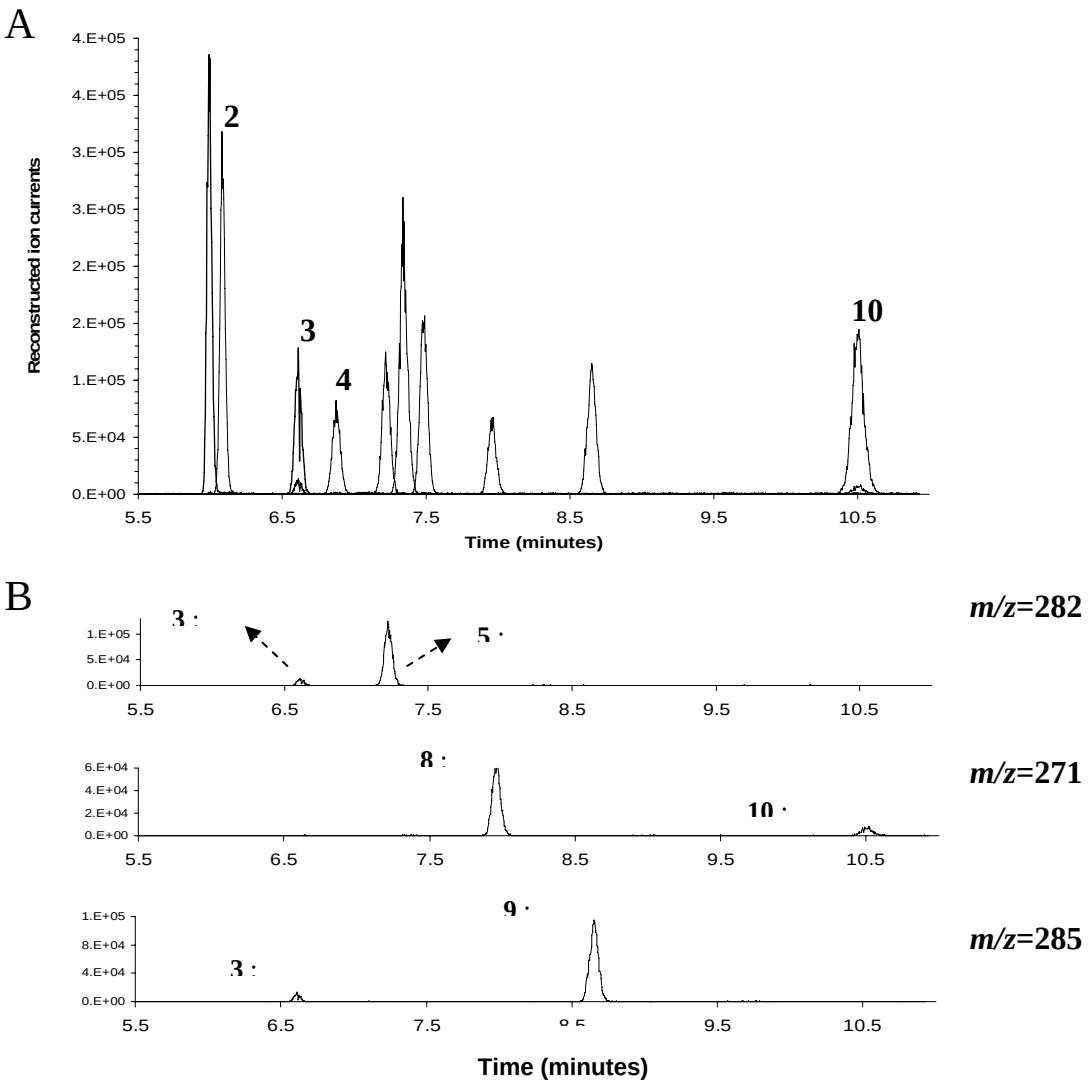


Figure 7: Reconstructed ion electrochromatogram of ten benzodiazepines (A) and selected extracted ion (*m/z*=282; 271 and 285) (B). Peak overlaps observed in the reconstructed ion electrochromatogram are due to benzodiazepines fragmentations. Hexyl monolithic column (L=50 cm). Mobile phase, 5 mM ammonium acetate pH 7.0–acetonitrile (30/70 v/v); running voltage, 20 kV; electrokinetic's injection, 10 kV for 4 s; benzodiazepines concentration, 10 µg/mL. 1-alprazolam, 2-triazolam, 3-chlordiazepoxide, 4-lorazepam, 5-nitrazepam, 6-clonazepam, 7-flunitrazepam, 8-clorazepate, 9-diazepam, 10-prazepam.

This method was then applied to the determination of benzodiazepines present in spiked urine samples at a concentration of 10 ng/mL. The preconcentration of the benzodiazepines at the head of the column was achieved, resulting in a 75–140 fold improvement in sensitivity due to the presence of the monolithic stationary phase.

Conclusions

The application of CEC/MS-TOF provided highly sensitive and efficient detection of a wide variety of benzodiazepines. By using inline extraction we achieve a sensitivity of under 10 ng/mL for all samples. In addition, the exact mass capability of the time of TOF system produces isotopic exact masses aiding in identification and confirmation of unknowns.

D. Conclusions and Future Work

In this project we have laid the groundwork for the application of microfluidic devices in drug detection. In particular we have demonstrated the applicability of fluorescence detection for highly sensitive screening of a wide variety of phenethyl amines and other basic drugs containing amine moieties. Secondly we have demonstrated on-chip indirect detection of flunitrazepam and other benzodiazepines. We have also developed an efficient and sensitive in-line preconcentration technique for the separation and detection of benzodiazepines which is applicable to the analysis of a wide variety of drugs implicated in DFSA. The monolithic CEC/MS system provides exceptional sensitivity and specificity of detection.

In the future, we will continue expand the application of chip based screening and **CEC/MS-TOF** techniques to other drugs implicated in DFSA. This will minimize sample requirements and provide sensitive, disposable analytical systems for high throughput analysis.

The development of microfluidic devices for the rapid isolation and detection of drugs of abuse

3. Introduction and Historical Overview

Drug facilitated sexual assault (DFSA) is a heinous crime which causes both physical and psychological distress to its victims. Along with the immediate physical harm there is a risk of contracting sexually transmitted diseases [Reynolds] and unwanted pregnancies (Holmes). But far worse for the victim is the emotional scarring. Victims may spend their entire lives trying to overcome this trauma, but never feel like the same person again. Further, this crime has a high rate of recidivism. It is also highly underreported and very difficult to prosecute, so offenders are often free to victimize again and again.

DFSA has been defined as the voluntary or involuntary ingestion of a drug by a victim that results in an act of sexual activity without consent. [Abarbanel] Some women are unconscious during part or all of an attack. Others, particularly those who have consumed benzodiazepines, may experience a phenomenon known as anterograde amnesia so that they are somewhat responsive during an attack, but their memory is distorted afterwards. Some women have spotty memories of an attack, while others have no memory and all. Sometimes they experience flashbacks after the fact of moments they could not initially recall.

The difficulty in prosecuting DFSA cases is compounded by society's gross misconceptions about the crime. First, the media's usage of the term "date-rape" for these cases has led most people to envision a young girl being slipped a mickey and assaulted by her date in

the resulting mind-altered state. However, the reality is that DFSA can be committed by anyone including strangers, but it is most commonly committed by someone the victim knows [Lebeau]. The second misconception is that date-rape drugs are consumed by a victim unwittingly. Many drugs are taken recreationally by a victim in a setting where she is surrounded by people she trusts. As long as the consumption of drugs leads to a mind-altered state DFSA can occur. This fact is closely tied to the third and final major misconception, which is what exactly constitutes a date-rape drug. A few illicit drugs have gained notoriety in the press as prevalent date-rape drugs, but they are not the only ones that are used, nor are they necessarily the most common.

Table 1 contains a comprehensive list of drugs that have been reported in DFSA cases [Lebeau, Julien]. It by no means precludes other substances from being encountered in prior or future cases. The long list of DFSA drugs includes illicit drugs, prescription drugs, and over-the-counter drugs which may be used alone or in potentially dangerous combinations. Alcohol is the most commonly encountered mind-altering substance involved in DFSA cases, and both victims and perpetrators may be under its influence. DFSA can involve voluntary or involuntary consumption of substances. Alcohol may be consumed by a victim, but may also be accompanied with other drugs that have been slipped into the drink or taken recreationally. It is important to note the difference between individuals who become slightly drunk and experience lowered inhibitions and those who lose control over their physical and mental faculties and are therefore legally unable to consent. DFSA can occur in the latter situation.

Table 1. Drugs that have been implicated in drug-facilitated sexual assault.

Drug Name	
Ethanol	Pentobarbital
Ethanol	Phenobarbital
GHB and Analogs	Primidone
Gamma-Hydroxybutyrate	Secobarbital
1,4-Butanediol	Thiopental
Gamma-Butyrolactone	**Over-the-Counter Medicines**
Benzodiazepines	Brompheniramine
Alprazolam	Chlorpheiramine
Chlordiazepoxide	Dextromethorphan
Clonazepam	Diphenhydramine
Diazepam	Doxylamine
Flunitrazepam	**Anti-Depressants**
Lorazepam	Amitriptyline
Oxazepam	Citalopram
Temazepam	Desipramine
Triazolam	Doxepin
Stimulants	Fluoxetine
Amphetamine	Imipramine
Cocaine	Paroxetine
Methamphetamine	Sertraline
Narcotics	**Miscellaneous Drugs**
Codeine	Carisoprodol
Fentanyl	Clonidine
Hydrocodone	Cyclobenzaprine
Hydromorphone	Ketamine
Meperidine	Methylenedioxyamphetamine
Methadone	Methylenedioxymethamphetamine
Morphine	Meprobamate
Oxycodone	Phencyclidine
Propoxyphene	Scopolamine
Marijuana	Valproic Acid
9-Tetrahydrocannabinol	Zolpidem
Barbiturates	Pentobarbital
Amobarbital	Phenobarbital
Butalbital	Primidone

A wide variety of compounds with a great variation in the mode of action are listed in Table 1. Some of these compounds are included as they are associated with risky behaviors such as rave parties where these drugs are self administered. Other compounds such as barbiturates, benzodiazepines, GHB and scopolamine are strong sedatives and have great potential use in the classic DFSA scenario in which an unsuspecting victim is given a compound in their drink [Negruz-NIJ].

Detection of Drug Facilitaed Sexual Assault Drugs

Detecting the presence of DFSA drugs can verify a victim's account when her memory cannot. Drugs may be identified in the victim's biological fluids such as urine and blood, or in the remnants of the drink the victim consumed. While numerous methods currently exist for detecting drugs, the ideal technology for analyzing DFSA specimens should have three crucial characteristics:

1) Because so many drugs may be utilized in DFSA, a screening method should be universal rather than a series of specific drug tests. Commonly used immunoassays may miss certain prescription drugs. GC/MS methods require volatility and temperature stability. For this reason methods based on electrophoresis show promise.

2) Since many date-rape drugs require very low-doses (ng) to bring about the desired effect and have fast clearance rates from the body, testing systems must be as sensitive as possible in order to test the biological specimens.

3) Techniques should be cost effective, as many testing laboratories may not have the resources for state of the art procedures.

Unfortunately these three goals are almost mutually exclusive. Highly sensitive techniques are often very specific and usually expensive. Clearly new analytical systems are needed that could be quickly applied in a variety of facilities including hospitals, crime laboratories and contract labs. Ideally they could quickly screen for the presence of these compounds and then allow a more advanced laboratory to confirm the result.

One potential solution is microfluidics. Microfluidic systems, also called labs-on-a-chip, have become of interest to forensic scientists and analysts of small, toxicological specimens because of their potential for high speed, highly sensitive detection [Verporte]. They separate compounds electrophoretically with a series of computer-controlled electrodes that manipulate a sample through a network of channels on a small chip. These miniature capillary electrophoresis (CE) systems are compatible with a wide range of analytes [Beard, Curre, Viskari]. Currently several commercial microfluidic systems exist for the detection of dsDNA based on capillary electrophoresis and fluorescence detection. Due to economy of scale, such systems can be made cheaply enough to be supplied to many laboratories. Issues with contamination are minimal because they are disposable. The key to their application is to develop appropriate analysis and detection methods.

In order to further improve the speed of analysis efforts have been made to incorporate sample preparation steps such as extraction and derivatization online [Beard 2004]. The ultimate goal is to create a *micro* Total Analysis Systems (μTAS). The μTAS would have all of the components necessary to complete an analysis from sample preparation to detection, and would be housed in a small portable unit. This would utilize microchips that are easy to use, inexpensive, and disposable. Under these circumstances, microfluidic systems could be a

valuable tool for performing initial screening of DFSA specimens. Since a majority of negative specimens can be ruled out in this manner, only the remaining few, potentially positive samples would need to undergo more expensive and time consuming confirmatory testing.

The goal of this project was to explore currently available microfluidic systems for the detection of illicit drugs, particularly date rape drugs, and examine various approaches for their analysis. In the development of these procedures, initial testing was performed using off-line techniques coupled with capillary electrophoresis. Systems which were proven useful were then further modified to an on-chip platform. Among the procedures developed in this work were protocols for the inline extraction and analysis of drugs of abuse from toxicological samples [Alnajjar] microfludic systems for the indirect detection of benzodiazepines [Bishop], microfluidic detection systems for the fluorescent detection of phenethyl amines, [Turner] CE/MS-TOF procedures for the direct detection of various drugs implicated in DFSA [Dehere] and monolithic stationary phases for the preconcentration and detection of benzodiazepines in toxicological samples [Blas].

4. Direct detection of phenethyl amines on microfluidic devices

Introduction

Laser induced fluorescence (LIF) is the most common detection method employed with microfluidic systems, primarily due to its high sensitivity. Although UV has been successfully used to detect drugs by traditional CE, its sensitivity on microchips is limited by short path lengths. The sensitivity of LIF is typically several orders of magnitude greater than UV. Although most drugs are not naturally fluorescent, many drugs found in DFSA cases such as phenethyl amines, opiates, cocaine and nitrobenzodiazepines contain amine moieties which may be derivatized with reactive fluorescent dyes.

In this work, the Micralyne µTK microfluidic system was tested for use as a portable screening device for detection of DFSA drugs. This system consists of a computer controlled electrode array coupled to a 532 nm υ-doubled green Nd:YAG laser for LIF detection. Because this laser was not in the fluorescence excitation range of the fluorescein dyes which we had were previously tested, we selected a new series of derivatizing agents, which utilized rhodamine and eosin based dyes. These dyes had absorption wavelengths ranging from 521 nm to 583 nm (Table 2). The dyes were coupled to a variety of reactive groups including isothocyantates, succinamidyl esters thionyl chlorides and dichlorotriazines. The various derivatizing agents were evaluated for reactivity and sensitivity when coupled to amine-containing drugs. In addition it was important to produce clean, easily- separated drug conjugates with good spectral and chromatographic characteristics.

Table 2: Amine-reactive dyes that absorb in the 540-590 nm range (Invitrogen)

Dye	**Abs. λ (nm)**	**MEC ($cm^{-1}M^{-1}$)**
Rhodamine B Isothiocyanate	540	
Tetramethylrhodamine-5-isothiocyanate	543	99,000
Lissamine™ rhodamine B sulfonyl chloride	568	88,000
5-carboxytetramethylrhodamine *N*-succinimidyl ester	546	95,000
Tesas Red® C_2-dichlorotriazine	583	87,000
Eosin-5-isothiocyanate	521	95,000

Four basic drugs which contain either primary (amphetamine) or secondary amines (methamphetamine, ephedrine, and ketamine) were initially selected for derivatization experiments. Other compounds examined included amine metabolites of nitrobenzodiazepines,

ketamine and methcathanone. Demethylated forms of tertiary amines such as morphine and cocaine were also examined.

Materials and Methods

Chemicals

Drug standards were purchased in 1.0 mg/mL of methanol standard solutions from Cerilliant (Round Rock, TX, USA). Some of the derivatizing agents, rhodamine B, rhodamine B isothiocyanate (RITC), and eosin 5-isothiocyanate were purchased from Sigma-Aldrich (St. Louis, MO, USA). 5-carboxytetramethylrhodamine (5-TAMRA), tetramethylrhodamine 5-isothiocyanate (5-TRITC), 5-carboxytetramethylrhodamine *N*-succinimidyl ester (5-TAMRA, SE), Lissamine™ rhodamine B sulfonyl chloride, and Texas Red® C_2-dichlorotriazine were purchased from Molecular Probes (Eugene, OR, USA).

Chemicals

Methanol and sodium bicarbonate were purchased from Fisher Scientific (Fairlawn, NJ, USA). Methyl sulfoxide (DMSO) and *N*,*N*-dimethylformamide also came from Sigma-Aldrich. β-cyclodextrins were purchased from TIC America (Portland, OR, USA) and sodium dodecyl sulfate (SDS) from Sigma-Aldrich (St. Louis, MO, USA).

Instrumentation

Capillary Electrophoresis Procedures

Resolution optimization experiments were run on a Beckman Coulter P/ACE 5000 series CE (Fullerton, CA, USA) equipped with a 488 nm argon-ion laser or a 532 nm solid state laser. Capillaries with a polyimide coating from Polymicro Technologies (Phoenix, AZ, USA) had 50 m ID and were 40 cm long, (32 cm to the detector). New capillaries were rinsed at high pressure

(20 psi) with 0.1 M NaOH for 3 minutes followed by distilled H_2O for 2 minutes. Before every run they were rinsed at 20 psi with run buffer. The NaOH/H_2O rinse was repeated as necessary if peaks became skewed or runs were not reproducible. In order to simulate microchip separations samples were injected at the cathode end of the capillary, 8 cm to the detector.

Microfluidic Procedures

A Micralyne µToolKit or µTK was used to perform the electrophoretic separations. It contained a high voltage power supply and an electrode stage which was controlled by LabView software from National Instruments (Austin, TX, USA). A 532 nm υ-doubled green Nd:YAG laser was coupled to the µTK for LIF detection in a confocal orientation. A PMT with a 568.2 nm bandpass filter collected the fluorescence emission which was recorded as an electropherogram in LabView.

Low fluorescence Schott Borofloat™ glass chips from Micralyne (Edmonton, Alberta, Canada) had a simple cross intersection (Figure 1a). The injection channels were 9.64 mm and the separation channels were 90.28 mm with 80 mm between the intersection and the end of the separation channel (effective length). Each channel was D shaped, 50 µm wide and 20 µm deep (Figure 1b). The wells had a diameter of 2 mm and held up to 3.46 µL of liquid each.

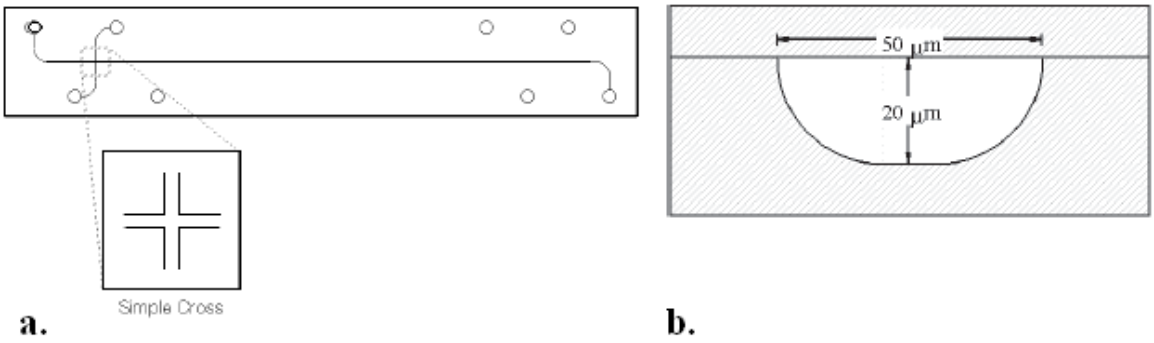


Figure 1. Micralyne's low fluorescence Schott Borofloat™ glass chip (Micralyne).

New chips were rinsed with 1.0M NaOH followed by H_2O for 5 minutes each. The chips were additionally rinsed with H_2O followed by run buffer for 5 minutes each between each run. When chips became clogged or dyes adsorbed onto the surfaces of the channels NaOH was also used to rinse between runs. The chips were rinsed by submerging them in a solution and applying suction to one well with a plastic syringe. Syringes were fitted with micropipette tips cut to form a tight seal with the microchip wells, and suction was applied by pulling back on the plunger for the desired amount of time.

Chips were filled by placing 3 µL of run buffer into three of the wells, the sample waste, buffer inlet, and buffer waste reservoirs with a micropipette. Capillary action pulled the buffer through the channels. Motion of the liquid was observed under a microscope until the channels were completely filled. Then 3 µL of sample was placed in the sample inlet reservoir and the chip was placed on the electrode stage. Voltages were applied to the electrodes in a two step process, [Crabtree]. The first step forms the sample plug in the intersection, and the second step performs the separation

Derivatization Procedure

The derivatization procedure was adapted from a method developed by Alnajjar, et. al. for derivatizing opiates with FITC. [Alnajjar] Stock solutions containing 1.0 mg/mL of each drug were prepared in methanol and were diluted 100 fold prior to derivatization. 2.5 mM solutions of each fluorescent dye were prepared in DMSO or DMF depending on manufacturer's recommendations and stored in 7 mL amber vials to protect the dyes from degradation. An aqueous solution of 20 mM sodium bicarbonate with the pH adjusted to 8.5 with NaOH was used as the reaction buffer. All solutions were stored in a refrigerator at 4°C.

To derivatize 100 µL of a drug solution, 200 µL of the dye solution, and 100 µl of reaction buffer were placed in a 600 µL PCR tube. Tubes were capped, shaken, and then stored in a thermocycler with an amber lid. Reactions were heated to the specified temperature using an ABI 480 thermocycler.

Results and Discussion

Rhodamine Dyes

Rhodamine isothiocyanate (RITC) and tetramethylrhodamine isothiocyanate (TRITC) were tested first because previous experiments had shown that isothiocyanates are reactive with primary and secondary amines. The three phenethyl amines, but not ketamine, were successfully reacted with both RITC and TRITC, and the derivatives were detected on the µTK. However, at room temperature the isothiocyanate reactions required about 24 hours to go to completion.

In order to increase reaction rates and improve throughtput for online screening tests, the reactions were examined at higher temperatures. Derivatizations were performed at 10°C intervals from 30°C to 80°C, but heating resulted in degradation of the dye. Between three and

eight peaks were observed on the electropherograms, the number increasing with the temperature applied.

Two additional reactive dyes were tested which to our knowledge have not yet been utilized as probes for small molecules. Lissamine™ rhodamine B sulfonyl chloride and 5-TAMRA, SE did not have the degradation problems observed with the isothiocyanates. However, the sulfonyl chloride was found to have poor reactivity with all of the targeted amines, and the reactivity of the succinimidyl ester was limited to primary amines (Figure 2).

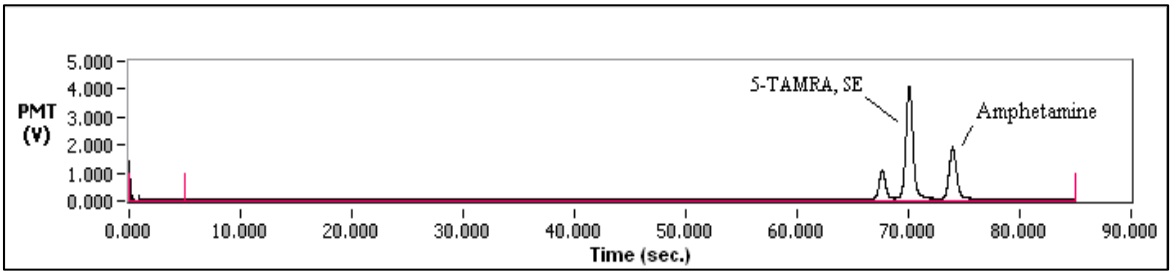


Figure 2. Microfluidic detection of methamphetamine, amphetamine, and ephedrine derivatized by RITC for 30 minutes at 80°C. Run buffer: 50 mM sodium phosphate, 15 mM β-cyclodextrin, pH = 3.5.

Because most drugs are either secondary or tertiary amines, neither of these probes would be appropriate. One final rhodamine based dye, Texas Red® C_2-dichlorotriazine was examined but had an excitation wavelength that was apparently too high for the 532 nm laser and produced no detectable fluorescence. Another problem observed with the rhodamine dyes was apparent adsorption to the capillary walls.

Eosin Isothiocyanate

As a result of the above problems, eosin isothiocyanate was selected as an alternate derivatizing agent for our experiments. Eosin, like fluorescein, lacks the cationic charge that caused adherence to channel walls and decomposition problems at elevated

temperatures. In addition isothiocyanates with both fluorescein[31] and rhodamine dyes have been proven to be reactive with primary and secondary amines.

Amphetamine, methamphetamine, and ephedrine were successfully derivatized with EITC with no degradation effects of the dye. The detection limit for amphetamine with this probe was 20 ppb. Completion of the derivatization reactions required about 24 hours at room temperature. This however can be reduced through increasing the temperature. [Alnajjar] No derivatization of ketamine was observed even when heat was applied and the reaction was allowed to proceed for 72 hours. It is probable that this is due to the steric hindrance of the amine group as compared to that on the phenethyl amines.

Buffer and pH effects

The pH of a run buffer influences the degree to which analytes are ionized and therefore the rate at which they travel through a capillary when an electric field is applied. Two buffers, one slightly acidic, and one basic, were analyzed to determine which would yield the best separation of the basic drugs. A 20mM aqueous solution of 2-(N-Morpholino)ethanesulfonic acid (MES) had a pH of 6.1. The second buffer was an adaptation of Alnajjar's buffer for separating FITC derivatives of opiates was used for our method [Alnajjar]. This basic buffer was composed of 20 mM $Na_2B_4O_7$, and the pH was adjusted to 9.8 with NaOH. While neither buffer yielded complete separation of the three phenethyl amines, resolution was best with the borate buffer (Figure 3)

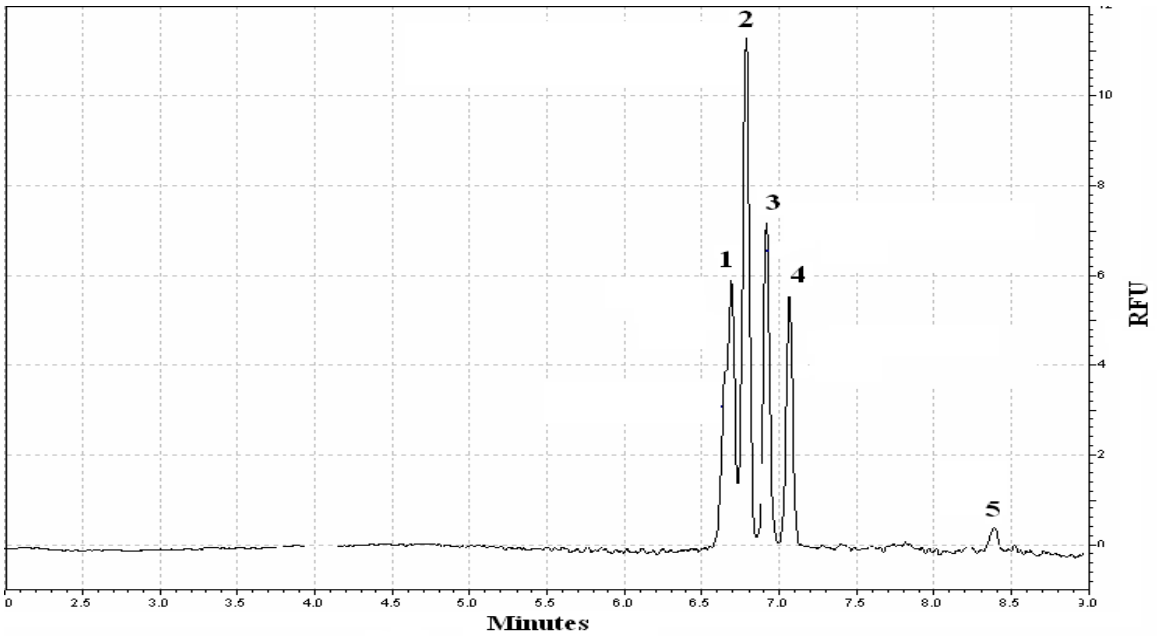


Figure 3. Separation of four phenethyl amines in a run buffer of 20 mM sodium borate, 20 mM β-cyclodextrin, 10% isopropanol, 10% acetonitrile, pH = 9.8. The peaks are 1. MDMA, 2. methamphetamine, 3. amphetamine, 4. ephedrine, and 5. EITC.

When dealing with compounds of a similar size and charge, a solution with a pH close to the pKa's of the analytes will create a difference in the extent to which each compound is ionized in the solution and therefore the speed at which they travel under an electric field. The pKa's of amphetamine, methamphetamine, and ephedrine are between 9.8 and 10.1. However, since the separation with a buffer in the target pH range was insufficient, further optimization was needed. The use of pseudo-stationary phases was therefore examined to improve separation

Pseudo-Stationary Phases

Pseudo-stationary phases travel through a capillary under the influence of the electroosmotic flow and if they are charged, an electrophoretic flow as well. Although these phases are not truly stationary they move at a rate independent of the analyte mobility. Neutral and ionic analytes partition themselves between the bulk solution and the pseudo-stationary

phases. When in the bulk solution, the analytes move at a velocity determined by their electrophoretic mobility. But, when they are in the pseudo-stationary phase they travel at a rate determined by the mobility of that phase. Each analyte has a different attraction or selectivity for the pseudo-stationary phase, so its overall speed is influenced differently, either faster or slower. Appropriate phases can be chosen to further separate analytes that are not fully resolved.

Micellar electrokinetic chromatography (MEKC) is an electrophoretic technique which uses micelles as the pseudo-stationary phase [Terabe]. Analytes partition between the aqueous buffer and the micelle phase. Sodium dodecyl sulfate, SDS, is a common surfactant used in MEKC that has negatively charged sulfate groups at the head and it moves counter to the osmotic flow in the capillary.

Another type of pseudo-stationary phase are the cyclodextrins. These cylindrical macromolecules are composed of sugar molecules connected to each other in a ring and contain polar hydroxyl groups on the exterior and hydrophobic groups on the interior. They are named for the number of sugar molecules used, and are called α(6)-, β(7)-, or γ(8)-cyclodextrins. Drug analytes also partition into cyclodextrins by forming inclusion complexes in the hydrophobic center [Lurie].

The effects of SDS and β-cyclodextrin on the resolution of amphetamine, methamphetamine, and ephedrine were compared utilizing two buffers which were previously optimized for separation of FITC derivatives [Alnajjar 2004]. SDS yielded complete coelution of amphetamine and ephedrine (Figure 4) while β-cyclodextrin yielded resolution of all three drugs (Figure 5). β-cyclodextrins also altered the selectivity of the run buffer so that the analytes eluted earlier, prior to the excess dye, and in a different order as compared to SDS. Because it yielded at least partial separation of all three analytes, β-cyclodextrin was selected as

the pseudo-stationary phase for our buffer. In addition it has the advantage of potential coupling with mass spectrometry for confirmatory testing. The surfactant nature of SDS and other micelles prohibits their use in mass spectrometry. However, further enhancement of selectivity using organic modifiers was still needed in order to achieve optimal resolution.

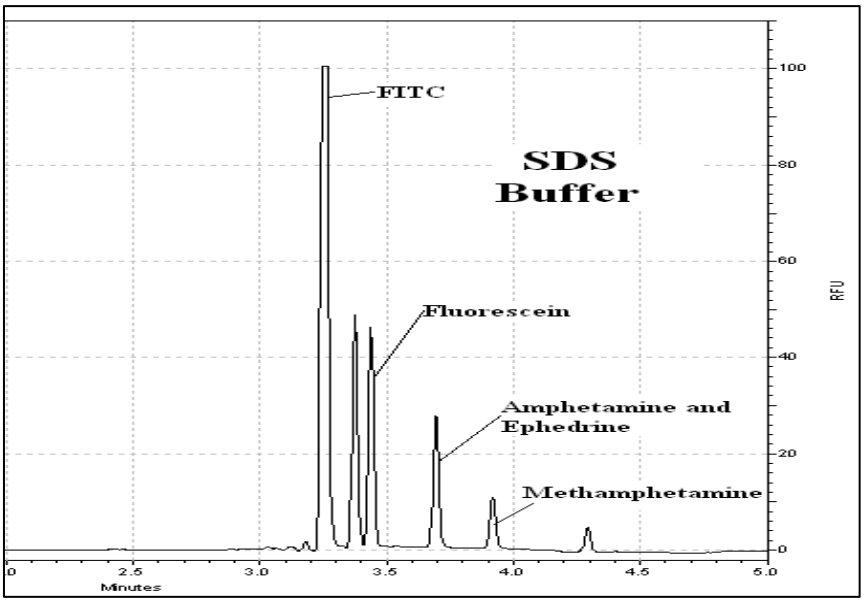


Figure 4. Separation of three phenethyl amines in a run buffer of 10 mM sodium phosphate, 6 mM sodium borate, and 75 mM SDS.

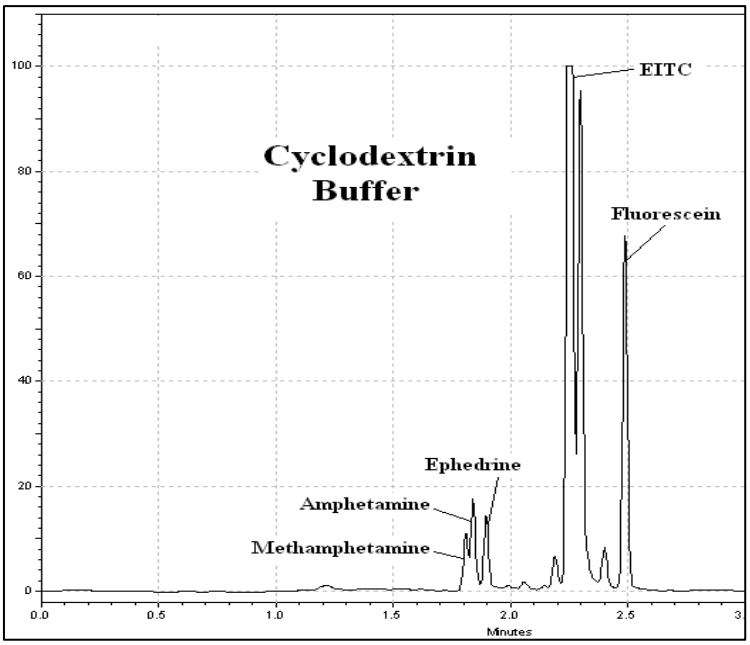


Figure 5. Separation of three phenethyl amines in a run buffer of 20 mM sodium borate, 20 mM β-cyclodextrin, 10% isopropanol, 10% acetonitrile, pH = 9.8.

Organic Modifiers

Small amounts of organic solvents may be added to a run buffer with a pseudo-stationary phase in order to influence the selectivity. The hydrophilicity of the bulk solution is affected thereby altering the attraction of each analyte for the pseudo-stationary phase and its mobility.

Acetone, acetonitrile, and isopropanol were independently added to the run buffer in 5% increments ranging from 5% to 25%. For all three solvents resolution improved further with increasing concentration until a plateau was achieved at approximately 20% (Figure 15). Also, significant peak tailing started to appear at concentrations above 20%. Optimal results were obtained with 20% isopropanol. In addition a 50/50 mixture of acetonitrile and isopropanol yielded resolution nearly equal to that of isopropanol alone with a lower overall run time.

This final buffer with 20% isopropanol was used to analyze a mixture of phenethyl amines including MDA, methamphetamine, amphetamine, ephedrine, and methcathinone. Other compounds observed to produce derivatives included amine metabolites of nitobenzodiazepines such as 7-aminoclonazepam and 7-aminoflunitrazepam, and demethylated tertiary amines such as normorphine and norcocaine.

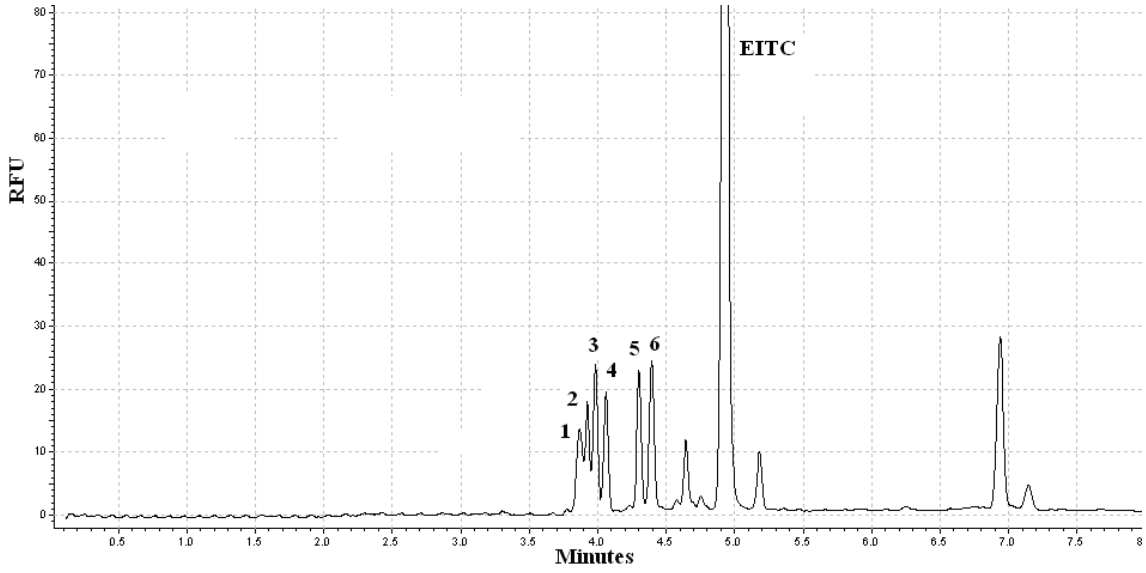


Figure 6. Separation of six drugs in a run buffer of 20 mM sodium borate, 20 mM β-cyclodextrin, 20 % isopropanol, pH = 9.8. The peaks are: 1. MDMA, 2. methamphetamine, 3. amphetamine, 4. ephedrine, 5. methcathinone, and 6. normorphine.

Microfluidic Resolution

While the optimized buffer yielded nearly complete separation of the three drugs, when samples were run on the µTK with the same buffer, poorer resolution was observed due to the shorter separation channel utilized. Methamphetamine, amphetamine, and ephedrine could be distinguished, but additional analytes in the mixture such as MDMA coeluted with these peaks and could not be distinguished. While this is less of a problem when the system is used as a general screening technique, further experiments focused on improving resolution of the microchip systems.

One particular improvement involved adding external reservoirs to the chips to minimize evaporation and permit longer run times. These reservoirs (nanoports) were glued with epoxy resin to the surface of the chips to create wells for holding sample and buffer. This created an opening that holds approximately 50 µL of fluid. The wells eliminate problems with

evaporation and siphoning effects. After several uses we noticed that the epoxy seals were breaking down and the bases were coming free from the microchips. This was due to the presence of acetonitrile in the run buffer. This problem was solved by switching to a run buffer which contained only isopropanol (20%) instead of an isopropanol/acetonitrile mixture. This was originally the preferred organic solvent (Figure 7).

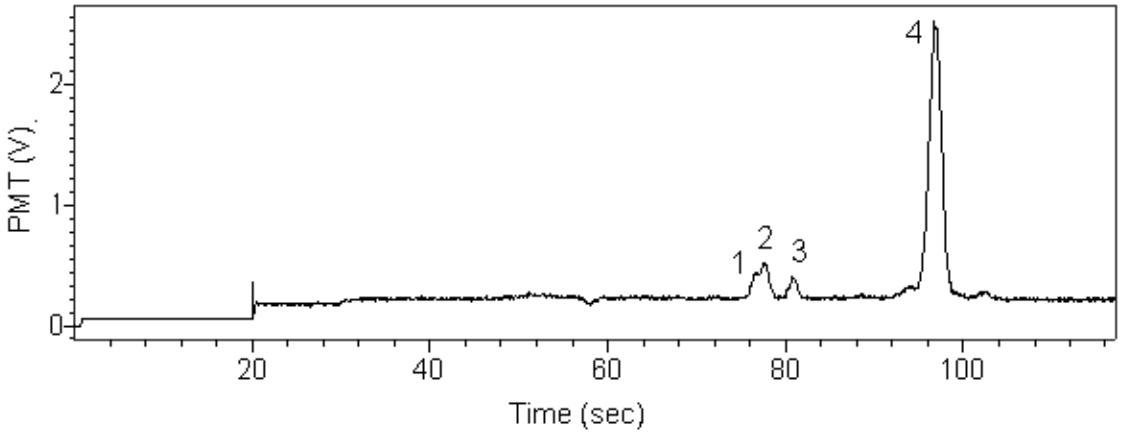


Figure 7. Separation on the μTK with the optimized run buffer of 20 mM sodium borate, 20 mM β-cyclodextrin, and 20% isopropanol, pH = 9.8, E = 250 V/cm. The peaks are 1. methamphetamine, 2. amphetamine, and 3. ephedrine.

Conclusions

A derivatization scheme for drugs containing primary and secondary amines was developed utilizing Eosin isothiocyanate. The technique involves mixing the drug and reactive dye in a 20 mM sodium bicarbonate solution at a pH of 8.5 and permits the detection of low levels of phenethyl amines (20 ng/mL on chip.) The reaction is suitable for trace detection of phenethyl amines in urine and other body fluids especially when combined with a suitable extraction procedure for further preconcentration. A run buffer with pH 9.8, close to the analytes' pKa's; a pseudostationary phase of β-cyclodextrins; and 20% isopropanol to alter the

selectivity yielded the best resolution. Future work will involve experiments with longer capillary channels to enhance resolution.

5. Indirect detection of benzodiazepines and GHB on microfluidic devices

While direct fluorescence is the most sensitive detection method for drugs of abuse, not all compounds produce fluorescence and derivatization is an additional step necessary prior to analysis. Alternatively, indirect laser induced fluorescence is a method where a fluorophore is added to the electrophoretic buffer so that the baseline is constantly elevated [Kuhr]. The presence of analytes will result in a decreased baseline, giving a negative peak as a signal.

Several papers have been published detailing the detection of explosives with indirect laser induced fluorescence [Bailey, Goodpasture, Wallenborg]. Bailey and Wallenborg did their initial work on a CE and then showed that the method could be translated to a microchip. Instead of the charge displacement that can occur, the decrease in fluorescence was determined to be due to the quenching effects of the nitroaromatic compounds.

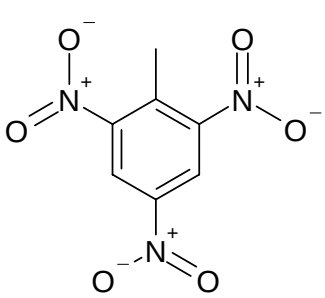
The mechanism here is thought to be dynamic quenching where an excited state charge transfer complex is formed between the fluorophore and the quencher. Normally, when the fluorophore absorbs a photon it relaxes from the excited state by releasing another photon. If an electron acceptor is present, an electron will be transferred from the fluorophore to the quencher. This decreases the energy of the fluorophore without releasing a photon and so no fluorescence is observed. Nitrated compounds are strongly electron withdrawing and will form

strong complexes with large quenching constants. This will happen if the free energy of the reaction is less than 0, or

$$\Delta G = E(F/F^{+}) - \Delta E_{F\text{-}F^{*}} - E(Q/Q^{-})$$

where $E(F/F^{+})$ and $E(Q/Q^{-})$ are the redox potentials of the fluorophore and quencher, and $\Delta E_{F\text{-}F^{*}}$ is the lowest singlet-singlet excitation energy of the fluorophore (Yang). Based on this equation, compounds with less negative reduction potential give a more favorable complex and are more likely to be detected.

As not much work has been done using the commercially available microfluidic system, the first step was to ensure that it worked as expected. In order to do this, the Bailey and Wallenborg method [Wallenborg] was reproduced. Eight nitroaromatic explosives, including TNT were detected on a microfluidic system by indirect laser induced fluorescence. Figure 8 compares the structure of TNT and flunitrazepam. Based on these results we believed it should be possible to adapt this method to the detection of nitro benzodiazepines. Thus flunitrazepam and its metabolite desmethylflunitrazepam, as well as clonazepam, and nitrazepam, should also be amenable to this approach and we examined their separation and indirect detection on microfluidic devices.



2,4,6-Trinitrotoluene

Flunitrazepam.

Figure 8: Structures of TNT and Flunitrazepam

Experimental

Reagents

Explosive standards were purchased from Radian International (Austin, TX). HPLC grade acetonitrile, boric acid, sodium dodecyl sulfate, and methanol were obtained from Fisher Scientific (Pittsburgh, PA). Monobasic sodium phosphate, dibasic sodium phosphate, and sodium tetraborate were purchased from Acros (Morris Plains, NJ). The benzodiazepines were purchased from Lipomed (Cambridge, MA). Cy5 Monohydrazide was purchased from Amersham Biosciences (Piscataway, NJ). The buffer for explosive analysis from the paper by Wallenborg and Bailey consisted of 50 mM boric acid, 10 mM sodium tetraborate, 50 mM SDS, and 1 μM Cy5 dye at pH 8.5 [Wallenborg]. The adjusted buffer was 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 buffer used in analysis of the benzodiazepines contained boric acid, sodium tetraborate, SDS in a 5:1:5 ratio and Cy5 dye. The explosives were dissolved in 47.5 μL buffer and 2.5 μL acetonitrile, as per the paper by Wallenborg and Bailey. The benzodiazepines standards were prepared by dissolving the drug in 50 μL buffer.

Instrumentation

All separations were conducted on a Micralyne (Edmonton, Alberta, Canada) μTK system with a 750 nm diode laser and photo multiplier tube (PMT) for detection. Simple cross microfluidic chips composed of borofloat glass from Micralyne were used. The channels were semicircular, measuring 50 μm wide at the top and 20 μm deep. The injection channels from the buffer, analyte, and analyte waste reservoirs were 9.64 mm long. The separation channel had a total length of 80.89 mm from the injection point and the effective lengths in the explosive and benzodiazepine studies were 55 mm and 45 mm, respectively. The sample reservoirs held 3 μL of buffer or analyte. New chips were preconditioning by flushing with 1.58 M HNO_3 followed by 1.0 M NaOH and deionized water. Chips were rinsed with 1.0 M NaOH at the beginning of each day and between runs they were rinsed with deionized water for 15 minutes and buffer for 1 minute.

In the explosive study, samples were injected for 60 seconds with 100%, 0%, 90%, and 90%, of the high voltage being applied at the analyte, analyte waste, buffer, and buffer waste reservoirs, respectively. To carry out the separation, the applied voltages to the analyte, analyte waste, buffer, and buffer waste reservoirs were 60%, 60%, 100%, and 0% of the high voltage, respectively. The same relative voltages were used in the benzodiazepine study, however the injection time was decreased to 20 seconds. Separations were carried out with the maximum voltage ranging from 1 to 4 kV. Labview software was used to record the separation data and ChromView used for all integrations.

Optimization of Method for Benzodiazepines

A standard containing 250 μg/mL of the four benzodiazepines and 25 μg/mL of 2,4-DNT as the internal standard was used in the optimization. The buffer used for the explosive analysis resulted in lengthy run times for the benzodiazepines and so further optimization was done. Buffers containing 29.25, 25, 20, and 15 mM SDS were run with maximum voltages of 2.0, 3.0, and 4.0 kV. Buffer solutions containing 15 mM SDS were prepared with 10.0, 15.0, 20.0, and 25.0 % acetonitrile as well as 10.0, 15.0, and 20.0 % methanol. These separations were performed at 3.0 and 4.0 kV. From the optimization, the best buffer was determined to consist of 15 mM boric acid, 3.0 mM sodium tetraborate, 15 mM SDS, and 20.0 % methanol.

Extractions

The solid-phase extraction columns were prepared by drawing 2 mL methanol, 2 mL deionized water, and 1 mL 100 mM phosphate buffer, pH 6, through the column. The sample was then loaded at 2 mL/min. The column was then rinsed by drawing 2 mL deionzed water through the column followed by 2 mL 20 % acetonitrile in 100 mM phosphate buffer. The column was dried and 2 mL hexanes drawn through with vacuum. The benzodiazepines were eluted with 2.0 mL CH_2Cl_2/IPA/NH_4OH (78/20/2). The eluate was then evaporated to dryness with N2 and reconstituted in 100 μL of buffer containing 2,4-DNT as an internal standard.

In the liquid-liquid extraction, 1.0 mL of a standard containing the four benzodiazepines at 10 μg/mL was used. 100 μL buffered phosphate was added before extracting twice with 0.50 mL ethyl acetate. The organic layer was isolated and dried with a stream of nitrogen. The sample was then reconstituted in 100.0 μL of buffer containing 2,4-DNT as an internal standard.

Results and Discussion

Initial work on buffer optimization used a standard that consisted solely of TNT, as this was the strongest quenching explosive. Separations were attempted with the buffer published by Wallenborg and Bailey for the explosive method, however these resulted in an over-current shutdown of the μTK system when a maximum voltage above 1 kV was used [Wallenborg]. A current that was higher than the system could handle suggested that Joule heating was occurring. By running the separation at a voltage of 1.0 kV, the TNT took over two and a half times longer to elute than had been reported. To fix this problem, the ionic strength of the buffer was reduced by decreasing the concentrations of the buffer components. This was performed by diluting the stock buffer solution so that it had a Cy5 content of 5 μM. While the reduction in the concentrations of buffer components decreased the run time it was still longer than might be desired. The detector was moved from 65 to 55 mm, reducing the retention times of the explosive by about 30 seconds and putting them in a more acceptable range.

Once it had been established that TNT was eluting at a reasonable time with the modified buffer, a standard consisting of eight explosives at 50 μg/mL was run with the method. This separation is shown in Figure 9. The explosives were determined to elute in the same order established by Wallenborg and Bailey [Wallenborg]. It can be seen that the peaks are not of uniform size. The size of the peak, and therefore the quenching effect, appears to be related to the number of nitro groups on the aromatic ring. TNT and tetryl, which have 3 nitro groups, have the strongest peaks in the separation. Those explosives with only one nitro group give the weakest signals. Three of the explosives with three nitro groups produce peaks of a similar size, however 2,4-DNT gives a much stronger signal. While stronger quenching abilities may be related to structure, they can mathematically be related to reduction potentials. Those

compounds with higher reduction potentials will quench the Cy5 more than others. TNT, NB, and NT have reduction potentials of -0.7, -1.15, and -1.2 V, respectively. The strength of the signals follows this order accordingly.

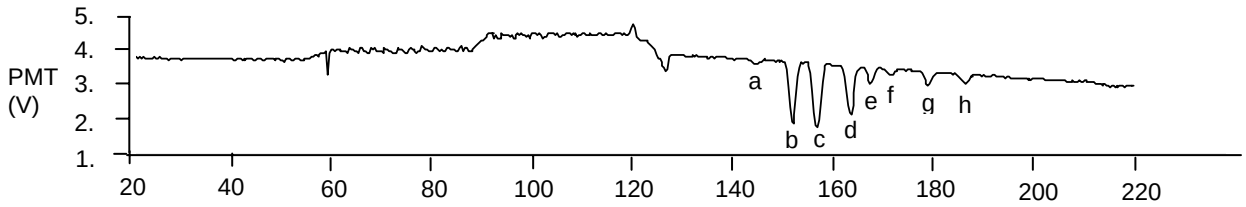


Figure 9: Separation of eight explsovies on a microfluidic chip: a) NB, b) TNT, c) tetryl, d) 2,4-DNT, e) 2,6-DNT, f) 4-NT, g) 2-Am-4,6-DNT, h) 4-AM-2,6-DNT. The buffer consisted of 29.3 mM boric acid, 5.9 mM sodium tetraborate borate, 29 mM SDS, and 5 μM Cy5 dye at pH 8.5. The run voltage was 2 kV and the detector was at 55 mm from the injection point.

Once it was established that the instrument functioned properly and a separation was possible, the analyte of interest was switched to benzodiazepines. The buffer used for the explosives was used initially for the benzodiazepines. This resulted in separation times that lasted about 147 seconds with a 20 second injection period. This was considered to be too long so further optimization was done. The capillary length was shortened to 45 mm, which reduced the run time by 10 seconds. As the pH of the explosive buffer was already optimized, this was maintained throughout the optimization. Lower concentrations of dye than used for the explosives were found to work if the PMT gain was raised. While keeping the maximum separation voltage at 2 kV, decreasing the concentration of buffer components resulted in an

increased separation time. The net mobility of ions in CZE is a function of the electroosmotic flow (EOF), which is driven by the electrical double layer formed by buffer cations and the negatively charged silanol groups on the capillary walls. Fewer cations in the buffer will slow down the EOF. The reduced buffer concentrations also decreased the ionic strength and therefore the current generated in the capillary. Therefore, the maximum voltage could be increased from 2 to 4 kV. The doubling of the voltage greatly decreased the separation time. Based on decreasing mobilities, the buffer containing 15 mM SDS, 15 mM boric acid, and 3 mM sodium tetraborate was determined to be optimal when the separation was carried out with a maximum voltage of 4 kV. The three benzodiazepines eluted within 80 seconds using this buffer system.

While desmethylflunitrazepam had a distinguishable mobility, flunitrazepam and clonazepam coeluted. Organic modifiers are known to affect the partition coefficients of neutral compounds into micelles, thus increasing the resolution between two peaks. While a number of different solvents were examined, a buffer containing 20% methanol was determined to be the best choice for a number of reasons. While acetonitrile tended to give a smoother baseline it had a system peak of considerable size. The methanol containing buffer produced a smaller system peak and had better efficiency. Figure 10 illustrates the results of some of these experiments.

The separation of the four benzodiazepines with this buffer is shown in Figure 11 as this buffer was used in the calibration and extraction of the benzodiazepines.

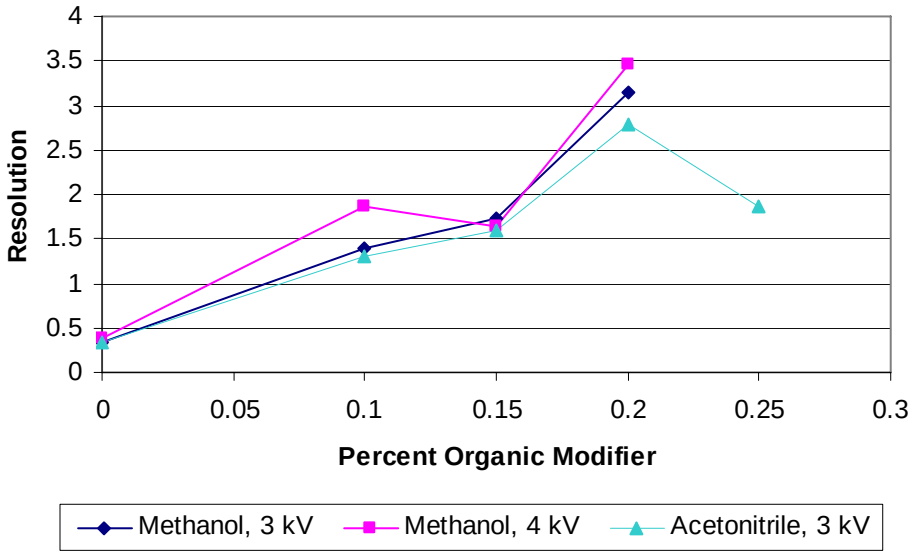


Figure 10. Resolution of flunitrazepam and clonazepam with adjusted concentrations of organic modifier. The conditions are the same as in Figure 3.4.

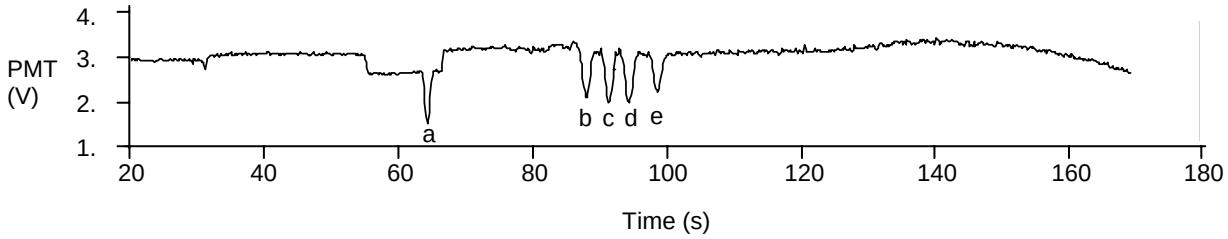


Figure 11: The separation of four benzodiazepines: a) 2,4-DNT, b) desmethylflunitrazepam, c) flunitrazepam, d) nitrazepam, e) clonazepam. The run buffer consisted of 15 mM SDS, 15 mM boric acid, 3 mM sodium tetraborate, 2.6 μM Cy5 with 20% methanol. The maximum voltage was set at 4.0 kV and the detector was at 45 mm.

Using the 20 % methanol buffer a calibration curve was constructed for four benzodiazepines using peak areas relative to that of the internal standard. The benzodiazepines were run at concentrations ranging from 25 to 250 μg/mL. The calibration data and limits of detection are listed in Table 3. Two of the benzodiazepines were highly linear, with R^2 values above 0.99.

Table 3: Calibration data and limits of detection for the benzodiazepines[a]

Compound	Slope	R^2	Detection limit L)
Desmethylflunitrazepam	0.0043 ± 0.0005	0.9620	66.7
Flunitrazepam	0.00391 ± 0.00005	0.9995	7.86
Nitrazepam	0.0037 ± 0.0002	0.9901	33.5
Clonazepam	0.0028 ± 0.0004	0.9490	77.7

[a] The conditions were the same as in Figure 11

As an estimate of reproducibility, mobilities of the four compounds were determined for four consecutive runs on three different days. The percent relative standard deviations for each drug are shown in Table 4.

Table 4: Run-to-run and day-to-day reproducibility[a]

Compound	Day 1 Mobility (% RSD)	Day 2 Mobility (% RSD)	Day 3 Mobility (% RSD)
IS	0.64	0.60	1.3
Desmethylflunitrazepam	0.89	0.82	1.4
Flunitrazepam	0.96	0.85	1.4
Nitrazepam	1.1	0.86	1.1
Clonazepam	1.2	0.92	1.4

[a] The conditions were the same as in Figure 11
% RSD = percent relative standard deviation

To examine the overall efficiency of the process in detecting benzodiazepines in beverages, several extractions were performed on benzodiazepines spiked in H_2O using a liquid/liquid extraction with buffered phosphate and ethyl acetate. The results from this study are shown in Table 5.

Table 5: Extraction efficiencies for the liquid-liquid extraction[a]

Compound	Percent Recovery
Desmethylflunitrazepam	81 ± 4
Flunitrazepam	79 ± 4
Nitrazepam	86 ± 6
Clonazepam	88 ± 6

[a] The buffer conditions were the same as in Figure 11. The maximum separation voltage was set at 3.0 kV

Conclusions

The use of microfluidics in the detection of nitro benzodiazepines by laser-induced indirect fluorescence has been shown. The reproducibility of the analysis is 1.4 % RSD or less. While the detection limits of the method are not sufficient for it to be used as a toxicological examinations, (8-80 ug/mL) the procedure should prove useful a screening method

for spiked beverages. Overall the work demonstrates the feasibility of using microfluidic devices as a rapid screening tool for the detection of benzodiazepines used in beverages during DFSA. Future work will involve expanding the range of compounds detected through the use of more generic indirect techniques such as ion displacement. For example Figure12 illustrates the detection of GHB using indirect fluorescence via ion displacement on a microfluidic device.

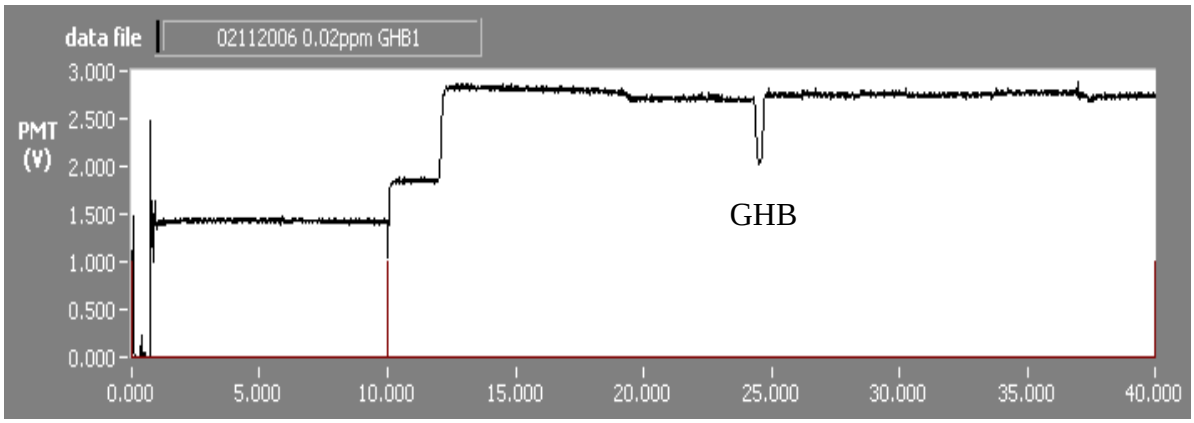


Figure 12: Indirect fluorescence detection of GHB. Buffer: 1.5mM sodium carbonate and 0.1mM eosin Y at pH9.0 6 kV separation on a micralyne glass chip. Detection occurs through the displacement of the eosin ion by GHB

6. CE/MS and Monolithic CEC/MS-TOF for inline extraction and ultratrace detection and confirmation of benzodiazepines

As the results above show, fluorescent techniques coupled with microfluidic detection can provide rapid and highly specific detection for screening drugs such as benzodiazepines and phenethyl amines. However for use in court, more definitive techniques are required. In addition, because the amount of sample is limited in microfluidic techniques, sensitivity is a key issue. For this reason we have investigated the combination of three advanced techniques,

monolithic capillary electrochromatography (CEC), electrospray CE/MS and TOF-MS to develop a separation tool for the trace detection and confirmation of the presence of a wide variety of low dose benzodiazepines implicated in sexual assault.

There are particular advantages to this approach from the standpoint of microfluidic detection. First of all, monolithic electrochromatography stationary phases are prepared by photoinitiation of a mixture of polymers and porogens pumped into a microchannel or capillary. Once a stable stationary phase is developed, these phases can be easily implemented on a wide variety of microfluidic devices without having to resort to specialized manufacturing techniques. Also, because these stationary phases are quite retentive for a wide variety of drugs, they permit preconcentration via in-line solid phase extraction followed by efficient separation using electrochromatography.

Once separated, the electrochromatography system can be coupled to electrospray TOF mass spectrometric detection. Electrospray permits efficient ionization of the eluted drugs, providing molecular ions and fragments for identification of the target drugs. Furthermore, the TOF system has high (3 ppm) mass resolution permitting further discrimination of the unknown drug based on its exact isotopic mass. The entire coupled design provides exceptional sensitivity and specificity. In this section of the report we discuss the initial development of the coupled system for the trace detection of benzodiazepines.

Detection of benzodiazepines using monolithic CEC/MS-TOF

Benzodiazepines are commonly used drugs in DFSA. [Drummer, Lebeau], mainly because they can produce anterograde amnesia. . These drugs 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 (Figure 1). Following an incident of DFSA, benzodiazepines may be present in very low

concentrations. A successful analytical method for the analysis of these compounds may require detection limits below 10 ng/mL [Lebeau 1999]. Thus a highly sensitive analytical method is required.

A diversity of procedures exist in the literature for the detection and the determination of 1,4–benzodiazepines in biological matrices. Gas chromatography–mass spectrometry (GC–MS) has often been used and has been reviewed [Maurer]. Nevertheless, (i) GC needs a derivatization step to increase the volatility of benzodiazepines [Borrey] and (ii) some benzodiazepines thermally decompose at high temperatures [Japp, Welston, Bugey]. To overcome these difficulties, the hyphenation of liquid chromatography (LC) with MS is a useful alternative to GC–MS [McClean, ElSohly]. The application of capillary electrophoresis (CE) combined with MS has also been envisaged for benzodiazepines analysis [Vanhoenacker]. However, benzodiazepine pKa values are low (1.3–4.0), which means that to perform CE analyses requires low pH. Analyses under such conditions can result in long analysis times due to weak electroosmotic flows .

In recent years, there has been a great deal of interest in exploring the potential of capillary electrochromatography (CEC), an analytical separation technique which combines the high peak efficiency of CE with the stationary phase selectivity of LC [Eeltink]. This technique proved to be suitable for the separation of benzodiazepines [Taylor, Kiyokatsu]. There are several solutions to the problem of introducing a stationary phase into a fused silica capillary. One option is to manually fill the capillary with modified silica particles using a slurry packing technique [Cahours]. However maintaining uniform packing in order to avoid bubble formation presents some difficulties. Special frits must also be developed in order to retain the phase within the capillary. Another option is to functionalize the capillary wall in order to perform open-

channel CEC. This technique suffers from low surface areas and concommitant poor loading capacities [Bruin, Ilfeffer]. A useful alternative to these two methods of column preparation involves the *in situ* polymerization of a monolithic stationary phase [Svec]. Examples of such phases includes silica sol-gels and porous organic polymers [Allen, Stulik]. Recently, Svec reviewed applications of monoliths as stationary phases for CEC [Svec, 2005]. The most popular materials used for organic monoliths are acrylate, styrene and acrylamide because their synthesis is fast and simple . For example, Barrioulet *et al.* demonstrated that the use of hexyl acrylate gives highly efficient monoliths (more than 300,000 plates *per* meter) [Barrioulet].

The hyphenation of CEC to MS combines the speed and efficiency of CEC with the selectivity and sensitivity inherent to MS. In spite of the numerous papers published on the development of monoliths, only few research groups have reported their use in CEC–MS analysis [Que]. Previously this technique has not been used in forensic science. Recently, Barcelo-Barrachna *et al.* reviewed the hyphenation of the CEC technique with mass spectrometry [Barcelo-Barrachina].

One of the main drawbacks of CEC–MS when compared to LC–MS is the low injection volume inherent in CEC, which decreases the sensitivity of analysis. However, a preconcentration step can be envisaged to improve the sensitivity. In the literature, monolithic media appear to be suitable for both extraction and preconcentration of the analyte [Svec 2006]. For example, a preconcentration step followed by a CEC separation of eight alkyl phenyl ketones and four polycyclic aromatic hydrocarbons (PAHs) was developed by Zare *et al.* [Quirino]. The preconcentration step permitted a 100-fold improvement in detection for the PAH mixture and a 1000-fold improvement in detection for test peptides when used with a gradient solvent. The principle behind the preconcentration step is to inject a dilute sample present in a noneluting

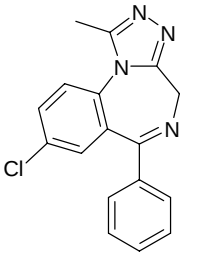
solvent such as water. Then, upon initation of CEC with and aqueous/organic buffer, the previously loaded sample is eluted and separation is initiated.

In this study, we have developed a CEC–MS(TOF) separation of benzodiazepines *via* optimization of the *in situ* synthesis and application of porous acrylate-based monoliths. The synthesis of these materials has been developed by Ngola *et al.* [Ngola] and studied by other groups [Barrioulet,Augustin]. In our studies the monolith was first characterized with a mixture of poly aromatic hydrocarbons (PAHs) to evaluate the chromatographic properties of this stationary phase. Then, this stationary phase was applied to the separation of benzodiazepines. High sensitivity and specificity were obtained by applying a preconcentration step and coupling the separation to a TOF-MS.

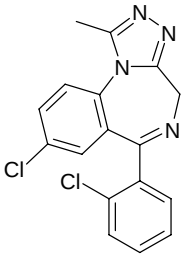
Material and methods

Reagents and materials

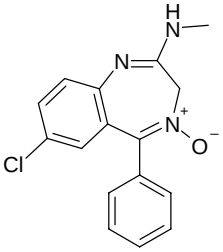
Fused-silica capillaries with a UV-transparent coating and an inner diameter of 100 µm were purchased from Polymicro Technologies (Phoenix, AZ, USA). Hexyl acrylate, 1,3-butanediol diacrylate, 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS), 2,2'-azobisisobutyronitrile (AIBN) were obtained from Acros Organics (Morris Plains, NJ, USA); trimethoxysilylpropyl acrylate, sodium phosphate and PAHs, from Sigma-Aldrich (St. Louis, MO, USA), ammonium acetate and ethyl acetate, from Ficher Scientific (Pittsburgh, PA, USA). Benzodiazepines and deuterated benzodiazepines were purchased from Cerilliant (Round Rock, TX, USA), Figure 13.



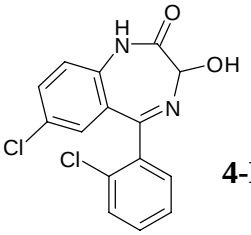
1-Alprazolam pKa=2.4[a]



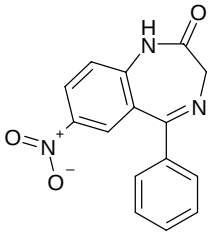
2-Triazolam



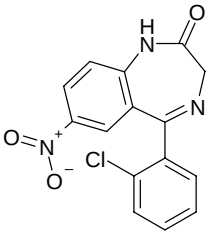
3-Chlordiazepoxide pKa=4.6[a]



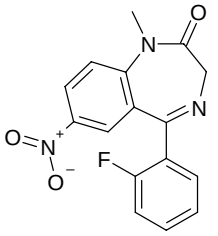
4-Lorazepam pKa=1.3, 11.5[a]



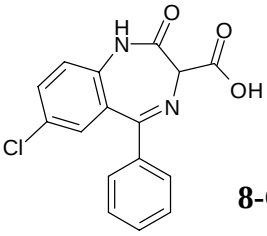
9-Diazepam pKa=3.3[b]



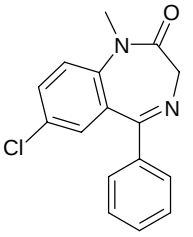
10-Prazepam pKa=2.7[a]



7-Flunitrazepam pKa=1.8[a]



8-Clorazepate pKa=3.5, 12.5[c]



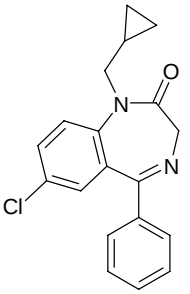


Figure 13: Structures of benzodiazepines.

Monolith polymerization

Monolithic columns were prepared in accordance with a procedure previously described by Ngola [Ngola]. Briefly, mixtures of monomers were prepared with the following molar ratio: hexyl acrylate/1,3-butanediol diacrylate (76/24 v/v), 0.5 vol% AMPS and, 0.2 mol% trimethoxysilylpropyl acrylate. A mixture of non-interactive porogens (used to create the permiable structure of the monolithic stationary phase) was made up of 60% v/v acetonitrile, 20% v/v 5 mM sodium phosphate buffer, pH 6.8 and 20% v/v ethanol. The ratio of monomer to porogenic mixture was 1:2 in the reaction mixture. AIBN (0.5%, w/w with respect to the monomers) was added to the solution as radical photo-initiator. The polymerization mixture was then sonicated to obtain a clear solution and to remove the dissolved air. The fused-silica capillary was pretreated according to the procedure described by Delaunay-Bertoncini *et al.* [Delaunay-Bertoncini]. The capillary (100 cm length, 100 µm I.D.) was filled with the polymerization mixture by immersing the inlet of the capillary into a reservoir and by pushing the polymerization solution through under gas pressure.

Afterwards, the ends of the capillary were sealed with pieces of rubber septa. To photoinitiate the polymerization mixture, the capillary was exposed for 1 h under a UV lamp (8 W, 365 nm, UVP inc., Upland, USA) at room temperature. Finally, the filled capillary was cut to the appropriate length (33 cm for UV detection experiments and 50 cm for MS detection) and was installed into the CE instrument (HP–3D, Agilent-Technologies). In order to remove the residual monomers and porogens, a voltage program ranging from 0 to 30 kV at 500 V/min, with a total duration of 120 min, was applied to the capillary.

Capillaries used for CEC–MS experiments (50 cm length) were coupled to the mass spectrometer via a sheath flow electrospray ionization (ESI) interface (Agilent, USA). For CEC–UV experiments, polymeric material was removed to create a detection zone at the capillary window. This procedure was performed through exposure at the detector interface to deuterium light from the UV source. 12 bars of pressurized nitrogen was applied to both ends of the capillary to minimize bubble formation during this step.

The analytes were composed of benzodiazepines or PAHs in the running buffer with acetone as an unretained marker. Separations were carried out at field strengths of 150–950 V/cm.

Instrumentation

The separation of the benzodiazepines was performed using a CE (Agilent Corp, Santa Clara, CA, USA). Column temperature was maintained at 25ºC. The CE system was connected to a TOF-MS (Agilent Corp, Santa Clara, CA, USA) equipped with an orthogonal electrospray interface, using the operational parameters included in Table 6 and a sheath flow liquid composed of methanol-water (50:50, v/v), containing formic acid (0.1%, v/v). The flow rate was 0.5 mL/min with a 1/100 split ratio.

The TOF–MS analysis was performed in positive ion mode using full scan mode with a mass range set at *m/z* 50–1000. The following reference masses were continuously monitored to maintain the instrument's calibration: 121.0509 and 922.0098 *m/z* (resolution of 9500±500 at 922.0098 *m/z*). Data analysis was performed using Applied Biosystems/MDS-SCIEX Analyst QS Software (Frankfurt, Germany).

The limits of detection (LOD) and the limits of quantification (LOQ) were determined *via* the analysis of a sample at 50 ng/mL and they were calculated as the analyte concentration that gave a signal-to-noise of 3 and 10, respectively.

Parameter	Value
Capillary voltage	3800 V
Nebulizer pressure	5 psig
Drying gas	5 l/min
Gas temperature	300 C
Fragmentor voltage	225 V
Skimmer voltage	60 V
Octapole RF	300 V
Mass range (*m/z*)	50–1000
Resolution	9500±500 (922.0098)
Reference masses	121.0509; 922.0098

Table 6

Liquid–liquid extraction

A liquid–liquid extraction method used to extract and concentrate benzodiazepines was previously described [Bishop]. Briefly 1.0 mL of urine was mixed with 100.0 µL of 100 mM buffered phosphate at pH 6.0 and extracted twice with 0.50 mL of ethyl acetate. The resulting mixture was vortexed and the organic layer (top one) was then dried under a stream of nitrogen and reconstituted in 200.0 µL of buffer (ammonium acetate 5.0 mM, pH 7.0) containing deuterated benzodiazepines (100 ng/mL) as internal standards (IS).

In order to calculate the extraction efficiency, two samples were prepared. For the first one, known quantities of the individual benzodiazepines were added to urine sample to reach the concentration of 10 ng/mL. The sample was extracted first and then the IS were added to the extracts. The second sample was obtained by added individual benzodiazepines and IS to the buffer to reach the concentration of 50 ng/mL. Sample 1 and 2 were then analyzed using the CEC–MS method.

Finally, the extraction efficiency was determined by comparing the drug/IS peak area ratio in the first sample to the ratio in the second sample.

Results and discussion

Monolith characterization

A monolith is a continuous porous bed which contains two pores size distributions, mesopores and macropores. Mesopores give the structure morphology with high surface area and macropores provide structural support and help to minimize back pressure. Figure 14 shows the hexyl acrylate monolith composed of microglobules, about 1 μm in diameter linked together. These pictures demonstrate that (i) the monolith is well linked to the capillary wall and (ii) the morphology of the monolith is similar to those usually presented in the literature [Barrioulet]. Regarding to the CEC proprieties, electroosmotic mobilities of $(2.5\pm0.2)\times10^{-4}$ $cm^2 V^{-1}s^{-1}$ were obtained using poly aromatic hydrocarbons which were quite similar to the values determined by Barrioulet *et al.* with the same mobile phase [Barrioulet]. Moreover, the reproducibility of all measured retention factors (k') was under 1.1%. This preliminary study shows that the morphology of the hexyl acrylate monolith synthesized in our group is able to generate high separation efficiency with good retention reproducibility. The monolith was then evaluated for benzodiazepine separation and detection using a TOF-MS.

A

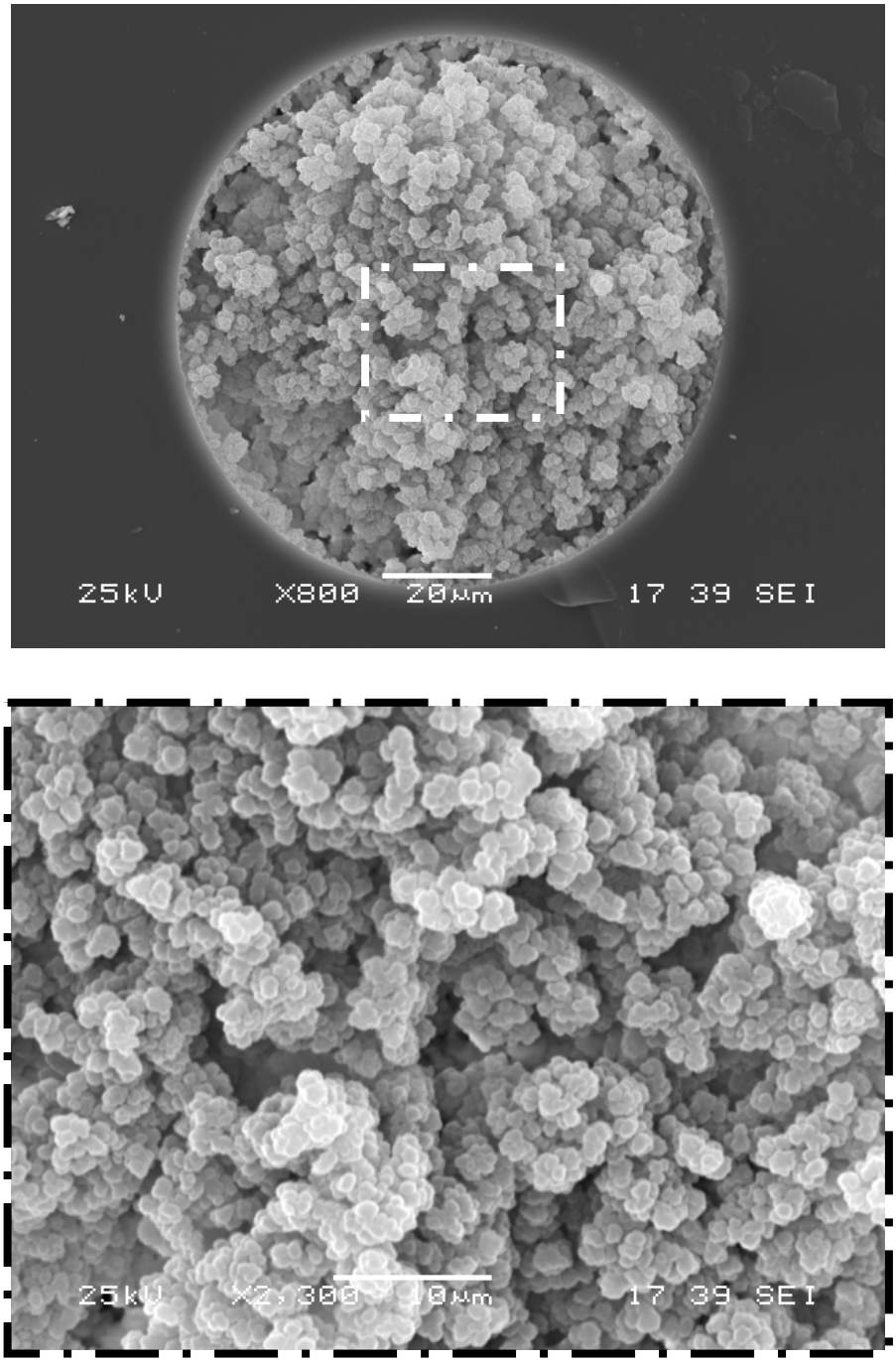


Figure 14: Scanning electron micrographs of hexyl acrylate monolithic column. A-entire capillary and, B-enlargement of the dotted zone.

CEC/MS-TOF of Benzodiazepines

In order to avoid electrostatic interactions between the stationary phase and benzodiazepines, the analysis of benzodiazepines was performed at pH of 7.0. At this pH, benzodiazepines are neutral and only hydrophobic interactions occur. For hyphenated CEC/MS, a volatile CEC buffer, compatible with the ESI source (ammonium acetate, 5 mM, pH 7.0) was used. An example of a CEC separation of the selected benzodiazepines with a 50 cm capillary length is presented Figure 15A. This electrochromatogram consists of the isolation and superposition of each protoned molecular ion. For clorazepate (peak 8), the major ion has a mass of 271.064±1mDa which corresponds to $[M+H-CO_2]^+$ (also observed by Wu *et al.* [Wu]). Certain peak overlaps can be observed in the reconstructed ion electrochromatogram presented in Figure 15A. The lower intensity ions observed under the large molecular ions in the figure result from daughter ions detected as a result of screening for other benzodiazepines in the standard. While their nominal masses are similar to those of the major ions, their exact masses and/or their chromatographic retention times can be used to distinguish them.

Figure 15B illustrates this process by providing extracted ion chromatograms from the standard, which correspond to the nominal masses of nitrazepam *m/z*=282, clorazepate ($[M+H-CO_2]^+$) m/z=271 and diazepam, m/z=285. The additional peaks at m/z=282 and 285 result from the decomposition of chlordiazepoxide (m/z=300.090±1mDa) into two fragments with molecular mass of *m/z*=282.080±1mDa, $[M+H-H_2O]^+$ and *m/z*=285.088±1mDa, $[MCl^{37}+H-HO]^+$ [53]. These fragments can be confirmed by examining the mass spectra of chlordiazepoxide (Figure 16A). The two peaks in the extracted ion of nominal mass 271 chromatogram include chlorazepate (retention time=8min) m/z=271.064±1mDa and the daughter ion of prazepam (retention time=10.5min) m/z=271.064±1mDa. These ions have the same structure and thus the same exact mass. They can be distinguished by retention time or by looking at the full spectrum of the peak presented Figure 16B. This study demonstrates that the combination of CEC separation with exact mass can quickly resolve issues in the identification of specific fragment ions.

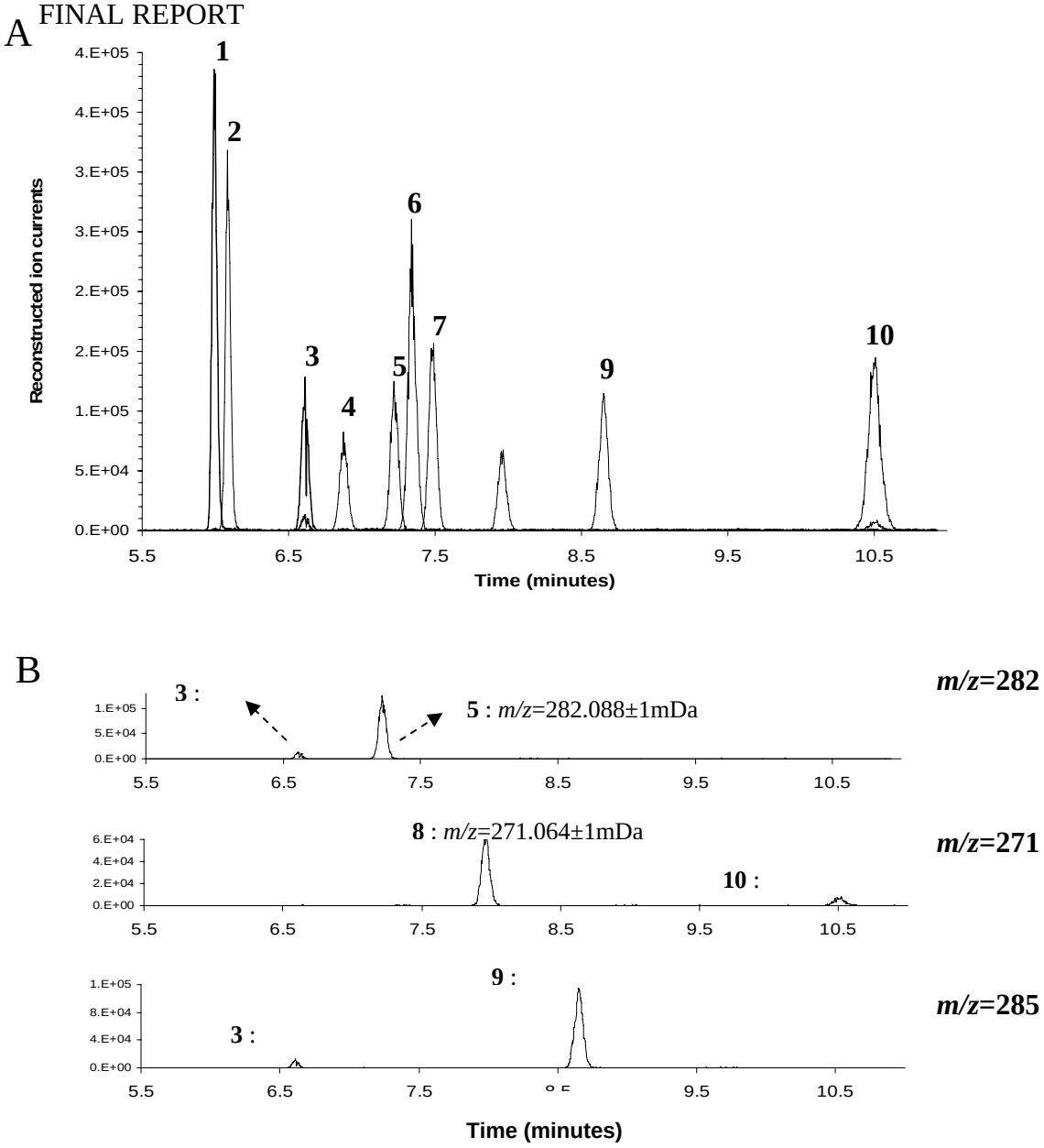
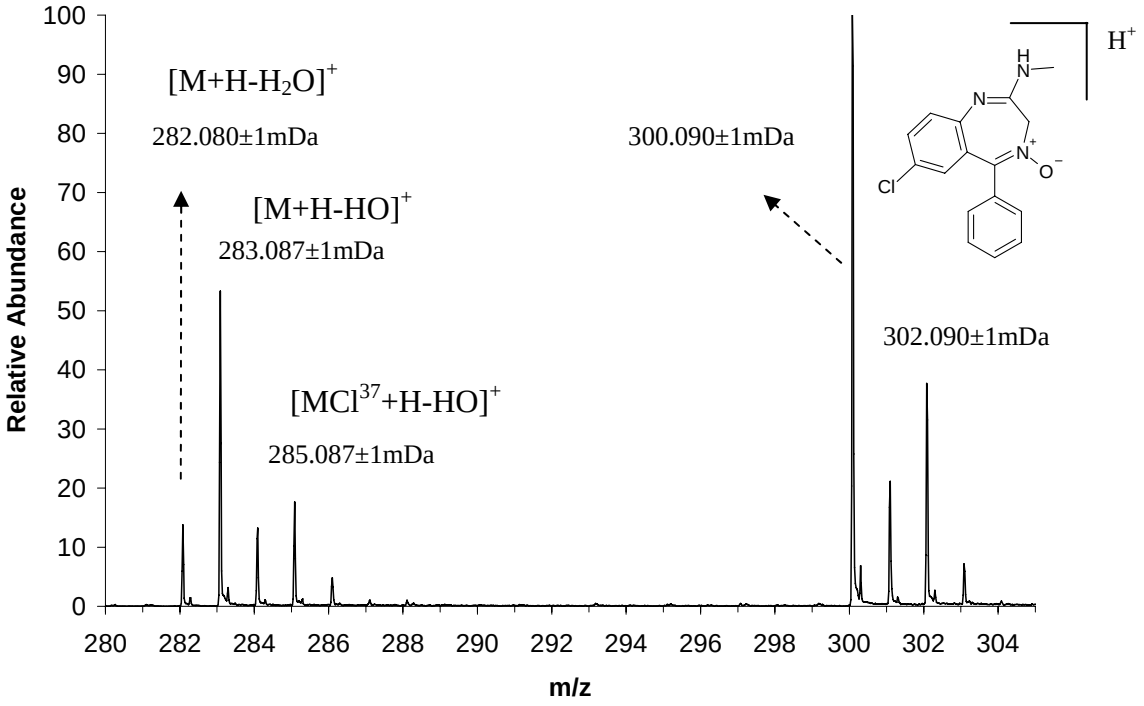


Figure 15: Reconstructed ion electrochromatogram of ten benzodiazepines (A) and selected extracted ion (*m/z*=282; 271 and 285) (B). Peak overlaps observed in the reconstructed ion electrochromatogram are due to benzodiazepines fragmentations. Hexyl monolithic column (L=50 cm). Mobile phase, 5 mM ammonium acetate pH 7.0–acetonitrile (30/70 v/v); running voltage, 20 kV; electrokinetic's injection, 10 kV for 4 s; benzodiazepines concentration, 10 µg/mL. 1-alprazolam, 2-triazolam, 3-chlordiazepoxide, 4-lorazepam, 5-nitrazepam, 6-clonazepam, 7-flunitrazepam, 8-clorazepate, 9-diazepam, 10-prazepam.



B- Prazepam

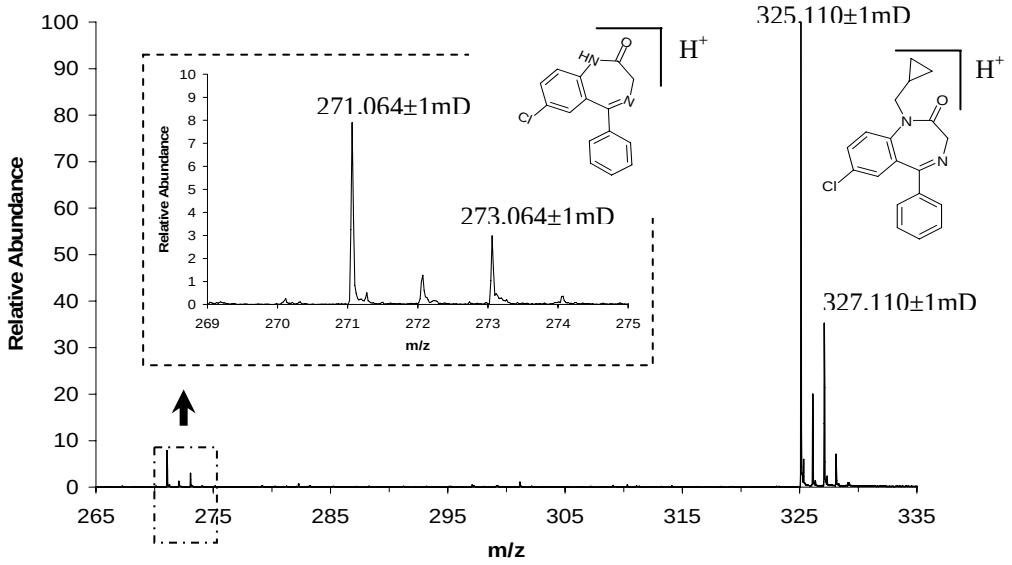


Figure 16: Full-scan ion spectra of (A) chlordiazepoxide (10 µg/mL) and (B) prazepam (10 µg/mL) obtained by continuous infusion of the analyte in MS. TOF system gives exact mass of parent ions, fragment ions and isotopic peaks, aiding identification.

The limits of detection for the different analytes ranged between 75 ng/mL for alprazolam and 700 ng/mL for lorazepam. These values were obtained using an electrokinetic injection of 10 kV for 15 s for a standard solution (10 μg/mL) diluted in the mobile phase (5 mM ammonium acetate pH 7.0–acetonitrile, 30/70 v/v). This sensitivity is not sufficient to quantify urine samples used to detect DFSA. Such samples should be detected at levels of benzodiazepines at 10 ng/mL or below [Lebeau]. Thus a preconcentration step is necessary to obtain adequate detection limits.

Given that CEC is a chromatographic technique, stacking of benzodiazepines at the head of the stationary phase can be performed. The application of this stacking technique consisting of the injection a large volume of the sample present in aqueous solution was discussed by Zare *et al.* [Quirino]. Because the distribution coefficient of the benzodiazepines is higher in aqueous solution than in the mobile phase (5 mM ammonium acetate pH 7.0–acetonitrile, 30/70 v/v), preconcentration of solutes occurs at the head of the column. Sample preconcentration was determined following a series of hydrodynamic injections at 12 bars from 1 min and 30 min (containing 100 ng/mL of each benzodiazepine in 5 mM ammonium acetate pH 7.0). Considering the permeability of the column (4×10^{-14} m^2), the sample plug length included between 1.1% and 33% of the column length for injection times of 1 min and 30 min, respectively. Figure 17 presents the LOD and the efficiency determined for lorazepam as a function of the injection time. Since lorazepam is the compound with the worst sensitivity, only results for this compound are presented. As expected, sensitivity increases with injection time. For injections of approximately 20 min, the sensitivity appears to reach an optimum. Moreover, the peak efficiency quickly decreases after injection times of 15 min or more. This phenomenon is the result of the injection contribution to the peak dispersion. Thus a 15 min-hydrodynamic injection with a pressure of 12 bars seems to be a good compromise between sensitivity and peak efficiency. This sample preconcentration provides a limit of detection of the various benzodiazepines in our standard mixture between 1.0 ng/mL (alprazolam) and 5.1 ng/mL (lorazepam). Table 8 presents LOD and LOQ achieved with a 15 min–preconcentration step. These LOD and LOQ were determined without the sample preparation (liquid-liquid extraction).

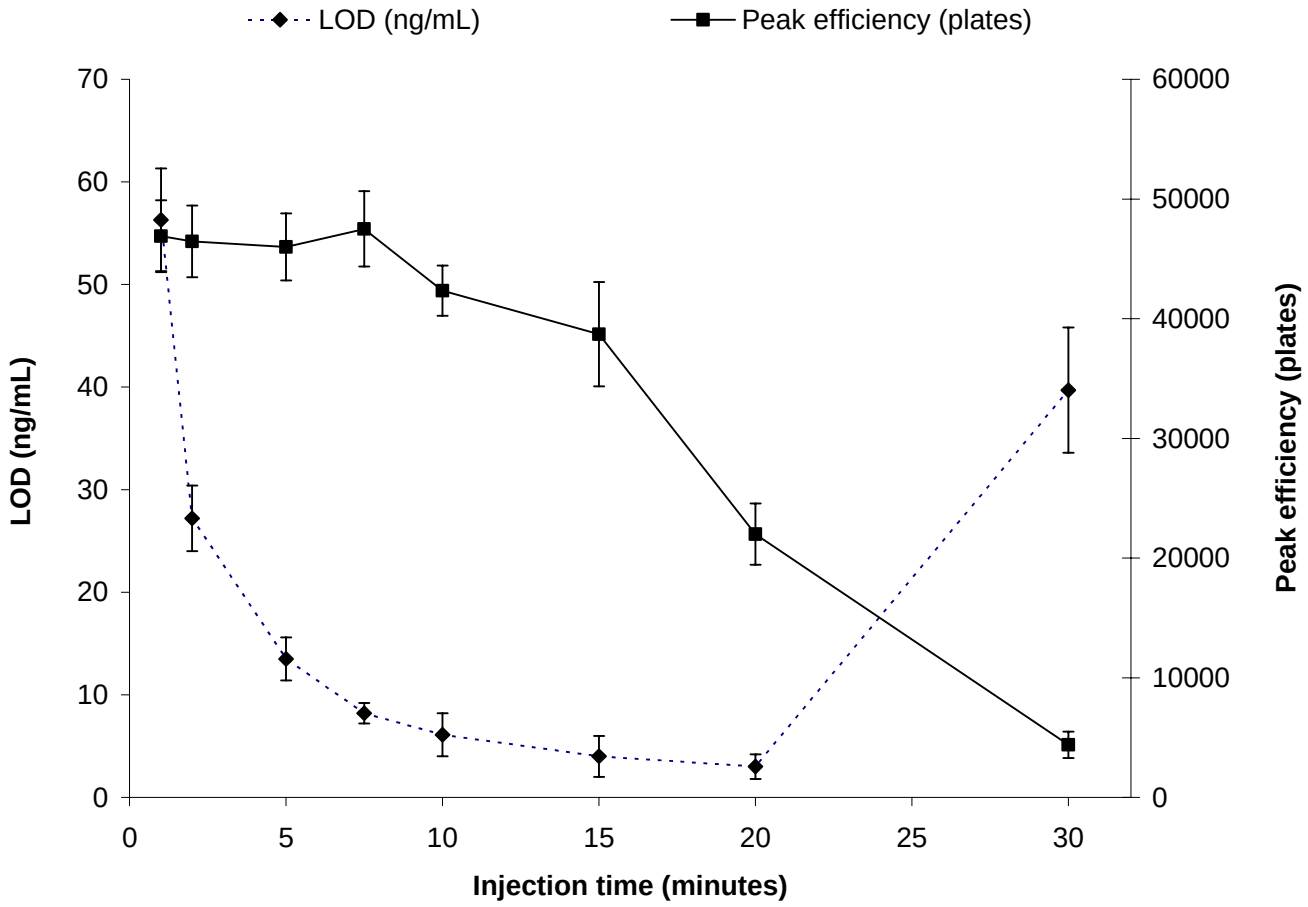


Figure 17: LOD and peak efficiency of lorazepam as a function of injection time for a standard mixture at 100 ng/mL. Other conditions, see Figure 4.

Accurate mass measurements

The accuracies obtained in the mass measurements of the protonated molecules of the benzodiazepines are shown in Table 7. The errors obtained were in the order of 3 ppm. Thus, this detection is able to determine exact mass of protonated benzodiazepines to three decimal places. The mass accuracy of the TOF–MS combined with the characteristic retention time provides a useful identification of benzodiazepines.

Table 7**:** CEC/MS accurate mass measurements.

Compound	**Formula**	**Selected ion**	***m/z*** **nental**	***m/z*** **ted**	**Error**	**Error**
Alprazolam	$C_{17}H_{13}N_4Cl$	$[M+H]^+$	309.0904	309.0907	– 0.3	0.97
Triazolam	$C_{17}H_{12}N_4Cl_2$	$[M+H]^+$	343.0509	343.0517	– 0.8	2.41
Chlordiazepoxide	$C_{16}H_{14}N_3OCl$	$[M+H]^+$	300.0889	300.0904	– 1.3	4.88
Lorazepam	$C_{15}H_{10}N_2O_2Cl_2$	$[M+H]^+$	321.0183	321.0198	– 1.5	4.54
Nitrazepam	$C_{15}H_{11}N_3O_3$	$[M+H]^+$	282.0861	282.0879	– 1.8	6.26
Clonazepam	$C_{15}H_{10}N_3O_3Cl$	$[M+H]^+$	316.0480	316.0489	– 0.9	2.83
Flunitrazepam	$C_{16}H_{12}N_3O_3F$	$[M+H]^+$	314.0929	314.0941	– 1.2	3.80
Clorazepate	$C_{16}H_{11}N_2O_3Cl$	$[M+H\text{-}CO_2]^+$	271.0636	271.0638	– 0.6	2.27
Diazepam	$C_{16}H_{13}N_2OCl$	$[M+H]^+$	285.0784	285.0795	– 0.9	3.74
Prazepam	$C_{19}H_{17}N_2OCl$	$[M+H]^+$	325.1098	325.1108	– 1.0	2.97

Quantification and spiked urine analysis

Quantification was carried out by using the extracted ion chromatogram of each protonated molecular ion of each benzodiazepine, except for the clorazepate, where the ion $[M+H\text{-}CO_2]^+$ was used. To minimize errors linked to the preconcentration and the detection steps, deuterated benzodiazepines were used as internal standards for each benzodiazepine, except chlordiazepoxide and clorazepate for which their IS were $[^2H_4]$–triazolam and $[^2H_7]$–flunitrazepam, respectively, because the deuterated forms are not available. The linearity was evaluated by using six concentrations ranging from 12.5 to 500 ng/mL and 25 to 500 ng/mL for lorazepam. For each benzodiazepine, the LOD, LOQ, and linearity are presented in Table 8. The linearity of the analytical response across the studied range results in correlation coefficients between 0.990 and 0.999. These values are similar to those determined in the literature for other analytes using LC–MS(TOF) [Ferrer]. The relative standard deviations for the migration times and relative peak areas were between 1.4–2.3% for retention times and 1.1–9.2% for relative areas.

Table 8 Validation data

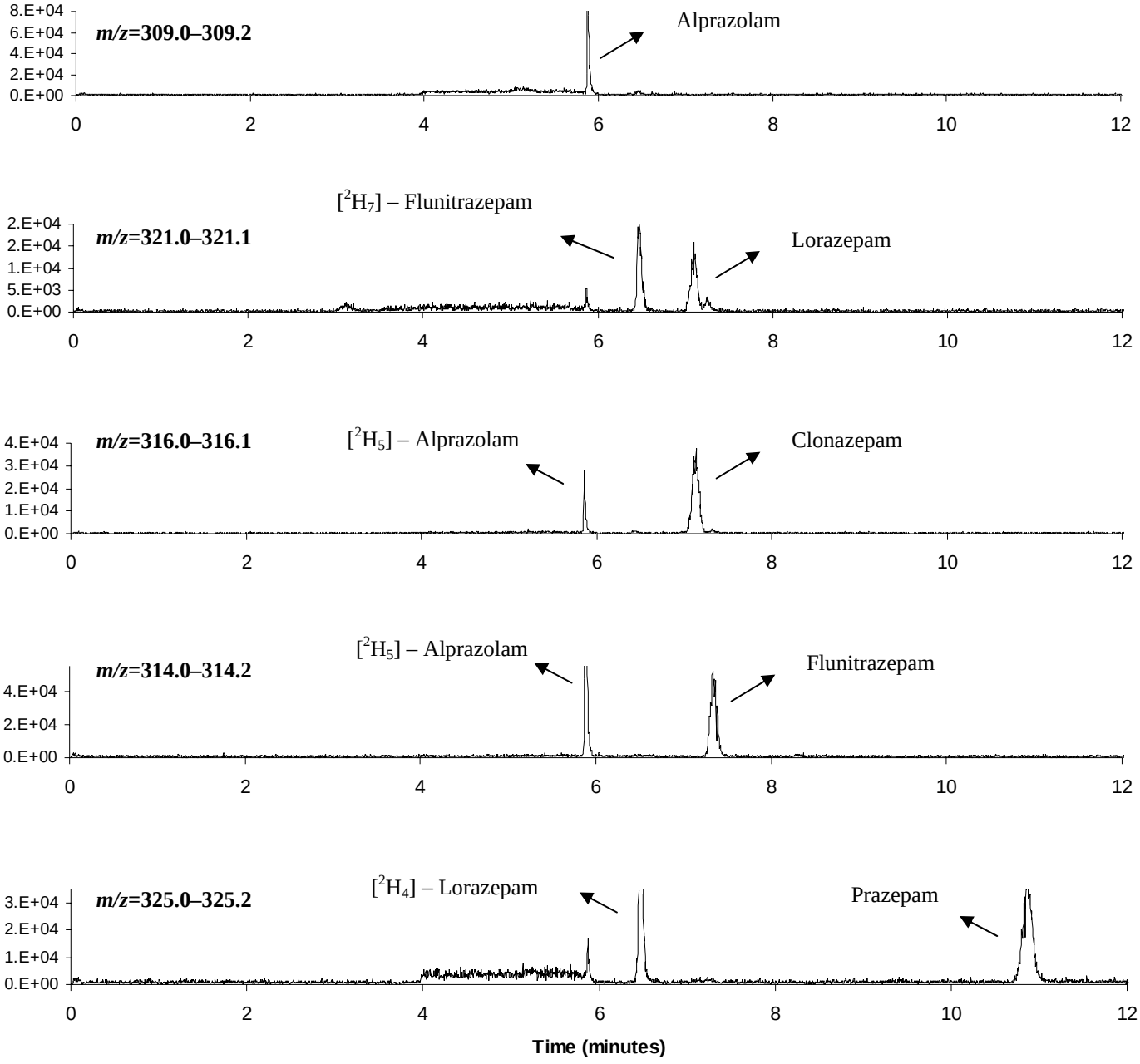
Benzodiazepine	**LOD (ng/mL)**	**LOQ (ng/mL)**	***y=ax+b* (R^2)**	**Linearity range (ng/mL) n=6**
Alprazolam	1.0	3.3	0.9969	12.5–500
Triazolam	1.0	3.3	0.9937	12.5–500
Chlordiazepoxide	3.5	11.7	0.9905	12.5–500
Lorazepam	5.1	17.0	0.9970	25–500
Nitrazepam	3.9	13.0	0.9991	12.5–500
Clonazepam	3.0	10.0	0.9953	12.5–500
Flunitrazepam	4.1	13.7	0.9956	12.5–500
Clorazepate	3.0	10.0	0.9911	12.5–500
Diazepam	2.1	7.0	0.9979	12.5–500
Prazepam	3.3	11.0	0.9972	12.5–500

LOD: limit of detection; LOQ: limit of quantification

This method was then applied to the determination of benzodiazepines present in spiked urine samples at a concentration of 10 ng/mL. The extraction efficiency ranged from 85-98% with RSDs of 3-9%. The extraction procedure, concentrates samples by a factor 5 prior to the injection. Figure 18 presents an example of the result of the extraction and illustrates extracted protonated molecular ions for each benzodiazepine and deutered benzodiazepine present in the spiked urine sample.

Table 9: Extraction efficiencies.

Benzodiazepine	**Extraction ıcy (%)**
Alprazolam	85±5
Triazolam	91±6
Chlordiazepoxide	81±9
Lorazepam	88±3
Nitrazepam	92±5
Clonazepam	98±6
Flunitrazepam	85±5
Clorazepate	92±6
Diazepam	95±3
Prazepam	89±6



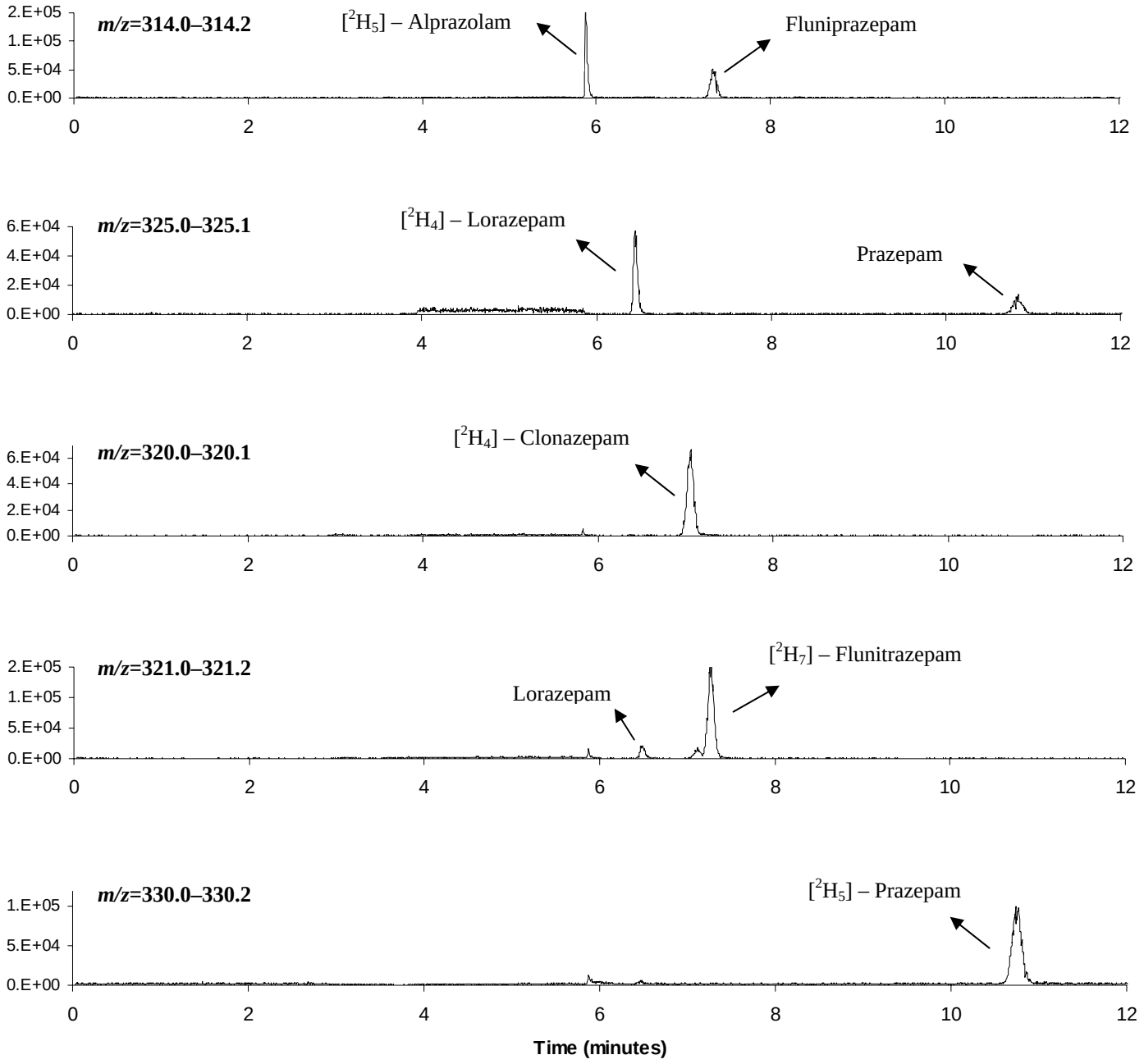


Figure 18: Extracted ion electrochromatograms of 5 benzodiazepines and their respective deuterated internal standards from a spiked urine analysis (10 ng/mL).

7. Concluding remarks

A sensitive CEC/MS-TOF method was developed that allows simultaneous analysis of ten benzodiazepines in urine. The preconcentration of the benzodiazepine at the head of the column was achieved, resulting in a 75–140 fold improvement in sensitivity due to the presence of the monolith stationary phase. This work shows that the CEC/MS-TOF technique may be a suitable approach for the trace determination of benzodiazepines in urine. However a full validation procedure is required for using this strategy in routine for the determination of benzodiazepines when they are used as a tools in DFSA.

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9. Conclusions and suggestions for future work

In this project we have laid the groundwork for the application of microfluidic devices in drug detection. In particular we have demonstrated the applicability of fluorescence detection for highly sensitive screening of a wide variety of phenethyl amines and other basic drugs containing amine moieties. Secondly we have demonstrated on-chip indirect detection of flunitrazepam and other benzodiazepines. We have also developed an efficient and sensitive in-line preconcentration technique in 100um capillaries for the separation and detection of benzodiazepines which is applicable to the analysis of a wide variety of drugs implicated in DFSA. The technique is highly sensitive and inline preconcentration minimizes sample consumption and sample handling. This technique can also be implemented in microfluidic channels. Lastly we have coupled this monolithic CEC system to an electrospray TOF-MS providing impressive sensitivity and specificity of detection.

In the future, we will continue to refine these procedures and work towards integration of the above techniques. Specifically, we will work to develop inline extraction and derivatization techniques to permit direct sample injection on chip. Additional sensors such as amperometric detectors will combined with fluorescence to increase the range and sensitivity of the screening techniques. Injection efficiency will also be improved to optimize chip based separations. We will also expand the application of the CEC/MS-TOF technique to other drugs implicated in DFSA and work to miniaturize the system to permit chip based CEC-MS systems,

minimizing sample requirements and providing sensitive, disposable analytical systems for high throughput analysis.

10. List of papers resulting from this work

Papers

Turner, Carla, McCord, Bruce, Separation and Fluorescent detection of Phenethyl amines using capillary electrophoresis and microfluidic systems, in preparation

Sacha Dehere, Maximilien Blas, Inge Corbin, and Bruce McCord, " A study of the electrospray ionization of benzodiazepines classified as low dose and their subsequent fragmentation using a time-of-flight mass spectrometer," in preparation.

Maximilien Blas[1], Tao Liu[2], Cristina Rodriguez[2], Roberto R. Panepucci[2], and Bruce R. McCord[1], Lab on a chip, submitted

Blas, Maximilien; McCord, B. "Determination of trace levels of benzodiazepines in urine using capillary electrochromatography – time of flight mass spectrometry," *Electrophoresis,* in press.

Bishop, Sandra C.; Lerch, Margaret; McCord, Bruce R. "Detection of nitrated benzodiazepines by indirect laser induced fluorescence detection on a microfluidic device," *J. Chromatogr. A* **2007,** *1154(1-2),* 481-484.

Alnajjar, A. ,Idris A. M., Multzenberg, M. and McCord, B. "Development of a capillary electrophoresis method for the screening of human urine for multiple drugs of abuse *J. Chromatogr. A,* **2007,** *856,* 62-67

Presentations

Carla Turner and Bruce McCord, Microfluidic detection of amphetamines using laser induced fluorescence detection, FACSS 2006, September 24-28, 2006, Lake Buena Vista, FL

Bruce McCord, invited speaker, The analysis of club drugs by CE and CE/MS; Bruce McCord, FACSS 2006, September 24-28, 2006, Lake Buena Vista, FL

Bruce McCord, plenary lecture, Development of methods for forensic drug screening using CE, CE/MS and microfluidic approaches, 8th Symposium on the Practical Applications for the Analysis of Proteins, Nucleotides and Small Molecules, October 2-6, 2006

Carla Turner and Bruce McCord, Sensitive detection of amphetamines and other basic drugs using eosin isothiocyanate, Annual Meeting of the American Academy of Forensic Sciences, Feb 19-24, 2007, San Antonio, TX

Sacha Dehere and Bruce McCord, Capillary Electrophoresis/electrospray ionization /time of flight mass spectrometry of low dose benzodiazepines, Annual Meeting of the American Academy of Forensic Sciences, Feb 19-24, 2007, San Antonio, TX

Bruce McCord, invited speaker, Application of microfluidic devices in the analysis of drugs of abuse, NIJ General Forensics Grantees Meeting, Annual Meeting of the American Academy of Forensic Sciences, Feb 19-24, 2007, San Antonio, TX

Bruce McCord (invited speaker), Carla Turner and Maiko Kusano, The detection of drugs of abuse by capillary electrophoresis and microfluidic systems, Pittcon 2005, March 12-17, 2006.

Carla Turner and Bruce McCord, Detection of drugs implicated in drug facilitated sexual assault on a microfluidic system, American Acaedmy of Forensic Sciences Annual Meeting, Seattle, WA, February 20-25, 2006

Maiko Kusano and Bruce McCord, The development of microfluidic approaches to the detection of GHB, American Acaedmy of Forensic Sciences Annual Meeting, Seattle, WA, February 20-25, 2006

Fluorescent derivatization for trace detection of opiates and other drugs of abuse by capillary electrophoresis, Bruce McCord, Ahmed Al Najjar, American Academy of Forensic Sciences,New Orleans, LA, Feb 21-26, 2005

The development of microfluidic devices for the rapid isolation and detection of drugs of abuse, Bruce McCord, General Forensics R&D Grantees Meeting, American Academy of Forensic Sciences, New Orleans, LA, Feb 21-26, 2005

Capillary Electrochromatography, Bruce McCord, FBI Laboratory Symposium on Forensic Toxicology, Washington, DC, August 28-29, 2004.

Capillary Electrophoresis methods for the detection of amphetamines and piperazine designer drugs, Sandra Bishop and Bruce C. McCord, Latin American Conference on Capillary electrophoresis, November 5-9, 2004, Toledo, Spain

11. List of Figures