ENHANCEMENT OF SENSITIVITY IN CAPILLARY ELECTROPHORESIS: FORENSIC AND PHARMACEUTICAL APPLICATIONS

A dissertation presented to

the faculty of

the College of Arts and Sciences of Ohio University

In partial fulfillment

of the requirements for the degree

Doctor of Philosophy

Ahmed Omer Al Najjar

November 2004

This dissertation entitled

ENHANCEMENT OF SENSITIVITY IN CAPILLARY ELECTROPHORESIS:

FORENSIC AND PHARMACEUTICAL APPLICATIONS

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AL NAJJAR, AHMED O. Ph.D. November 2004. Analytical and Forensic Chemistry

Enhancement of Sensitivity in Capillary Electrophoresis: Forensic and Pharmaceutical Applications (171pp.)

One of the most important issues facing the technology of capillary electrophoresis (CE) is sensitivity. In general, when compared with high performance liquid chromatography (HPLC), CE is a less sensitive technique. This arises from the small dimensions of the CE capillary, which restrict both the volume of sample that can be injected and the optical path length when spectrophotometric detection is used. In this research project, several approaches to improve the sensitivity of CE for the analysis of illicit drugs were investigated and will be discussed. In developing this methodology, we had three main objectives: (1) the development of universal detection techniques for drugs of abuse using systems based on ultraviolet absorption (UV) or native fluorescence and sample stacking; (2) the development of rapid high-sensitivity screening methods for specific classes of drugs using fluorescent derivatization; and (3) the design of more advanced microfluidic systems that permit the coupling of functions such as extraction and/or fluorescent derivatization into a single device. Initial techniques were developed for the analysis of seized drugs and then followed by the analysis of more complex samples, such as blood, urine, or saliva.

The application of electrokinetic injection with field amplified sample stacking for opiate drugs analysis is reported herein. Separation was achieved

using an uncoated (50 μm I.D.) fused-silica capillary, 77 cm long, containing the detector window 10 cm from the outlet end. The running buffer (pH 6) contained 50 mM sodium phosphate and 0.015 M ß-cyclodextrins (ß-CDs). Biological samples spiked with the opiate drugs were first extracted and pre-concentrated using an off-line mixed-mode solid-phase extraction procedure and then analyzed by CE. The UV absorbances of these samples were monitored at 214 nm. The effect of the concentration of β-CDs and pH on separation efficiency was also evaluated. The application of electrokinetic injection with field amplified sample stacking resulted in low detection limits, 40-50 ng/mL, and the method had good reproducibility, precision, accuracy, and high recovery.

The native fluorescence properties of major opiate drugs were also examined. Normorphine, morphine, 6-acetyl morphine (6-AM), and codeine were analyzed by CE-native fluorescence without any derivatization and detected at an excitation wavelength of 245 nm with a cutoff emission filter of 320 nm. This technique provided a rapid and simple analysis. Separation conditions, analytical characterization, method optimization, and validation were reported. The detection limits were in the range of 200 ng/mL, which was higher than the UV absorbance method. However, the native fluorescence detection proved to have better specificity than UV detection, especially for the analysis of biological samples.

For a highly sensitive analysis, laser-induced fluorescence (LIF) detection was performed using a two-step pre-column derivatization procedure. In this method, drugs extracted from human urine were first subjected to a *N*-

demethylation reaction involving the use of 1-chloroethyl chloroformate (ACE-Cl), derivatized using fluorescein isothiocyanate isomer I (FITC), and analyzed by CE coupled to a LIF detector. Variables affecting this derivatization include the yield of the demethylation reaction, the concentration of FITC, and the reaction time and temperature. The estimated detection limits of the FITC-derivatives were in the range of 50-100 pg/mL, using LIF detection with excitation and emission wavelengths of 488 nm and 520 nm, respectively. The linearity, reproducibility, and reliability of the methods were also evaluated. In addition, a comparison of the characteristics for both native fluorescence and LIF detections will be discussed.

Finally, a number of chromatography-based pre-concentration techniques were investigated. These techniques offer two significant advantages: (1) the ability to pre-concentrate the analytes, and (2) the ability to eliminate most of the interfering matrix. Several methods were developed and validated for the determination of illicit or abused drugs in biological fluids using various extraction techniques, including solid-phase extraction, membrane pre-concentration, and solid-phase microextraction. A comparison of the analytical characteristics of each method will be discussed. The proposed CE methods allow on-line extraction, pre-concentration, and separation of abused drugs in a single run.

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Acknowledgments

First and foremost, I would like to thank my advisor, Dr. Bruce McCord, for giving me the opportunity to be a part of his research group and for his patience, support, and encouragement. Thank you for your help and encouragement in my academic progress and research pursuits.

I would also like to thank the members of my dissertation committee, Dr. Howard Dewald, Dr. Tadeusz Malinski, Dr. Martin Tuck, and Dr. Peter Jung, for their efforts and time given to this project. Special thanks go to Dr. Jared Butcher for his assistance on the organic part of this work. Also, special thanks go to Ira Lurie from the US Drug Enforcement Administration for his useful discussions on methods development and Dr. Robert Powers from the Hamilton County Coroners Office-Forensic Toxicology Laboratory for his collaboration and donation of some of the biological samples.

I want to thank Brittany Hartzel for her detailed reviews of all my published papers and this manuscript. Thank you, Brittany, for your support, and friendship. I would also like to thank all the previous and current members of Dr. McCord's research group.

I gratefully acknowledge the faculty and staff in the Department of Chemistry and Biochemistry. Special thanks to Dr. Peter Harrington, Dr. Gary Small, Rollie Merriman, Bascom French, Paul Schmittauer, Carrie Linscott, and Carolyn Khurshid.

Also, I would like to thank my family in Saudi Arabia, who provided me with love, prayer, and support.

Finally, this work was supported, in part, by a grant from the National Institute of Justice. I also acknowledge Ohio University (USA) and King Faisal University (SA) for support of this project.

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List of Abbreviations

ACE-Cl – α-chloroethyl chloroformate

CE – capillary electrophoresis

CZE – capillary zone electrophoresis

EOF – electroosmotic flow

FAI – field-amplified sample injection

FITC – fluorescein isothiocyanate isomer I

GC – gas chromatography

HPLC – high performance liquid chromatography

IS – internal standard

LIF – laser-induced fluorescence

LLE – liquid-liquid extraction

LOD – limit of detection

MEKC – micellar electrokinetic chromatography

MS – mass spectrometry

mPC – membrane pre-concentration

RFI – relative fluorescence intensity

SDS – sodium dodecyl sulfate

SPE – solid-phase extraction

SPME – solid-phase microextraction

UV – ultraviolet absorption

β-CDs – β-cyclodextrins

Chapter 1 General Introduction

1.1 Brief history

Capillary electrophoresis (CE) is a relatively new technique for the separation and analysis of chemical compounds. It is also a method that is being used with increasing frequency in the field of analytical chemistry. CE methods have applicability in a variety of venues including biochemistry, biomedical, forensic, and pharmaceutical sciences. A comparison of the review articles that have been published on capillary electrophoresis over the last two-and-a-half decades illustrates the nearly exponential growth in the number of papers on the subject, which reflects the technique's expanded acceptance and usage (Figure 1-1).

Electrophoresis as an analytical technique was first introduced by Tiselius[1] in 1937. In his thesis, he described the separation of blood plasma proteins (albumin from α-, β-, and γ -globulin) in free solution using electrophoresis. For this pioneering work, Tiselius was awarded the Nobel Prize in 1948. Since then, many advancements in electrophoresis, such as paper[2] and gel electrophoresis,[3, 4] have been published. In 1967, Hjerten[5] showed that it was possible to carry out an electrophoretic separation in a 300 μm glass tube and to detect the separated compounds by ultraviolet absorption (UV). He called this method free zone electrophoresis. Although other researchers[6, 7] had used electrophoresis in glass, teflon, and tubes, electrophoresis in a tube did not

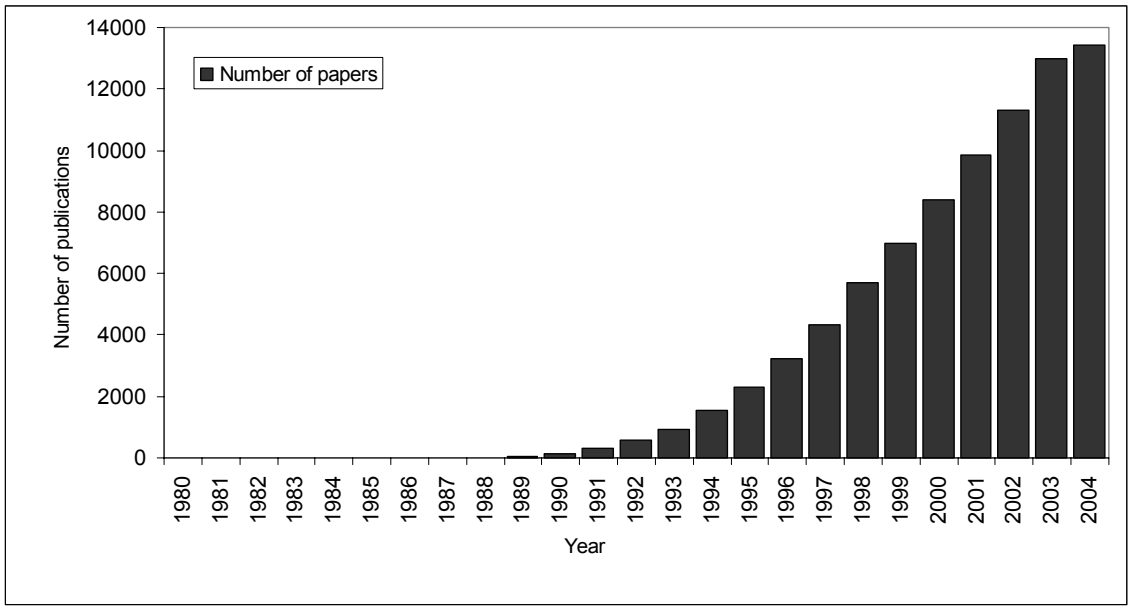


Figure 1-1: Plot of the growth of CE literature over time. Search engine, “Science Citation Index Expanded (ISI)”; search words, “capillary electrophoresis”; years, 1980-2004.

become popular until 1981 when Jorgenson and Lukacs[8] published their work, in which they demonstrated the high resolving power of capillary zone electrophoresis (CZE). They used narrow internal diameter (ID) fused-silica capillary (< 100 μM), voltages as high as 30 kV and on-line UV detection for the separation of ionic species. These authors also gave a brief description of some of the theoretical aspects of CE and showed that electroosmosis played an important part in determining the mobility of ionic species, affecting both resolution and analysis time. In 1984, Terabe et al.[9] introduced micellar electrokinetic chromatography (MEKC) for the separation of neutral compounds by adding a micelle, sodium dodecyl sulfate (SDS), to the running buffer. In 1988, the first commercial instrument was marketed by the late Bob Brownlee's company, Microphoretics. Since then, many advances and applications of this technique have taken place.

1.2 General comments about CE

1.2.1 Description of separation mechanism

In CE, separation is driven by two factors. The first is the movement of the solute in the capillary under the influence of an electric field, also called electrophoretic velocity. The second is the bulk flow of the buffer solution due to the surface charge on the capillary wall, also called electroosmotic flow (EOF). A detailed account of these theoretical aspects is given by Jorgenson and Lukacs.[10]

The movement of a charged solute through a conductive solution toward or away from an electrode is dependent upon the mobility of the solute and the magnitude of the applied electric field. This movement is called the electrophoretic velocity (V_{EP}) and is given by:

$$V_{EP} = \mu_{EP} E \qquad (1\text{-}1)$$

where μ_{EP} is the electrophoretic mobility and E is the field strength (obtained by dividing the applied voltage by the length of the capillary). Electrophoretic mobility is dependent on the solute and the buffer properties and is given by:

$$\mu_{EP} = q / 6\pi\eta r \qquad (1\text{-}2)$$

where q is the charge of the analyte, η is the buffer viscosity, and r is the solute radius. This means that the actual elution order for cations and anions is based on their charge-to-size ratio. Thus cationic solutes with the largest charge-size ratio have the highest net mobility and elute first. For anions the opposite occurs; that is, solutes with the largest charge-size ratio elute last because of their greater attraction to the anode. Finally, neutral solutes, being uncharged, elute as a single peak .The form of CE just described is free-zone capillary electrophoresis.

Electroosmotic flow[11] is the second factor that affects the movement of solute through the capillary. Surface charges on the interior of the capillary induce the formation of a double layer upon application of the electric field. In a capillary composed of fused silica, the surface silanol (Si-OH) groups are ionized to negatively charged silanoate ($Si\text{-}O^-$) groups at pHs above three. These

negatively charged silanoate groups attract positively charged cations from the buffer solution, which form an inner layer of cations at the capillary wall. These cations are not of sufficient density to neutralize all the negative charges, so a second, outer layer of cations forms. While the inner layer is tightly held by the $Si\text{-}O^-$ groups, the outer layer of cations is not tightly held because of its larger distance from the silanoate groups. Under the influence of an electric field, the outer layer of cations is pulled toward the negatively charged cathode. Since these cations are solvated, they drag the bulk buffer solution with them, thus causing EOF, as represented in Figure 1-2. The electroosmotic flow can be described in terms of velocity, V_{EOF}, or mobility, μ_{EOF}:

$$V_{EOF} = \varepsilon\zeta E / 4\pi\eta \quad (1\text{-}3)$$

$$\mu_{EOF} = \varepsilon\zeta / 4\pi\eta \quad (1\text{-}4)$$

where ε is the dielectric constant of the buffer, ζ is the zeta potential that arises on the surface of the capillary, and η is the viscosity of the buffer.[2, 11, 12] The overall mobility, that is, apparent mobility, μ_a, of a solute is the sum of the electrophoretic mobility and the electroosmotic mobility:

$$\mu_a = \mu_{EP} + \mu_{EOF} \quad (1\text{-}5)$$

1.2.2 Capillary electrophoresis system

A schematic diagram of a typical CE system is shown in Figure 1-3. A narrow capillary typically between 25 and 100 μm is immersed between two buffer reservoirs that contain electrodes, across which a high voltage is applied.

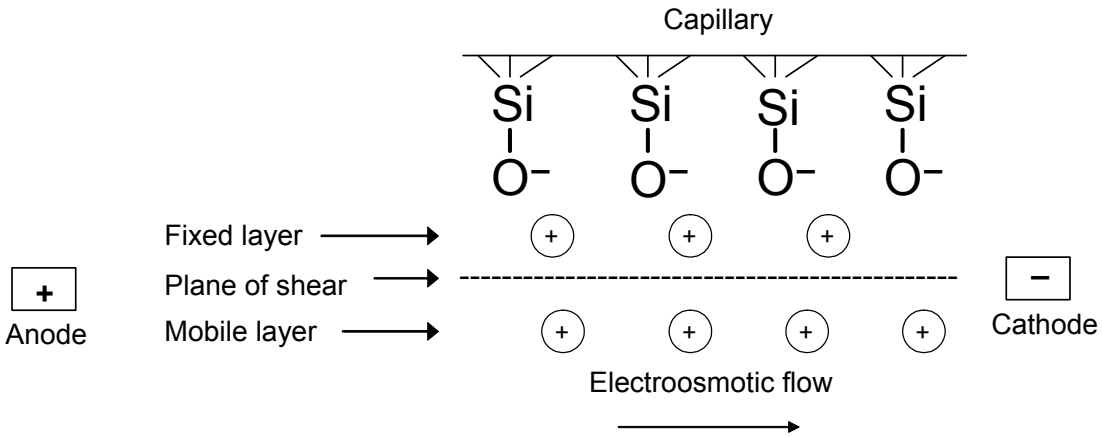


Figure 1-2: Representation of electroosmotic flow in a capillary. Electrolyte cations are attracted to the capillary wall, forming an electrical double layer. When voltage is applied, the net movement of electrolyte solution toward the cathode is known as electroosmotic flow. Acidic silanol groups impart a negative charge on the wall. Counter ions migrate toward the cathode, dragging solvent along. Adapted from 11.

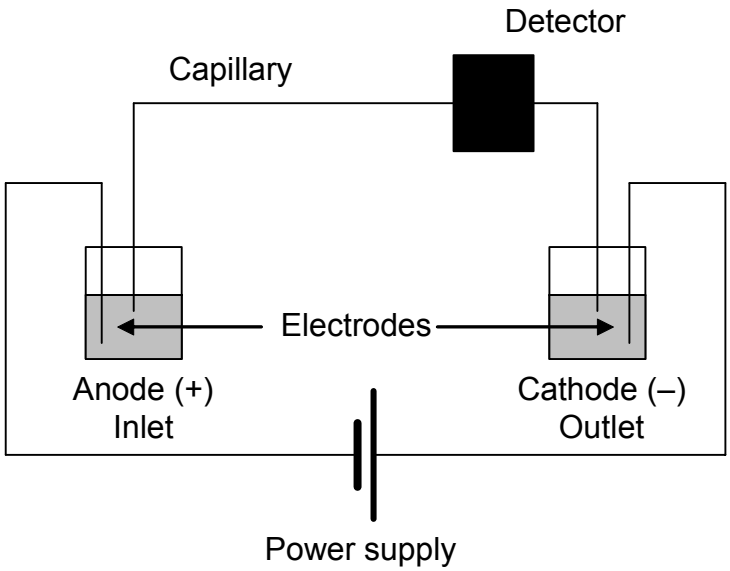


Figure 1-3: Schematic representation of a capillary electrophoresis system. Adapted from 11.

The power supply is generally capable of supplying up to 30 kV. Most commercial instruments utilize UV detection, which is accomplished by burning off a portion of the polyamide coating on the fused silica capillary to form a detector window. The path length is essentially the internal diameter of the capillary. Analytes can be injected into the capillary in several ways: (1) gravity injection, in which the sample enters the column by capillary action, (2) pressure injection, in which applied pressure on the sample forces analyte into the capillary, and (3) electrokinetic injection, in which an applied electric field forces the sample to enter the capillary as it is attracted to the electrode at the opposite end of the capillary.

The basic steps in a CE analysis consist of: pretreating the capillary, source and destination vials with run buffer, conditioning the capillary with the buffer, injecting the sample into the capillary and applying an electric field across the capillary. The solutes can then migrate through the capillary, become separated, and then are detected near the end of the CE capillary. The output of the detector is a plot of detector response versus time, and is called an electropherogram.

1.3 Why capillary electrophoresis

In recent years, a number of forensic laboratories have begun to use CE as an alternative to the gas chromatography/nitrogen-phosphorous ionization detection (GC/NPD) screening methods for the separation and detection of illicit drugs. CE is useful for the analysis of the wide variety of solutes found in illicit

drug seizures,[13] especially for those compounds which are otherwise difficult to analyze via GC and high-performance liquid chromatography (HPLC). GC can be problematic for the analysis of nonvolatile, thermally labile, and highly polar drugs, while HPLC often lacks sufficient resolving power for complex mixtures. In addition, although drugs of forensic interest can be analyzed by either GC[14-16] or HPLC,[17-19] derivatization and/or the use of expensive, specialized columns are usually required. CE offers high efficiency, high selectivity, and low cost operation. Therefore, it has great potential for the forensic chemist. The economy of operation arises from the low flow rates (nL/min) and capillary costs (~ $5/capillary).

1.4 Forensic toxicology of opiate alkaloids

The opiate alkaloids are derived from opium, which is considered the oldest drug on record.[20] The source of opium is the opium poppy, *Papaver somniferum*. The plant may have been in use for over 6000 years, and there are accounts of its use in ancient Egyptian, Greek, and Roman documents. Interestingly, it was not until the 18th century that the addiction liability of opium began to cause concern.

Opium is obtained from the opium poppy by incision of the seed pod after the petals of the flower have dropped. The white latex that oozes out turns brown and hardens on standing. This sticky brown gum is opium. It contains about 20 alkaloids, including morphine, codeine, thebaine, and papaverine. Thebaine and papaverine are not analgesic agents, but thebaine is the precursor of several

semisynthetic opiate agonists (i. e., etorphine, a veterinary agent 500-1000 times as potent as morphine) and antagonists (i. e., nalorphine). The principal alkaloid in opium is morphine, present in a concentration of about 10%. Codeine is present in less than 0.5% concentration; it is synthesized commercially from morphine.[20, 21] Cultivation of the opium poppy is now restricted by international agreement, but illicit production of opium is widespread and difficult to control.

The characteristic pharmacologic effects of opioids (used to describe natural and semisynthetic alkaloids prepared from opium as well as their synthetic surrogates with actions that mimic those of morphine) are the result of selective receptor binding at several sites in the central nervous system[22] (Table 1-1). Relative small molecular alterations may drastically change the pharmacology of these compounds and their potency as an analgesic. Opioids produce an analgesic effect by blocking the transmission of painful stimuli. The interaction between the opiate and specific receptors at terminal nerve endings impedes the release of neurotransmitter, thus interrupting the pain. This prevents the recognition of painful sensations and inhibits the negative emotional component of pain, and can produce euphoria in some instances.

Opioid analgesics are indicated to relieve moderate-to-severe pain, such as the pain associated with myocardial infarction, cancer, and labor. They are used as preanesthetic medications, as analgesic adjuncts during anesthesia, and occasionally as a primary anesthetic agent. Opioids are also used clinically for their antitussive and antidiarrheal properties and for detoxification of patients after opioid intoxication.[20]

Table 1-1: Mode of action and metabolism of some opioids.

Drug	Mode of action	Analgesic potency (morphine=1)	Metabolites
Codeine	Weak μ agonist, weak δ agonist	0.1	Codeine*c*, morphine*c*, norcodeine*c*
Dihydrocodeine	μ Agonist	0.3	Dihydromorphine*c*, nordihydrocodeine*c*, dihydrocodeine*c*
Fentanyl	Strong μ agonist	100-200	Despropionylfentanyl, norfentanyl, hydroxyfentanyl, hydroxynorfentanyl
Heroin	Strong μ agonist	1-5	6-Acetylmorphine*c*, morphine*c*, normorphine
Hydrocodone	μ Agonist	1-2	Hydromorphone*c*, norhydrocodone, hydrocodol, hydromorphol*c*
Hydromorphone	Strong μ agonist	7-10	Hydromorphol*c*, hydromorphone*c*
Methadone	Strong μ agonist	1	EDDP*c*, EDMP*c*, methadone*c*, methadol, normethadol
Morphine	Strong μ agonist, weak *k* and δ agonist	1	Morphine*c*, normorphine*c*
Oxycodone	μ Agonist	1-2	Noroxycodone, oxymorphone*c*, oxycodone*c*

μ receptor interactions result in central depression, clinically manfested as supraspinal (μ_1) and spinal (μ_2) analgesia, respiratory depression, miosis, euphoria, reduced gastrointestinal motility, hypothermia, bradycardia, and physical tolerance and dependence.
k receptor interactions produce spinal analgesia, sedation, miosis, diuresis, mild respiratory depression, and low addiction liability.
δ receptors are the binding sites for most endogenous peptides. Interactions at these sites mediate spinal analgesia, dysphoria, delusions, hallucinations, and respiratory and vasomotor stimulation.
c indicates that conjugation prior to elimination occurs.
2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), 2-ethyl-5-methyl-3,3-diphenylpyrroline (EMDP).

Opioids exert their major pharmacological effect on the central nervous system. The effects are a combination of depression and simulation and include increased pain tolerance, suppression of anxiety, and sedation. Higher doses result in drowsiness, mood changes, mental clouding, nausea, and respiratory depression. Opioid poisoning occurs most commonly following heroin administration or oral methadone overdoes by addicts or nontolerant infrequent users. Severe overdose is characterized by apnea, circulatory collapse, convulsions, cardiac arrest, and death.[21] The pharmacologic properties[22] of major opioids are summarized in Table 1-2.

1.5 Metabolic pathways of heroin, morphine and codeine

The confirmation and identification of heroin drugs in biological matrices often may lead to more questions than answers. The presence of codeine and morphine may be indicative of pain therapy, heroin abuse, or poppy seed ingestion.

Heroin is rapidly degraded to 6-acetyl morphine (6-AM) by both chemical and enzymatic processes. Heroin is labile in aqueous solutions, and deacylation is accelerated in biological fluids. In addition, there is extensive organ metabolism. 6-AM is more stable than heroin; it undergoes conversion to morphine by enzymatic and nonenzymatic deacylation.[23-25] Morphine may be further metabolized by demethylation to normorphine, and both forms can be conjugated before excretion. About 80% of the drug is eliminated in the urine

Table 1-2: Pharmacokinetics of major opioids.

Drug	Half-life (h)	Volume of distribution (L/kg)	Duration of analgesia (h)	Plasma protein binding (%)	Therapeutic conc. (ng/mL)	Toxic conc. (ng/mL)	Addiction and abuse liability
Morphine	1.3-6.7	2-5	4-5	20-35	10-80	>200	High
Codeine	1.9-3.9	3.5	3-4	7-25	10-100	>200	Moderate
Heroin	1-1.5	25	3-5	20-35	NA	NA	High
Hydrocodone	3.4-8.8	3.3-4.7	3-5	25	2-24	>100	Moderate
Hydromorphone	1.5-3.8	2.9	4-5	19	1-30	>100	High
Oxycodone	4-6	1.8-3.7	3-4	NA	10-100	>200	Moderate
Methadone	15-25	4-5	4-6	87	100-400	>2000	High
Fentanyl	3-12	3-8	1-1.5	79	1-3	3-28	High

NA, not applicable.

within 24 h, mostly as morphine-3-glucuronide (required acid or enzyme β-glucuronidase hydrolysis for deconjugation) and only about 0.1% as the free drug.[22]

Codeine is metabolized by demethylation to morphine or norcodeine followed by the formation of glucaronide conjugates. The rate of conversion of codeine to morphine varies widely among individuals. Codeine is often present in the starting material for illicit heroin.[26] Additionally, metabolic conversion of morphine to codeine has been reported.[27] Thus, a user of heroin may produce a specimen that is positive for both codeine and morphine. Dietary sources of opiates, such as poppy seeds, further complicate the differentiation of hard-core drug abusers from the general population.[23, 28] Poppy seeds may contain morphine and codeine and therefore, ingestion of poppy seed food may unwittingly expose the individual to a significant quantity of opiates. Urinary concentrations of morphine and codeine peak within 2-4 h of ingestion of such food. However, opiates may remain detectable for up to 72 h, which poses a particular problem for employment or rehabilitation drug-testing programs.[20-22] The metabolic pathways of heroin and poppy seeds are shown in Figure 1-4 and Figure 1-5, respectively.

Because of these complications, attention has lately been focused on morphine precursors. Several studies have proposed using 6-AM as the primary marker for heroin abuse because there is no natural source, it is not a codeine metabolite, and it is comparatively stable relative to heroin.[26, 29, 30] However, it is normally excreted in the first few hours following heroin use, is present in

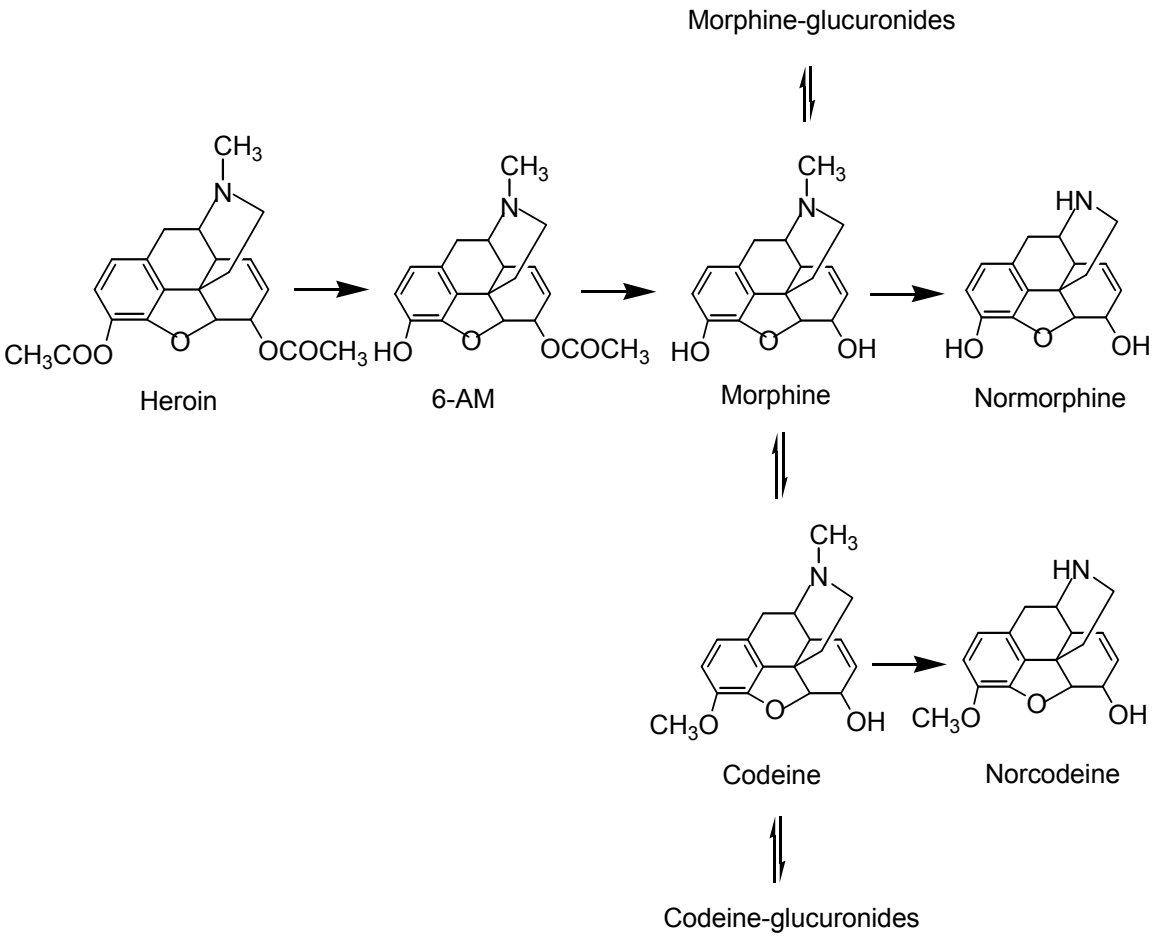


Figure 1-4: Metabolic pathways of heroin, morphine and codeine. Adapted from 31, 32.

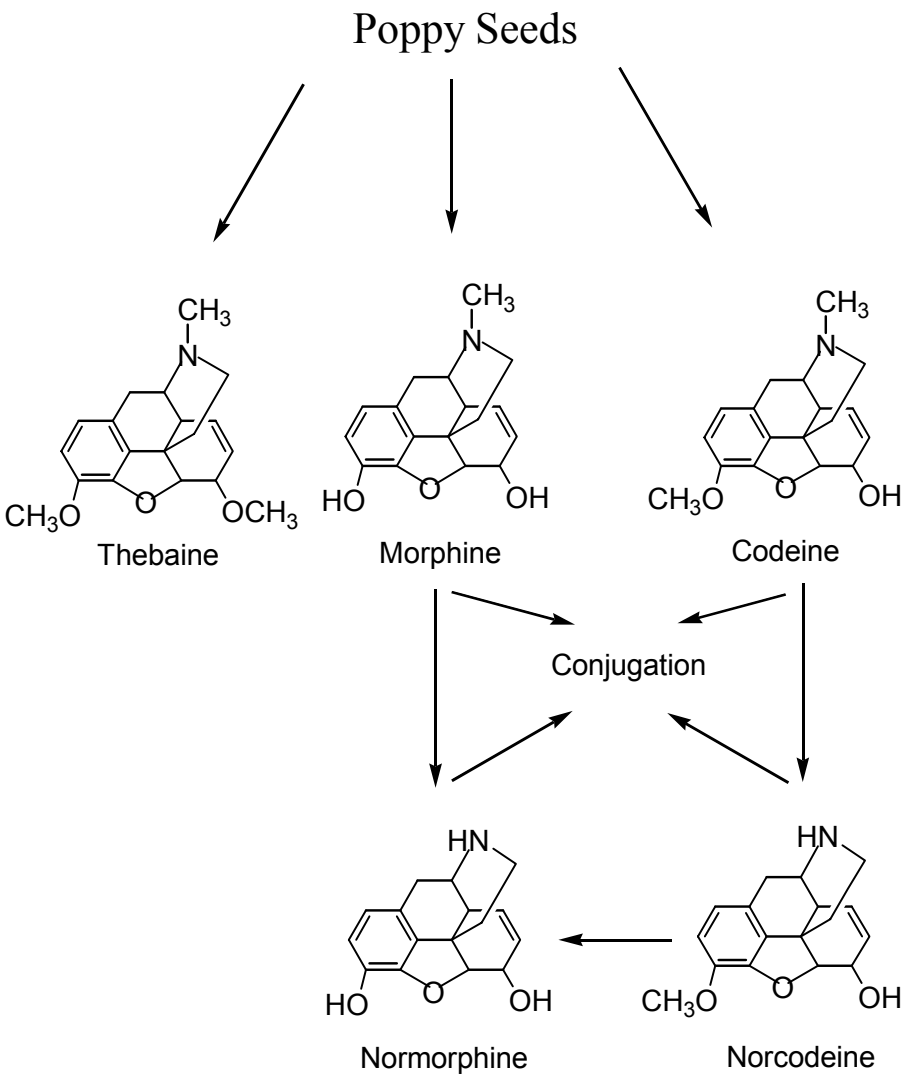


Figure 1-5: Poppy seed metabolism. Poppy seeds contain varying concentrations of morphine, codeine, thebaine, and other compounds. A number of common biotransformations such as oxidation, hydroxylation, *O*-demethylation, and *N*-demethylation can tacke place. The *N*-demethylation pathway yields a nor-derivative, which frequently undergoes conjugation prior to elimination. Glucuronidation is the major metabolic route for drugs that contain an available hydroxyl group, such as morphine and codeine. After conjugation, these products primarily undergo biliary and urinary excretion. Adapted from 32.

trace concentrations, and has limited chemical stability. The window of detection for 6-AM in urine is less than 8 h.[33] The concentration of 6-AM has been reported to typically be 1-3% of the concomitant total morphine concentration and the highest 6-AM/morphine ratio reported in the literature was less than 8%.[34, 35]

1.6 CE applications in the analysis of illicit drugs

The use of CE in the analysis of illicit drugs and especially opiates, both in drug forms and in body fluids, represents one of the most rapidly growing applications of this technique. The first application of CE in the forensic field was introduced in the early 1990s by the pioneering work of Weinberger and Lurie,[13] who first reported the simultaneous separation and determination of illicit drugs by using MEKC. The authors used 50 μm I.D. bare silica capillaries, 25-100 cm in length, and a hydroorganic buffer consisting of 85 mM SDS, 8.5 mM phosphate, 8.5 mM borate, and 15% acetonitrile at a pH of 8.5. Detection was by UV absorption at 210 nm. Under the described conditions, it was possible to separate with high efficiency acidic and neutral impurities in an illicit seizure of heroin, as well as heroin degradation products and adulterants such morphine, phenobarbital, O^6-monoacetylmorphine, O^3-monoacetylmorphine, methaqualone, acetylcodeine, papaverine, and noscapine.

The separation of enantioners of amphetamine, methamphetamine ephedrine, pseudoephedrine, norephedrine, and norpseudoephedrine with application to forensic samples was later reported by Lurie.[36]

Recently, Trenerry et al.[37] reported the use of MEKC with 50 mM cetyltrimethylammonium bromide (CTAB) as a micellar agent for an analysis of the illicit heroin seizures. Also, Krogh et al.[38] applied CE to the analysis of seized heroin and amphetamine.

In 1991, Wernly and Thormann,[39] reported the first use of MEKC with a borate/phosphate buffer, pH 9.1, containing 75 mM SDS, for the separation of illicit drugs and metabolites in human urine, including benzoylecgonine, morphine, heroin, 6-acetylmorphine, methamphetamine, codeine, amphetamine, cocaine, methadone, methaqualone, and benzodiazepines. Sample pre-concentration was performed by means of solid-phase extraction using 5 mL of urine followed by the dissolution of the dried extract in 100 µL of running buffer. The method allowed the determination of the concentrations of these drugs at levels as low as 100 ng/mL, without relevant interferences from the matrix. Peak identification was obtained not only on the basis of the retention times, but also by plotting the absorbance spectra of the peaks and comparing them to computer-stored library spectra. This approach showed a sensitivity comparable to common non-isotopic immunoassays and was proposed by the authors as a technique for confirmatory testing following toxicological screening by the usual enzyme-immunoassay procedures.

As an alternative to MEKC, Chee and Wan[40] used CZE with a simple phosphate buffer at pH 2.35 to achieve the baseline separation of 17 basic drugs of potential forensic interest. Detection was by UV absorption at 214 nm. Since

then, a relatively vast literature has been published for the analysis of heroin, opium, opiates, cocaine, amphetamines, ring substituted amphetamines, LSD, and anabolic steroids, both in seized material and biological samples (blood, urine, and hair).

In both CZE and MEKC separation modes, cyclodextrins (CDs) have been used extensively as buffer additives to enhance the selectivity of both charged and neutral species.[41, 42] CDs enhance selectivity through the formation of inclusion complexes with the analyte that are highly dependent on chemical structure.[43] In 1994, Lurie et al.[44] first reported the use of a mixture of neutral and anionic CDs for the separation of various drugs of forensic interest, including the principal phenalkylamine constituents of khat, amphetamine, cathinone, methamphetamine, methcathinone, cocaine, and propoxyphene. Also, Gong and co-workers[45] reported the use of β-cyclodextrin (β-CD) to modulate the separation of morphine, 6-monoacetylmorphine, and heroin in a CZE method with chemiluminescence detection. More recently, Macchia and colleagues[46] reported the use of β-CD in CZE for the separation of the components of clandestine heroin preparations.

Most of the previously mentioned studies used UV absorbance for drug detection. However, a crucial disadvantage of using CE with UV detection is that the sensitivity of this method for a given analyte is typically 10^{-4}-10^{-6} M (i.e. about 1 μg/mL). This limit of detection can be too high for certain analyses in forensic and pharmaceutical studies, especially for trace level analysis. The goal of this

research project was to provide strategies to overcome this problem by using sample pre-concentration techniques and/or improved detection schemes, such as fluorescence derivatization. To our knowledge, in the field of forensic chemistry, the application of electrokinetic injection with field amplified sample stacking for opiate drug analysis has not been previously reported. Also, no single paper in the literature has been published concerning the analysis of opiate drugs using CE and direct fluorescence detection. It is the goal of this dissertation to investigate the potential of these techniques for trace level analysis. In these studies, both UV and fluorescence detection were used. In addition, a number of chromatography-based pre-concentration techniques were examined for the further improvement of CE sensitivity. Finally, an on-line automatic clean-up and pre-concentration unit for basic drugs in human urine was developed.

Chapter 2 On-line Pre-concentration Methods for Capillary Electrophoresis

The main limitation of CE analysis of trace compounds in complex matrices, such as biological samples, is its low concentration sensitivity. Sample pre-concentration is a useful strategy to overcome this problem. A number of techniques have been developed to pre-concentrate such samples and to increase the amount of analytes that can be loaded onto the column without loss of CE efficiency. The focus of this chapter is to explain the different approaches that have been developed over the last fifteen years. These approaches can be categorized into two groups depending on the mechanism involved: electrophoretic and chromatographic techniques. Recent applications for the detection of illicit drugs are included, and the advantages and drawbacks of the techniques are discussed.

2.1 Introduction

Capillary electrophoresis has a relatively high limit of detection when compared to traditional HPLC.[47] This difference lies primarily in two areas. The first is detector path length. In HPLC, the path length of the detector cell is generally between 5-10 mm. However, in CE, since detection is performed on-column, the portion of a CE capillary used for detection typically has a length of 100 μm and a width of the capillary inner diameter (20-100 μm), resulting in a detector cell path length that is about 100 times less than that of HPLC. Because absorbance is directly proportional to path length according to Beer's Law,

sensitivity greatly suffers. The second difference is the capacity of the method to analyze samples at low concentrations. In HPLC, large volumes of a sample at a low concentration can be loaded and concentrated at the head of a column. Sample is later eluted with a gradient giving greatly enhanced sensitivity. However, in CE the application of large sample volumes results in a deterioration in the quality of the separation and a distortion of peak shape.

Several approaches to improve CE sensitivity have been developed. The use of different detection schemes, such as fluorescence,[48] mass spectrometry,[49] and electrochemistry[50] have been reported that enhance sensitivity for compounds that are amenable to these types of selective detection (Chapter 3). Extended path length detector cells, such as bubble-shaped flow cells and Z-shaped flow cells that are part of the fused silica capillary, have also been employed; however, they provide only a 3-10 fold sensitivity enhancement and the sensitivity improvement is offset by a reduction in separation efficiency.[51]

Sample pre-concentration methods are another possibility for increasing sensitivity. Pre-concentration can be combined with the CE in different ways. Four types of interfaces between sample pre-concentration and CE separation can be used: (1) off-line, where pre-concentration and CE separation are performed independently using methods that include manual solid-phase extraction (SPE),[52] manual liquid-liquid extraction (LLE)[53] and manual solid-phase microextraction (SPME);[54] (2) at-line, where a robotic system joins the pre-concentration and the separation steps;[55] (3) on-line, with direct transport taking

place by connecting capillaries;[56] and (4) in-line, where concentration takes place in the CE capillary.[57]

Pre-concentration techniques combined with CE for the analysis of a wide number of analytes in biological samples have already been reviewed.[58-60] Instead, this section will discuss past and present electrophoretic and chromatographic based pre-concentration procedures that are used for the analysis of illicit and/or abused drugs in biological fluids.

2.2 Electrophoretic methods

Electrophoretic pre-concentration methods for CE are usually easier to implement than other pre-concentration methods, as most do not require instrumental modification. There are three main electrophoretic pre-concentration mechanisms: sample stacking, field-amplified sample injection, and isotachophoresis. A brief discussion of these techniques follows:

(a) Sample stacking is the most straightforward method by which pre-concentration can be achieved in CE and was first explained by Mikkers et al.[7] in 1979. In this method, the sample is prepared such that it has a lower conductance than the buffer solution. The sample is injected hydrodynamically into the capillary and the pre-concentration effect occurs when voltage is applied after injection. In general, if a sample is dissolved in pure water, solvent, or diluted buffer, then the ionic strength of the sample zone will be considerably lower than that of the rest of the capillary. The low conductivity sample zone will, therefore, have a higher resistance than the rest of the capillary. When a voltage

is applied across the capillary, the field strength experienced in the sample zone is higher than the rest of the capillary. The sample ions will then initially move rapidly, but will slow down when they reach the buffer interface in the capillary because of the decrease in field strength. Therefore, when the voltage is applied, the contents of the sample zone are electrically focused (stacked), which reduces the length of the sample zone and produces on-capillary concentration (Figure 2-1). Macchia et al.[46] applied this method for separation of the components of clandestine heroin preparations. Heroin samples were first dissolved in $CHCl_3$-CH_3OH (96:4, v/v) and injected by pressure (0.5 p.s.i.) after evaporation of the organic mixture and reconstitution in aqueous buffer. The detection limits were in the range of 0.5 μg/mL using UV absorption at 200 nm.

(b) The principle of field-amplified sample injection (FAI) is similar to that of normal stacking.[61] The only differences are the injection procedure and the focusing process. In normal stacking, hydrodynamic injection is used and the focusing process occurs when the separation voltage is applied. In FAI, electrokinetic injection is used and the focusing process occurs during injection. Therefore, in this technique, both electrophoretic migration of the charged sample ions and electroosmotic flow of the sample solution contribute to the introduction of the sample into the capillary. This mode of injection increases the introduction of charged compounds while the introduction of non-charged compounds decreases. The low conductivity of the sample enhances the amount of charged analytes introduced into the capillary. Therefore, the ions are

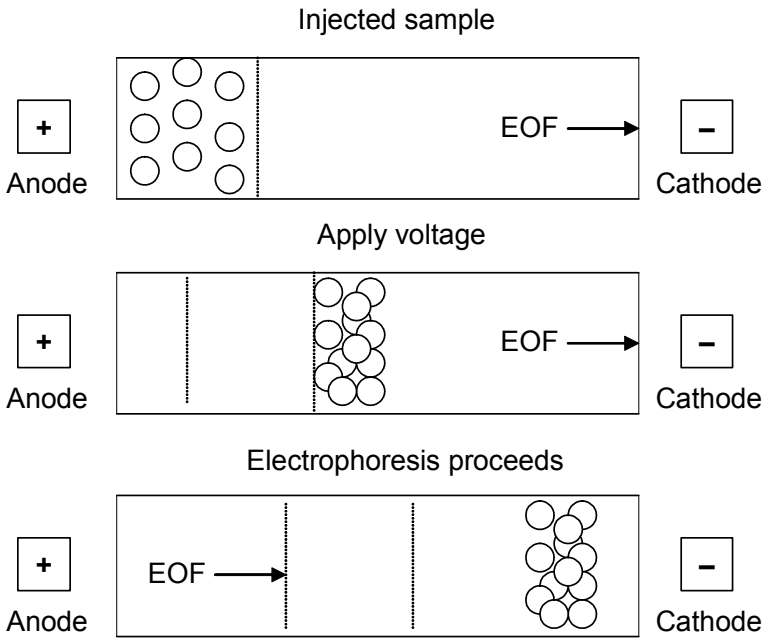


Figure 2-1: Sample stacking with a sample dissolved in a solution that has a lower conductivity than the electrophoresis run buffer. The circles represent a cationic solute. Top: the sample plug is injected. Middle: voltage is applied and since the electric field in the sample solution is higher than in the rest of the capillary, the cations rapidly migrate through the sample solution until they reach the low electric field in the buffer, where they slow down and become stacked at the boundary between the solutions. Bottom: the stacked ions migrate through the capillary as a zone that is narrower than the sample plug. Adapted from 11.

concentrated into a thin zone of the electrolyte front that possesses higher conductivity. It has been reported from measurements on antimalarial drugs that electrokinetic injection results in increased sensitivity over hydrodynamic injection.[62, 63]

(c) In isotachophoresis (ITP), the analyte is positioned between two different buffers--the leading electrolyte which contains ions with a higher mobility than the analytes (at the detector side) and the terminating buffer which contains ions with a lower mobility than the analytes (at the injection side).[64] When a high voltage is applied, a potential gradient is created throughout the capillary. Analytes are then distributed into zones on the basis of their mobilities. ITP is performed mainly in two modes--coupled-capillary ITP and transient ITP. In the first mode, two on-line coupled capillaries are utilized, where the first capillary is used for the ITP procedure and the second is used for the CE procedure. In transient ITP, both the ITP and CE procedures are completed in the same capillary.

2.3 Chromatographic methods

Several methods based on different chromatographic mechanisms are available for on-line pre-concentration.[65-67] These methods have at least one advantage over the previously described electrophoretic methods--the ability not only to enrich but also to clean-up the sample. This is extremely useful in the analysis of biological samples, such as blood, urine, or saliva. Using this methodology, the sample can be purified from interfering and clogging components, such as proteins and salts, which can disturb the electrophoretic

process. In addition, with appropriate modification it is possible to use an electrophoretic pre-concentration method (such as FAI) after chromatographic pre-concentration, thus providing further enhancement in sensitivity. A brief discussion of the most used techniques follows:

(a) Solid-phase extraction (SPE) is commonly used off-line for the extraction and separation of a wide variety of compounds in biological mixtures.[55] This is a useful technique that allows a large volume of a low concentration sample to be loaded onto the solid-phase and eluted into a smaller volume, providing concentrations that can be easily detected. Since this technique obviously consumes more analyst time, on-line methods have been investigated for CE.[68, 69] One method is to pack a short segment, about 2 mm, from the injection end of the capillary with a liquid chromatography stationary phase (Figure 2-2). This material is kept in place by using frits at each side of the packing. Therefore, the pre-concentration column is directly connected to the CE capillary. The sample is loaded onto the stationary phase by hydrodynamic injection and then eluted from the packing by the injection of a small amount of organic solvent--usually 50-100 nL (Figure 2-3). Subsequently, the CE separation process is carried out. While this technology is very useful for cleaning and concentrating analytes from biological samples, a number of problems may arise,[60] including tailing, loss of CE efficiency, peak broadening, interference between the organic elution solvent and the CE electric field, and disturbance of the electroosmotic flow. Analysis time is also longer with this method than with normal CE. These limitations are

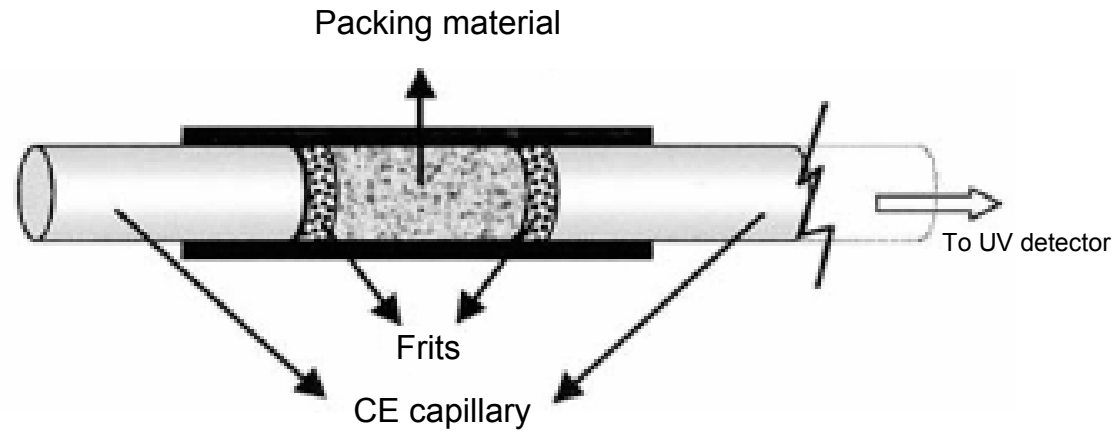


Figure 2-2: Configuration of the on-line SPE tip and its attachment to the inlet of the CE separation capillary. Adapted from 70.

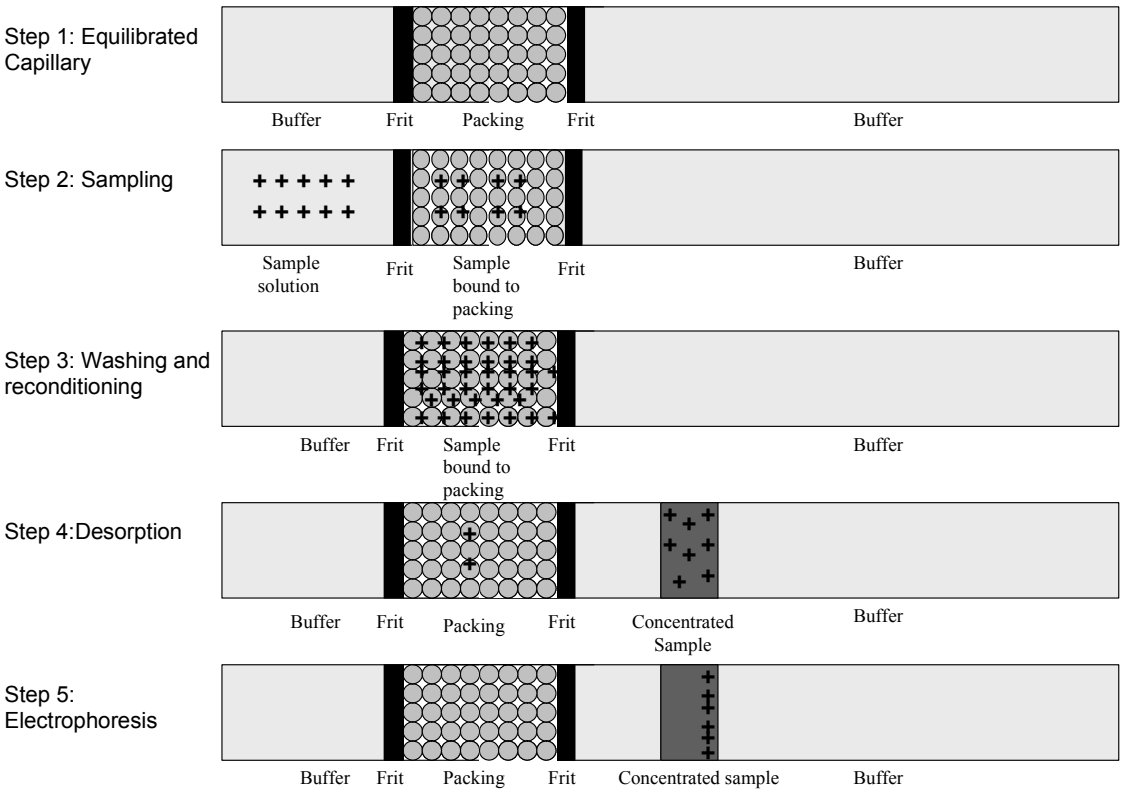


Figure 2-3: On-line pre-concentration using chromatographic techniques.

mostly caused by the packing material and the frits and can be, at least partially, solved by reducing the size of the solid-phase or completely removing the adsorptive phase from the CE capillary during electrophoresis by means of a switching valve.

(b) Membrane pre-concentration (mPC) was first developed by Naylor and co-workers[71-73] in 1995 to remove, or at least decrease, some of the problems arising from the use of large packed beds in SPE-CE. This technique is designed to improve the CE efficiency by minimizing the bed volume of the adsorptive phase at the inlet of the pre-concentration capillary. In mPC, a thin polymer membrane is installed in the center of a cartridge. Two pieces of fused silica capillary are inserted into each end of the cartridge and subsequently sealed with a solvent-resistant epoxy resin (Figure 2-4). Polymeric phases, such as styrene-divinyl benzene, C_2, C_8, and C_{18} have all been used for protein analysis.[60, 71-74] Although, the application of this pre-concentration method partially improves the detection limit for on-line CE analysis, the CE efficiency is also greatly affected. Furthermore, the analysis time is longer and the capillary is subjected to clogging, especially when urine samples are analyzed.

(c) Unlike the previous on-line chromatographic pre-concentration techniques, flow injection systems (or automated SPE-CE) are among the most powerful tools for implementing pretreatment and conditioning samples in an automated fashion.[75, 76] Various automated SPE-CE on-line assemblies have recently been used for this purpose with excellent results.[77-79] As can be seen in Figure 2-5, the

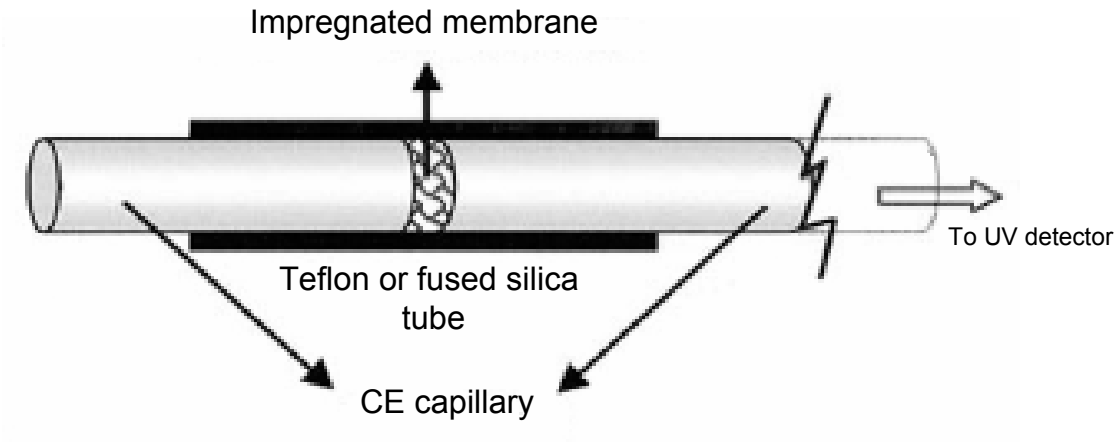


Figure 2-4: Configuration of the on-line mPC tip and its attachment to the inlet of the CE separation capillary. Adapted from 70.

system consists of a multi-channel peristaltic pump and an injection valve. The injection valve allows adsorption of the sample onto a C_{18} cartridge for its pre-concentration and clean-up. In the load position, samples are loaded onto the C_{18} column and cleaned with an organic solvent, followed by a water rinse. Analytes can then be eluted with a small amount of organic solvent and transferred directly to the CE system for analysis. The potential use of this technique for the analysis of abused drugs in biological samples has been investigated and will be discussed (Chapter 7).

(d) Solid-phase microextraction (SPME) was developed in the late 1980s by Arthur and Pawliszyn.[80, 81] A sample (or its headspace for volatile analytes) is exposed to a coated fiber for a finite period of time, and analytes can then partition between the sample and the fiber phase. Depending on the length of exposure, the amount of analyte extracted is either based on its equilibrium distribution between the two phases or, when there is insufficient time for equilibrium to be reached, it is proportional to the initial concentration and the time of extraction. The strongest features of SPME are its simplicity, rapid extraction time, solvent-free nature, and its ability to be automated. This technique is most commonly used in environmental research for the extraction of organic compounds from water samples.[82, 83] However, there are a few reports of the use of SPME coupled with GC for the analysis of abused drugs, such as amphetamine and cocaine, in biological matrices.[84, 85] The reported techniques proved to be very sensitive; however, they are less sensitive when applied to the

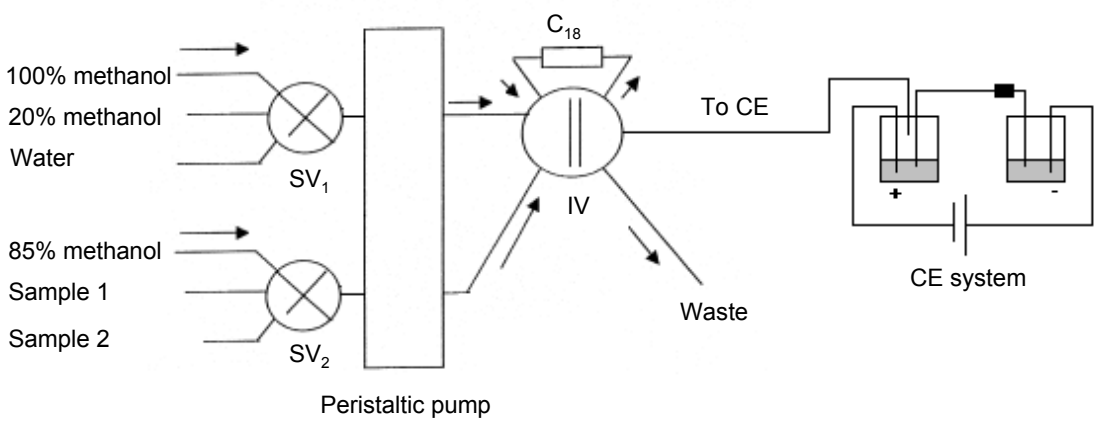


Figure 2-5: Automated SPE-CE designed for the pre-concentration and clean-up of urine samples. SV: switching valve and IV: injection valve. Adapted from [76].

detection of polar drugs such as morphine and codeine especially since derivatization reagents are not available. The application of SPME-CE for the analysis of abused drugs has never been previously reported and will be given in Chapter 7.

Chapter 3 Reactive Fluorescent Dyes

Derivatization of analytes with fluorescent dyes, in combination with laser induced fluorescence detection (LIF), has the potential for greatly enhancing sensitivity. In this chapter, a number of the amine and hydroxyl reactive fluorescent dyes for opiate drug analysis are reviewed and the advantages and drawbacks of these methods were discussed.

3.1 Detection schemes in capillary electrophoresis

There are a number of different approaches that can be used for detection in capillary electrophoresis. These include the use of absorbance,[86, 87] fluorescence,[48] mass spectrometric,[49] or electrochemical[50] detectors. Typical ranges of detection limits for each method are listed in Table 3-1. Unfortunately, absorbance measurements suffer from minimal path lengths and poor UV transparency. Electrochemical detectors suffer coupling problems with the electric fields used in separations. The use of mass spectrometers eliminates the natural advantages of the device; i.e. low cost and simplicity. Unlike these methods, the fluorescent approach has natural advantages when used with CE since fluorescence output increases with excitation light intensity yielding highly sensitive detection. In forensic and pharmaceutical analyses, most CE applications utilizing fluorescence detection employ laser sources, as these provide incident light that is monochromatic and easily focused. While lamp sources can be utilized for fluorescence detection in CE, the detection limits are

Table 3-1: Limit of detection for different detection techniques in CE.

Detection technique	Limit of detection (LOD M)	Comments
UV absorption	10^{-5}–10^{-6}	Direct and indirect UV[12, 88]
Fluorescence	10^{-10}–10^{-11}	Native fluorescence[89]
	10^{-13}	Chemical derivatization[90, 91]
	10^{-16}	Post-column LIF[92]
	10^{-5}–10^{-7}	Indirect LIF[93]
Electrochemical	10^{-7}–10^{-8}	Conductivity[94]
	10^{-7}–10^{-8}	Amperometry[95]
Mass spectrometry	10^{-8}–10^{-10}	

not as low as those of laser sources due to reduced intensity. There are three approaches to fluorescence detection coupled with CE. These involve the measurement of indirect fluorescence, native fluorescence, or the fluorescence of derivatized compounds. Few applications of indirect fluorescence detection for abused drugs have been reported[93, 96] because these methods typically suffer from poor sensitivity (LODs are in a range of 10^{-5}-10^{-7} M).

Fluorescence detection of compounds that possess native fluorescence, while highly useful, is limited by the number of compounds that will fluoresce at wavelengths obtainable with commercially available capillary electrophoresis-laser-induced fluorescence (CE-LIF) systems. Compounds that have been detected by native fluorescence include lysergic acid diethylamide (LSD),[97] zolpidem,[98] zopiclone,[99] naproxen,[100] and heroin impurities.[101] Unfortunately, the excitation bands of major opiate drugs such as morphine, codeine, 6-AM, and normorphine are in the UV range, where laser excitation is not available in a simple, inexpensive, commercially available CE instrument. In this case, chemical derivatization and/or conventional multiple excitation wavelength fluorescence detectors can be utilized.

Most reports of the use of capillary electrophoresis with LIF detection for the analysis of forensic and pharmaceutical compounds involve chemical derivatization of the compounds of interest prior to analysis.[102, 103] The application of so-called “fluorogenic reactions” by which non-fluorescent compounds can be converted into fluorescent derivatives by chemical means,

has long been accepted as an effective modification technique in various separation methods, such as GC and HPLC.[104-106] These reactions improve the overall specificity, the chromatographic performance, and the sensitivity for trace analysis.

Chemical derivatization for fluorescence detection can be performed before or after the separation. Each method has distinct advantages. With pre-column derivatization, the tagging reaction may be more complicated. However, the tag itself can be fluorescent since excess reagent will simply be separated from the tagged derivatives. Therefore, pre-column detection limits are often better due to lower background levels. In addition, because the reaction chemistry occurs prior to the separation, no band broadening results from the tagging protocol. However, because the fluorescence tag is usually large relative to the analyte of interest and is often charged, tagged analytes may appear more similar in size and charge than untagged analytes. This makes separation more difficult. The high efficiency of CE will often solve this problem and, as a result, most of the CE separations of forensic and pharmaceutical compounds utilizing fluorescence detection have employed pre-column derivatization.[48, 107]

The advantages of post-column derivatization[108] are that the separation is based solely on the properties of the analyte of interest and not the fluorescent tag and that a working post-column system is easier to operate because the tagging chemistry is performed on-line. One disadvantage of post-column systems is that the tagging reagent must not be intrinsically fluorescent or there

will be a large background interference due to the tag. A second disadvantage of post-column derivatization techniques for CE is that the analytes separated by the applied voltage are diluted during mixing with the derivatization reagents, which also results in higher limits of detection. In addition, the derivatization reaction must be fast, and the reaction products must be stable long enough to reach the detector. Lastly, post-column systems are more difficult to develop because of their complicated assembly.

Before a fluorescent derivative can be considered for CE, it needs to meet certain criteria. The drug/derivative complex must be soluble in aqueous solution and possess a charged functional group. The fluorophore also needs to have good sensitivity (i.e., high quantum yield) and an excitation wavelength that falls within the capabilities of available laser systems. However, a number of derivatization procedures exist which may be suitable for this purpose. With proper optimization, these procedures should provide a sensitive and specific screening mechanism for the analysis of illicit and/or abused drugs.

3.2 Fluorescence derivatization of basic drugs

Among the most common fluorescence derivatizing reactions are those which target reactive species, such as acids, alcohols, and primary and secondary amines. Unfortunately, many drugs of abuse (i.e., cocaine and heroin) are tertiary amines and are not compatible with the most commonly utilized amine reactive fluorescent dyes. These dyes include fluorescein isothiocyanate

isomer I (FITC),[107] 4-(4,6-dichloro-s-triazin-2-ylamino)fluorescein (DTAF),[109] 4-fluoro-7-nitrobenzofurazan (NBD-F),[110] 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA),[111] and 5-carboxyfluorescein succinimidyl ester (FSE)[112] (Figure 3-1).

However, compounds with tertiary amine groups can be derivatized for fluorometric analysis by utilizing a mixture of malonic acid and acetic acid anhydride reagent (MAA).[113, 114] Rama-Rao and Tandon[115] described the application of MAA reagent for the spectrofluorometric determination of the opium alkaloids, including morphine, codeine, thebaine, papaverine, and narcotine. The reaction solution was heated with anhydride reagent at 80-85 ºC After cooling and the addition of ethanol, the fluorescence was measured. The derivatized drugs have excitation and emission wavelengths of 480 nm and 520 nm, respectively. The fluorescent product formed by condensation of the MAA reagent with the various drugs has not as yet been identified. Thin-layer chromatography indicated that there was one or two products. However, the chromatograms also had a number of other spots that seemed to be associated with the MAA reagent.[114] HPLC studies performed in our laboratory support this view. Unfortunately, the fluorescence of the reaction product deteriorates in the presence of water and other hydroxylated solvents, leading to unsuitable conditions for CE analysis.

Hara et al.[116] reported the reaction of the hydroxyl group of morphine with benzylamine in the presence of potassium hexacyanoferrate (III) in neutral

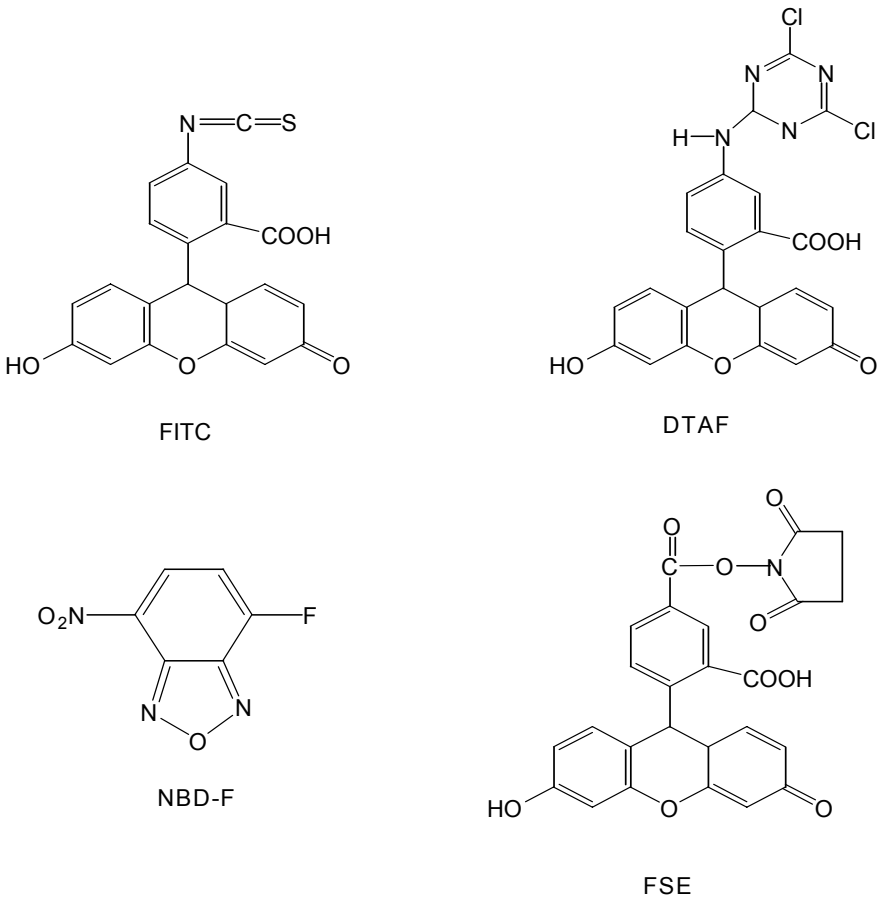


Figure 3-1: Amine reactive fluorescent dyes. Fluorescein isothiocyanate isomer I (FITC), 4-(4,6-dichloro-s-triazin-2-ylamino)fluorescein (DTAF), 4-fluoro-7-nitrobenzofurazan (NBD-F), and 5-carboxyfluorescein succinimidyl ester (FSE).

media (borate buffer, pH 7.0) to produce an intensely fluorescent product (Figure 3-2a). Separation was performed using a HPLC system equipped with a fluorescence detector operated at an excitation wavelength of 348 nm and an emission wavelength of 467 nm. This reaction was extensively examined in our laboratory for major opiate drugs; however, no fluorescence derivatives were obtained under the described conditions even at high concentrations.

In another set of experiments, Barrett and colleagues[117] described the derivatization reaction of major opiate drugs, such as morphine, codeine, 6-AM, and normorphine with dansyl chloride (Figure 3-2b). The reaction was performed in the dark at 45 ºC for 20 min and the resulting reaction mixture was purified by liquid-liquid extraction with toluene. Analysis was carried out using normal-phase HPLC equipped with a fluorescence detector, operated at an excitation wavelength of 340 nm and an emission wavelength of 500 nm. The detection limit was 10 ng/mL for morphine and 25 ng/mL for 6-AM. These detection limits are not low enough to be used for trace level analysis.

The oxidation of morphine to its fluorescent dimer, pseudomorphine, by alkaline potassium ferricyanide has been extensively used for the detection of morphine in biological samples.[118] This reaction has been applied as a pre- and post-column fluorescence derivatization method for HPLC (Figure 3-3). The reaction has excitation and emission wavelengths of 322 nm and 432 nm, respectively. The detection limit was 140 ng/mL. As indicated above, this detection limit is considered to be too high for use in forensic toxicology.

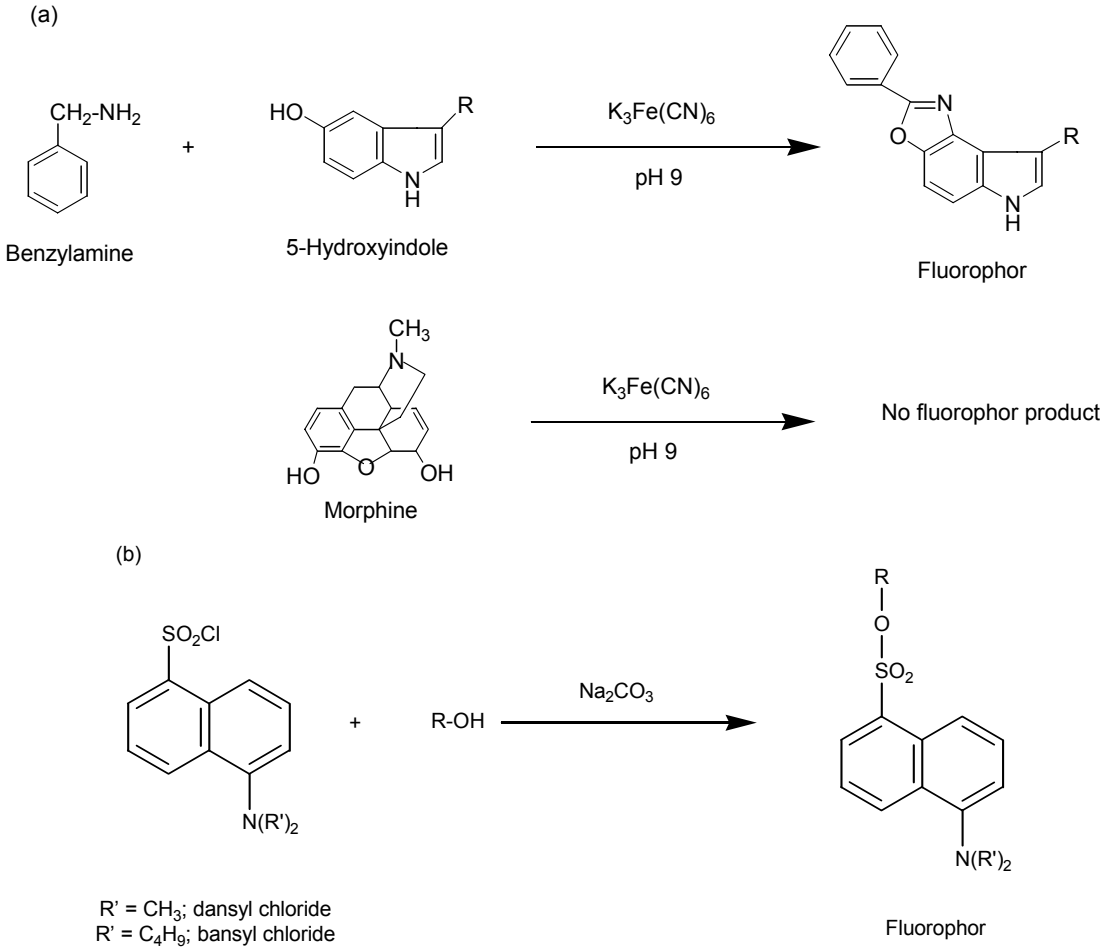
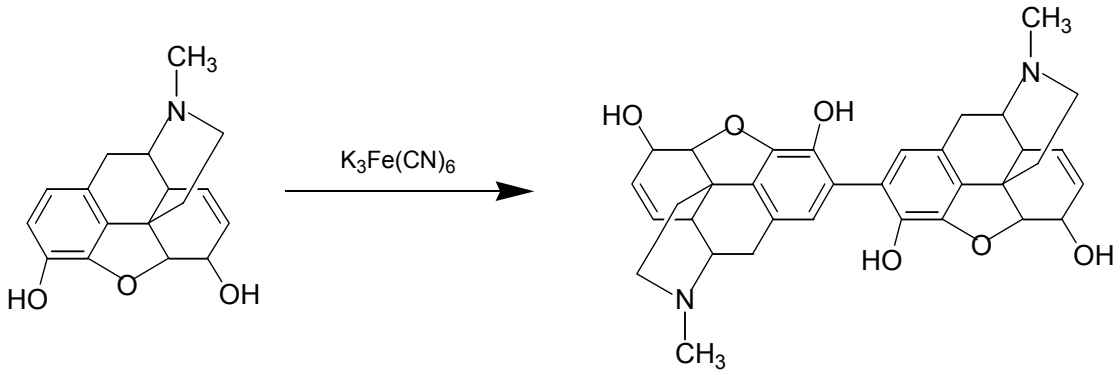


Figure 3-2: Hydroxyl reactive fluorescent dyes: (a) the reaction of benzylamine,[119] (b) the reaction of dansyl chloride.[120]



Morphine

Pseudomorphine
(Fluorophore)

Figure 3-3: Oxidation dimerization of morphine to the fluorescent pseudomorphine (2,2- bimorphine).[118, 120]

Additionally, the excitation and emission wavelengths of this reaction are considered to be unsuitable for the argon-ion lasers utilized in commercially available CE-LIF systems.

An alternative to the chemical derivatization procedures previously mentioned is to convert the tertiary amine group on these drugs to a secondary amine. This conversion allows for the use of a large variety of sensitive fluorescent dyes that target secondary amines, including FITC, DTAF, FSE, and NBD-F. Using this methodology, a more sensitive and selective CE-LIF detection technique for drug analysis could be obtained.

Several methods have been applied to the *N*-dealkylation of tertiary amines drugs, including reactions with cyanogen bromide,[121] photochemical degradation,[122] ruthenium catalyzed reactions with alkyl- and hydrogen peroxides,[123] oxidation with *m*-chloroperbenzoic acid,[124] and reaction with chloroformates.[125] Of these reactions, α-chloroethyl chloroformate (ACE-Cl) gives the best selectivity and highest yields for the *N*-dealkylation of tertiary amines.

Olofson[126] first reported the reaction of *N*-ethylpiperidine with ACE-Cl to give piperidine hydrochloride in a 99% yield. The reaction (Figure 3-4) was performed by adding ACE-Cl to *N*-ethylpiperidine in the presence of dichloroethane at 0 ºC and then refluxing the mixture for 1 h. The intermediate ACE-piperidine was then demethylated to form piperidine hydrochloride by evaporating the reaction mixture in vacuo and then heating the residue in

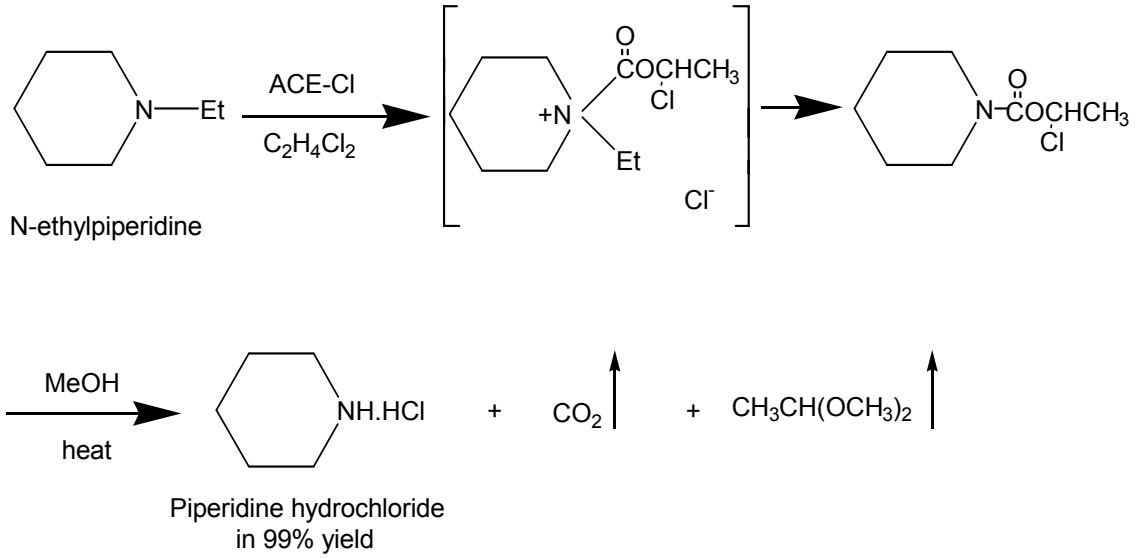


Figure 3-4: The reaction of the tertiary amine drug of *N*-ethylpiperidine, with α-chloroethyl chloroformate to give a secondary amine, piperidine hydrochloride.[126]

methanol. The reaction was tested on a number of tertiary amine compounds, such as arecoline, O-acetyltropine, 6-acetylcodeine, and oxycodone. The yields of the *N*-demethylated products of these compounds were in the range of 95-97%.

Recently, Pelander et al.[127] reported the use of ACE-Cl for *N*-demethylation of tertiary amine compounds of forensic interest, such as promazine, levomepromazine, chlorprothixene, clomipramine, and orphenadrine. The reaction was performed as stated above. The mixture was refluxed for 24 h under nitrogen and the yield of the purified *N*-demethylated products was in the range of 42-60 %. Later, Ferguson and co-workers[128] reported the demethylation of morphine-6-glucuronide (M-6-G) to produce normorphine-6-glucuronide as its hydrochloride salt in a 73% yield, using the ACE-Cl reagent. The refluxing time necessary for this reaction was 6 h.

Following demethylation, various fluorescent derivatization reagents[48, 107-112] are readily applicable to CE-LIF detection. FITC was chosen to derivatize the synthesized nor-drugs because of (1) its efficiency for the derivatization of secondary amines, (2) the match of its excitation and emission wavelengths with the 488/520 nm argon ion laser optical system, and (3) its high fluorescence quantum yield and molecular absorption coefficient. FITC has been widely used as a fluorescent derivatization reagent in forensic and pharmaceutical fields, providing good sensitivity for secondary amines.[48, 107, 129] The first use of this reagent in CE was reported by Cheng and Dovichi[130] in 1988 when subattomole

analysis of some amino acids was demonstrated. The principle of the derivatization is based on the reaction of the amino groups of the demethylated drugs with the isothiocyanate functionality of FITC.

Figure 3-5 depicts the two reactions used for the fluorescent of the abused drugs analyzed in our studies. The reactions are (1) the demethylation of the tertiary amine using ACE-Cl and (2) the fluorescent derivatization of the secondary amine using FITC. These reactions greatly enhance the sensitivity and selectivity of CE for the analysis of drugs in biological fluids. A detailed description of the fluorescence derivatization mechanism and the parameters effecting these reactions for trace level analysis will be given in Chapter 6.

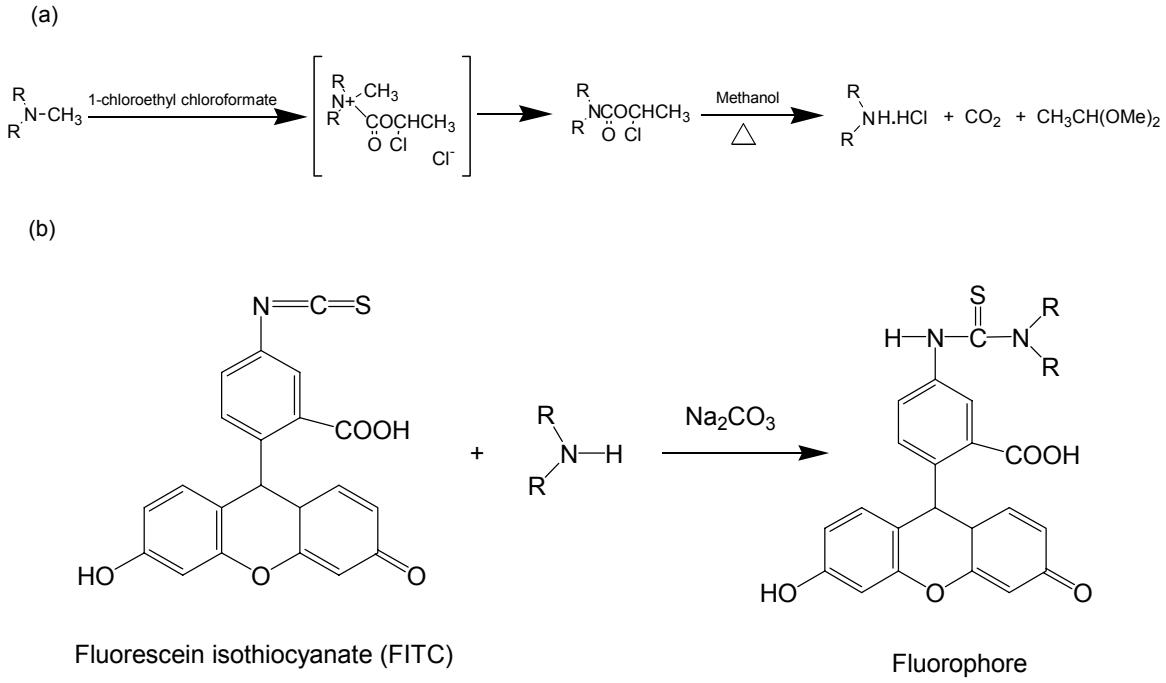


Figure 3-5: Two-step pre-column derivatization reaction: (a) the first step was based on the *N*-demethylation of a tertiary amine using 1-chloroethyl chloroformate;[127] (b) the second step was based on the derivatization of the secondary amine using fluorescein isothiocyanate isomer I.[107]

Chapter 4 Experimental Procedures

4.1 Field amplified sample injection

4.1.1 Chemicals

6-acetylmorphine.HCl, normorphine.HCl, noscapine.HCl, papaverine.HCl, and nalorphine.HCl were obtained from Lipomed Inc. (One Broadway, Cambridge, MA). Morphine-sulfate, codeine-sulfate, thebaine, opium, and levallorphan were purchased from Sigma-Aldrich. Native β-cyclodextrins are commercially available from TIC America. Sodium phosphate and phosphoric acid were purchased from Spectrum Quality Products, Inc. (Gardena, CA). Bond Elut Certify SPE columns were obtained from Varian Sample Preparation Products (Harbor City, CA). All other solvents and reagents were of analytical grade.

4.1.2 Apparatus and conditions

The P/ACE 5000 SERIES CE (Beckman Instruments, Inc. Fullerton, CA) was used with a UV absorbance detector operated at 210 nm. Control of the instrumentation, data acquisition, and processing was performed with Chrom Perfect software version 3.5 (Justice Laboratory Software, Palo Alto, CA). Separation was achieved using an uncoated (50 μm I.D.) fused-silica capillary (Polymicro Technologies) with a length of 77 cm and the detector window located 10 cm from the outlet end. The capillary was washed sequentially with distilled water (1 min), 0.1 M NaOH (1 min), and distilled water (2 min) at high pressure

(20 p.s.i.), followed by reconditioning with running buffer (3 min) at high pressure between runs. After daily use, the capillary was washed with distilled/deionized water for 5 min at high pressure.

The CZE system was operated using “normal” polarity (the cathode was located on the detector side). Samples were injected electrokinetically. The electrokinetic injection was made by placing the capillary and the anode into the source vial and applying a voltage (5 kV) for 10 s. After the sample was introduced, the anode and capillary were placed back into the source vial, an electric field was applied, and electrophoresis was initiated. The system was run at 20 ºC and at a constant field strength of 325 V/cm. Under optimized electrophoretic conditions, separations were carried out using a running buffer that contained 50 mM sodium phosphate (adjusted to pH 6 with phosphoric acid) and 15 mM ß-CD.

4.1.3 Preparation of standard solution and FAI conditions

Standard mixtures were prepared by adding 50 µL of codeine, morphine, normorphine, and 6-AM (1 mg/mL in methanol) to 10 µL (1% HCL in methanol). The resulting mixture was evaporated to dryness under a stream of N_2. The dried residues were reconstituted in 200 µL of slightly warm H_2O. The resulting mixture was diluted in water to prepare six different concentrations: 500, 400, 300, 250, 150, and 100 ng/mL, for calibration. Nalorphine (internal standard, IS_1) and levallorphan (IS_2) were diluted in water to the desired concentration and were added to each sample. All standard solutions were stored at 4 ºC. Calibration

curves were constructed by plotting the ratio of the peak area of each of the drugs to the nalorphine against the known concentrations of drugs in the standards and fitted by linear regression analysis.

4.1.4 Urine samples preparation

Due to the tendency of opiates and their metabolites to undergo conjugation prior to elimination, sample pretreatment is necessary to determine the total amount of drug in a specimen. Acid hydrolysis was used in these experiments to cleave off the conjugated group at elevated temperatures. However, acid hydrolysis should be used with caution because 6-AM may be degraded in the process.[22, 32]

(a) Tests for 6-AM: Levallorphan and 2 mL of 10 mM phosphate buffer (pH 6) were added to 5 mL human urine samples spiked with 6-AM. The pH was then adjusted to 8.0-8.5 with KOH.

(b) Tests for normorphine, morphine, and codeine: Levallorphan and 1 mL concentrated HCl were added to 5 mL human urine samples spiked with the above metabolites. The samples were immersed in a hot water bath for at least 30 min at 100 ºC and cooled to room temperature before proceeding. 2 mL of 0.1 M phosphate buffer (pH 6) was added and the samples were vortexed. The pH was then adjusted to 8.0-8.5 with KOH.

4.1.5 Extraction procedure

A 300 mg Bond Elut Certify SPE column was used for the extraction. The SPE columns were conditioned by the sequential passage of 2 mL of methanol and 2 mL of 10 mM phosphate buffer (adjusted to pH 6-6.5). The supernatant layers from the samples were applied to the SPE columns. The columns were washed with 2 mL of distilled water, 2 mL of 10 mM phosphate buffer adjusted to pH 4.0 with phosphoric acid for extraction of 6-acetylmorphine or 100 mM acetate buffer (pH 4) for extraction of the other opiates, and 2 mL of methanol. The drugs were eluted with a solution consisting of a single phase mixture of dichloromethane/ isopropanol/ ammonium hydroxide (78/20/2) and collected in glass tubes. The elution solvent was evaporated to dryness under nitrogen. The dried residues were then reconstituted in slightly warm water and nalorphine was added before the injection of the sample. Sample preparation and the solid-phase extraction takes less than 40 min. In addition, as many as 12 samples can be simultaneously extracted using the Visiprep vacuum manifold (Supelco, Bellefonte, PA).

4.2 Fluorescence detection

4.2.1 Chemicals

The derivatizing agents fluorescein isothiocyanate isomer I and 1-chloroethyl chloroformate were purchased from Sigma-Aldrich. 1, 2 dichloroethane, sodium carbonate, and sodium borate were purchased from Spectrum Quality Products, Inc. Acetonitrile was purchased from GFS Chemical,

Inc. (Columbus, OH). Isopropyl alcohol was obtained from EMD Chemical Inc. (Gibbstown, NJ). The drug standards were also supplied by Sigma-Aldrich. Deionized water was used to prepare all buffers. All other solvents and reagents were of analytical grade.

4.2.2 Apparatus and conditions

For CE-native fluorescence analysis, a laboratory designed capillary electrophoresis instrument was built. This system utilized a FL 750B fluorescence detector (McPherson, Chelmsford, MA). Detection was performed at an excitation wavelength of 245 nm with a cutoff emission filter of 320 nm. Capillaries with a length of 77 cm (70 cm to detector) × 50 μm I.D. were used. Samples were electrokinetically injected using a high voltage power supply at 5 kV for 10s. The system was run at room temperature and at a constant field strength of 325 V/cm. Separations were carried out using a running buffer containing 50 mM sodium phosphate (adjusted to pH 6 with phosphoric acid) and 15 mM ß-CDs.

For the CE-LIF system, a P/ACE 5000 SERIES CE fitted with an argon-ion laser (National Laser Company, Salt Lake City, UT) possessing an excitation wavelength of 488 nm and an emission wavelength of 520 nm was used throughout the experiments. Capillaries with a 47 cm length (40 cm to detector) and 75 μm I.D. were used. Samples were injected hydrodynamically. The system was run at 20 ºC and at a constant field strength of 532 V/cm. Separations were

carried out using a running buffer containing 20 mM sodium borate with 10% isopropanol, 10% acetonitrile, and 20 mM ß-CDs.

The capillaries for both applications (native fluorescence and fluorescence derivatization) were washed sequentially with distilled water (2 min), 0.1 M NaOH (2 min), and distilled water (2 min) at high pressure (20 p.s.i.), followed by reconditioning with running buffer (2 min of high pressure rinsing) between runs. After the end of each day, the capillary was washed with 0.1 M NaOH for 5 min followed by distilled/deionized water for 5 min. The CE systems were operated using “normal” polarity. Control of the instrumentation, data acquisition, and processing was performed with Chrom Perfect software version 3.5.

4.2.3 Sample preparation

A mixture of stock solutions containing 1000 mg/L of each drug were prepared in methanol, stored in a refrigerator at 4 ºC, and used after dilution to their required concentrations. The preparation and solid-phase extraction of a spiked human urine sample were performed, as previously described.[32, 57] A flow chart of the complete assay procedure is shown in Figure 4-1.

4.2.4 Derivatization procedure

The ACE-Cl procedure was a modification of the method of Olofson.[126] The illicit drugs were dissolved in 2 mL dry 1, 2-dichloroethane. Excess ACE-Cl was added (50 µL), and the mixture was then refluxed for 3-4 h under nitrogen. After refluxing, the solvent was evaporated and the residue was dissolved in

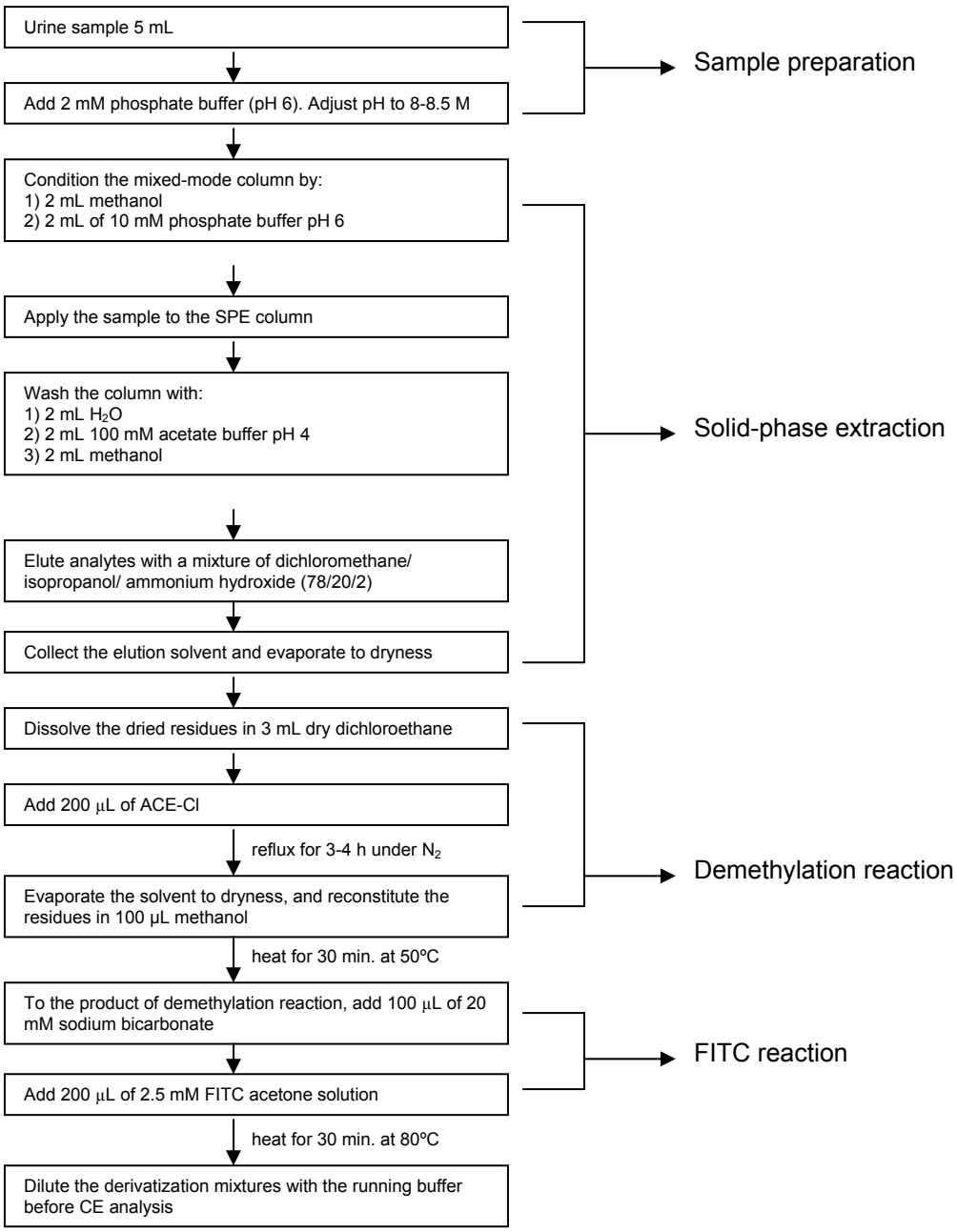


Figure 4-1: Flow chart of the complete assay for codeine, 6-AM, and morphine.[32, 48, 107, 126, 129, 130]

methanol (1 mL). The solution was kept in a water bath at 50 ºC for 30 min to hydrolyse the intermediate carbamate and then concentrated to a volume of 100 µL before the FITC fluorescence derivatization step.

To the product of the demethylation reaction, 100 µL of 20 mM sodium bicarbonate (pH 8.5) and 200 µL of 2.5 mM FITC acetone solution were added. After stirring, the mixture was heated at 80 ºC for 30 min in darkness. Before CE analysis, the derivatization mixtures were diluted with the electrophoretic buffer. To prepare the reagent blank, 100 µL of water in place of the test solution was used throughout the procedure. Limits of detection were calculated on the basis of the minimum analyte concentration that provided a chromatographic signal three times higher than peak-to-peak noise.

4.3 Automated solid-phase extraction

4.3.1 Chemicals

All chemicals and solvents were of analytical grade. Amphetamine, methamphetamine, ephedrine, psilocin, cocaine, cocaethylene, methaqualone, canuabiol, benzoylecognin, normorphine, hydrocodone, hydromorphone, caffeine, methadone, pentachlorophenol (PCP), pheniramine, diphenhydramine, oxycodone, thebaine, fentanyl citrate, codeine, morphine, 6-AM, quinine, heroin, noscapine, papaverine, and Morphine-3-glucuronide (M-3-G) were purchased from Sigma-Aldrich. β-CDs are commercially available from TIC America. Sodium phosphate and phosphoric acid were purchased from Spectrum Quality Products, Inc. Stainless steel column blanks were purchased from Supelco

(Bellefone, PA). Modified silica particles (C_{18}, 45 µm, 60 °A) were also purchased from Supelco.

4.3.2 Apparatus and conditions

The experiments were performed using a P/ACE 5500 CE system equipped with a UV detector operated at 210 nm. A SP 8800 ternary HPLC pump (Spectra-Physics), six port injection valve (Valco instrument Co. Inc., Houston, Texas), and PTFE tubing of 0.05 mm I.D. were used to construct the manifold. Control of the instrumentation, data acquisition, and processing was performed with Chrom Perfect software version 3.5. Unless otherwise stated, the separation was performed on a 67 cm (effective length 60 cm) × 51 µm I.D fused-silica capillary. The capillary was washed sequentially with distilled water (2 min), 0.1 M NaOH (2 min) and distilled water (2 min) at high pressure (20 p.s.i.), followed by reconditioning with running buffer (4 min of high pressure) between runs. The CE systems were operated using “normal” polarity. Samples were electrokinetically injected (5 kV for 10 s). The system was run at room temperature and at a constant field strength of 373 V/cm. Separations were carried out using a running buffer containing 100 mM sodium phosphate (adjusted to pH 6 with phosphoric acid), 20 mM ß-CD, 5% acetonitrile, and 20% isopropanol.

4.3.3 Urine samples preparation

Sample pH should be adjusted to 9.5 in order to neutralize all of the basic compounds present and maximize reversed-phase retention (this procedure also

served to remove proteins). Therefore, 4.00 mL of human urine sample spiked with basic drugs was adjusted to pH 9-9.5 with KOH. The sample was then vortexed, centrifuged, and the precipitate was discarded.

4.3.4 Extraction procedure

The assembly used to pre-concentrate and clean-up the urine samples is depicted in Figure 2-5. The switching valve (SV_1) allowed selection of 100% methanol and water to condition the C_{18} column and 20% methanol for sample clean-up. Valve SV_2 allowed switching among the different samples. The injection valve (IV) permitted injection of the sample into the C_{18} column for its pre-concentration and clean-up. In the load position, samples were loaded onto the C_{18} column and cleaned with a 20% methanol solution, followed by water. Drugs were eluted with a small volume of methanol when the injection valve was in the elution position. The carrier flow rate was 0.8 mL/min throughout the experiment. The extraction process was started by conditioning of the C_{18} cartridge with 100% methanol for 2 min and water for 2 min. A 4 mL urine sample (or working standard) was then introduced into the system. After this introduction, the column was flushed with 20% methanol for 2 min, followed by water for 2 min. Finally, the drugs were eluted with 85% methanol for 40 s (100 µL) and manually transferred to the CE system. However, a programmable arm controlled by a microcomputer through an electronic interface could also be used to transfer the eluted drug to the CE instrument in an automated fashion.[131]

Chapter 5 Field Amplified Sample Injection Capillary Zone Electrophoresis Applied To the Analysis of Heroin Metabolites In Biological Fluids

The present chapter discusses the application of electrokinetic injection with field amplified sample stacking as a tool to improve the sensitivity of capillary electrophoresis for the analysis of heroin metabolites in biological matrixes. The importance of β-CD concentration and pH adjustment in obtaining selectivity have been investigated and will be discussed, along with other factors found to be significant in obtaining high resolution and optimum sensitivity. Also, a relatively new solid-phase extraction technique, which provides clean extracts with high recoveries for all tested drugs, will be utilized for extraction of urine samples.

5.1 Optimization of the separation conditions

In the first stage of our work, we used a buffer containing 50 mM sodium phosphate and 100 mM phosphoric acid at pH 6. A typical electropherogram for a mixture of heroin metabolites is shown in Figure 5-1. Under these conditions, analytes were only partially separated in the following order: normorphine + codeine, morphine, nalorphine (internal standard), and 6-AM. The lack of resolution may be attributed to the fact that all analytes migrate according to their charge-to-size ratios in CE, which in this case are very similar. To enhance this separation, further experiments were performed by changing the pH and the buffer concentration. However, no improvements were seen in the results.

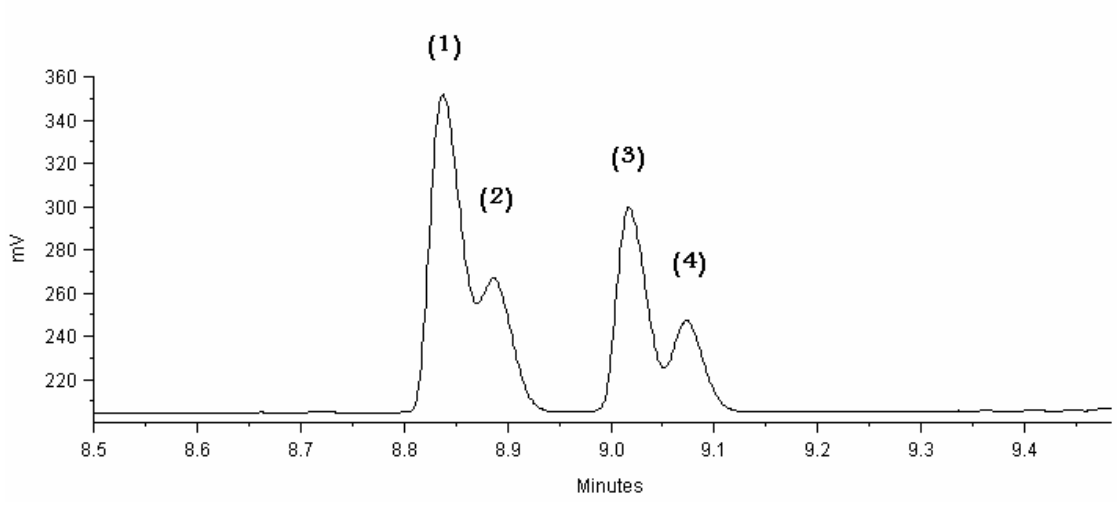


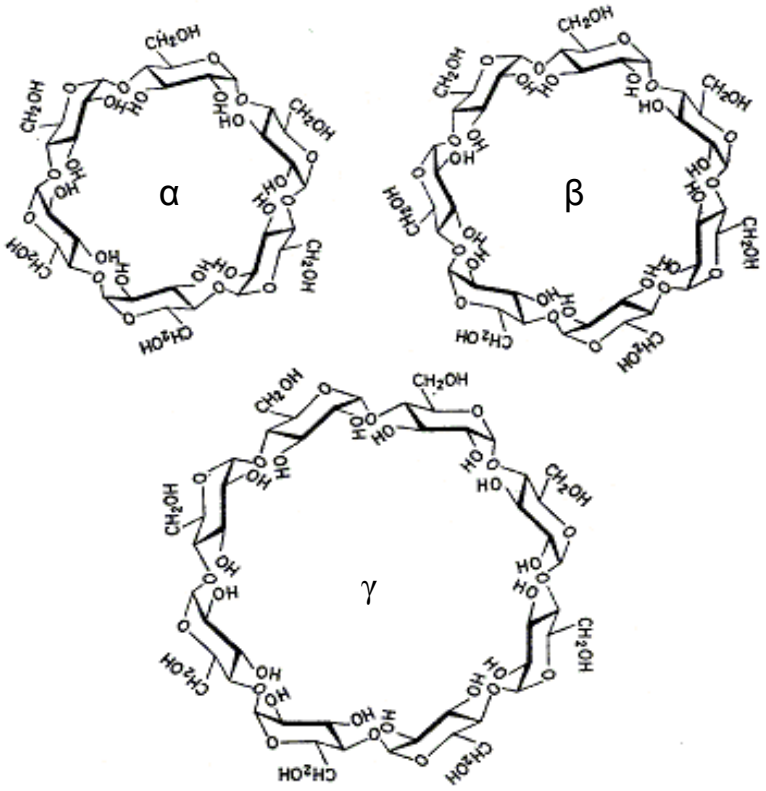
Figure 5-1: Typical electropherogram of 500 ng/mL mixture of heroin metabolites: (1) normorphine, codeine; (2) morphine; (3)1000 ng/mL nalorphine and (4) 6-AM. Conditions: injection, electrically (5 kV) for 10 s; capillary, uncoated (50 μm I.D.) fused-silica capillary, 77 cm long, and containing the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

5.1.1 Effect of the addition of cyclodextrins (CDs)

CDs are sugar molecules that have the structure of a hollow truncated cone with a hydrophobic cavity. These compounds, as shown in Figure 5-2a, consist of six, seven, or eight glucopyranose units attached by (1→4) linkages (α, β, and γ, respectively). They contain a hydrophilic outer rim and a hydrophobic inner core, as depicted in Figure 5-2b. Run buffers containing CDs have proven invaluable for forensic drug applications, including the analysis of enantiomers[44] and diastereomers.[132, 133] This mode of CE analysis is particularly attractive because derivatization is not required.

The effect of the addition of CDs is shown in Figure 5-3. When ß-CDs were added to the buffer solution, clear separation of the standard mixture of four opiates was achieved. In this case, base-line separation was accomplished with a running buffer composed of 50 mM sodium phosphate (pH 6) and 0.015 M ß-CDs. Under these conditions, analytes were separated in the following order: normorphine, morphine, 6-AM, codeine, nalorphine, and levallorphan. This order can be attributed to the influence of the hydrophobic cavity of the ß-CDs. The rate that solutes partition into and out of the cavity will vary with their structure, polarity, and size. Concomitantly, the mobility of these solutes will be affected as well. When the solutes partition into the cavities, their velocities are retarded, but when they are in the bulk phase, their mobilities are unaffected. It is this differing partitioning behavior among the various drugs that leads to greater differences in their mobilities and, therefore, an improved separation when CDs are used.

(a)



(b)

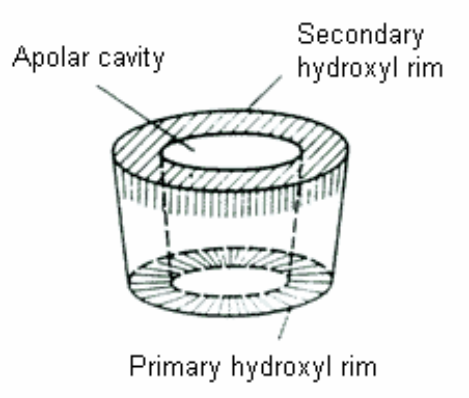


Figure 5-2: (a) Native cyclodextrin α, β, and γ. (b) basic feature of the three native cyclodextrins. The primary hydroxyl rim is made up of hydroxyls attached to the C_6 carbons, and the secondary hydroxyl rim is made up of hydroxyl groups attached to the C_2 and C_3 carbon atoms.[134]

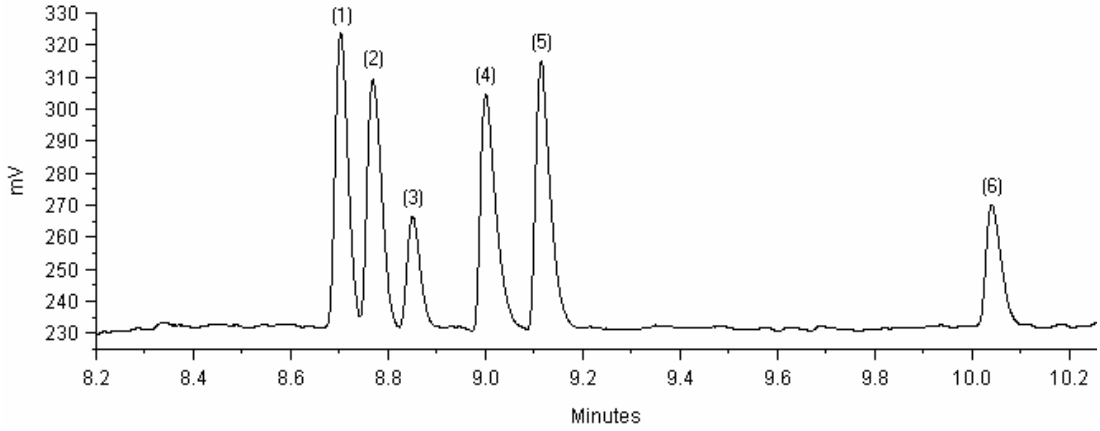


Figure 5-3: Typical electropherogram of 500 ng/mL mixture of heroin metabolites: (1) normorphine; (2) morphine; (3) 6-AM; (4) codeine; (5)1000 ng/mL nalorphine and (6) 1000 ng/mL levallorphan. Conditions: buffer, (pH 6) contained 50 mM sodium phosphate and 0.015 M ß-cyclodextrins, injection, electrically (5 kV) for 10 s, detection, UV absorbance at 214 nm, other conditions as in Figure 5-1.

The theoretical basis for this improvement arises from the general resolution equation in capillary electrophoresis:

$$R_s = 0.177\Delta\mu_{EP} [EL / (\mu_{EP} + \mu_{EOF})D_m]^{1/2} \quad (5\text{-}1)$$

where R is the resolution, $\Delta\mu_{EP}$ is the mobility difference between the solutes, μ_{EP} is the average electrophoretic mobility, μ_{EOF} is the electroosmotic mobility, E is the field strength, L is the capillary length, and D_m is the diffusion coefficient. This equation states that resolution will improve when the EOF is low. However, the EOF is only one adjustable parameter affecting resolution. If a neutral complexing reagent is added to the buffer, this expression becomes:

$$R_s = 0.177\Delta\mu_{EP} [EL / (\mu_n + \mu_f + \mu_{EOF})D_m]^{1/2} \quad (5\text{-}2)$$

where μ_n is the average mobility of the solute-CD complex and μ_f is the average mobility for the uncomplexed solute. Wren and Rowe[135] derived an equation for the apparent mobility, μ_a, for neutral CDs:

$$\mu_a = \mu_f + \mu_n + K_1 [C_1] / 1 + K_1 [C_1] \quad (5\text{-}3)$$

where μ_f and μ_n are the electrophoretic mobilities of the uncomplexed and complexed solutes, respectively, K_1 is the equilibrium constant for the CD inclusion complex, and $[C_1]$ is the CD concentration. Based on this equation, resolution occurs when solutes have different equilibrium constant.

Figure 5-4 shows the plots of the mobility of heroin metabolites versus ß-CD concentration. It is apparent that mobility decreases as ß-CDs concentration increases until the limit of 0.015 M, above which the solubility of the cyclodextrins

decreases, resulting in a reduction in the reproducibility of the method. On this basis a concentration of 0.015 M was chosen as optimum.

ß-CDs were also used in our lab to separate a standard mixture of morphine, codeine, thebiane, nalorphine (IS), papaverine and noscapin. The results were compared with a prepared opium sample (Figure 5-5).

Interestingly, while ß-CDs were effective in providing an increase in resolution, α- and γ-CDs did not. Since these molecules only differ in the dimensions of their inner cavities, these results emphasize the importance of analyte complexation inside the cyclodextrin cavities.

5.1.2 Effect of pH

Figure 5-6 shows the importance of pH adjustment in obtaining selectivity. Buffer pH has a significant effect on electroosmotic flow because it changes the degree of surface charge on the capillary wall. As pH increases, the dissociation of Si-OH to $Si\text{-}O^-$ on the inner capillary wall increases and, consequently, the electroosmotic velocity increases. At lower pH values, there is less surface ionization and lower electroosmotic velocity. On the other hand, the pH of the buffer will influence the degree of ionization of the drug under study. At values 2 units above and below the pK_a, the largest changes in migration times are expected because the solutes change from the free base to ionized form. In this study, at low pH values the opiates are all protonated and their relative mobilities

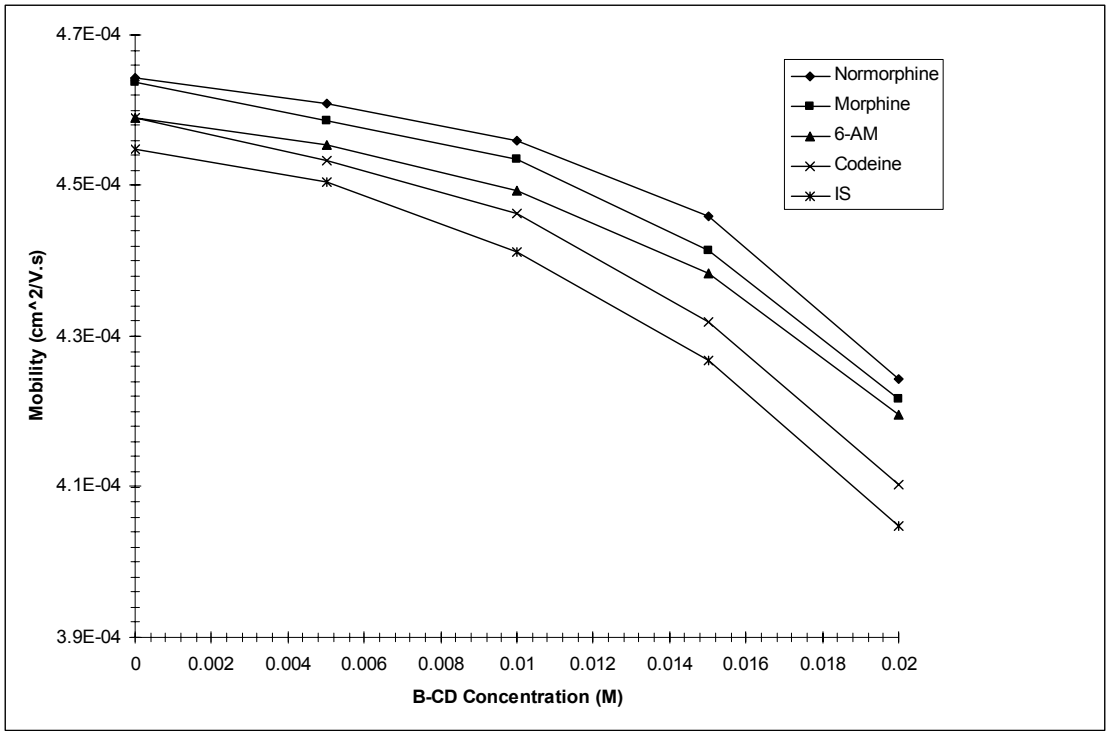


Figure 5-4: Plots of mobility versus ß-CD concentration of mixture of heroin metabolites; analytical conditions as in Figure 5-3.

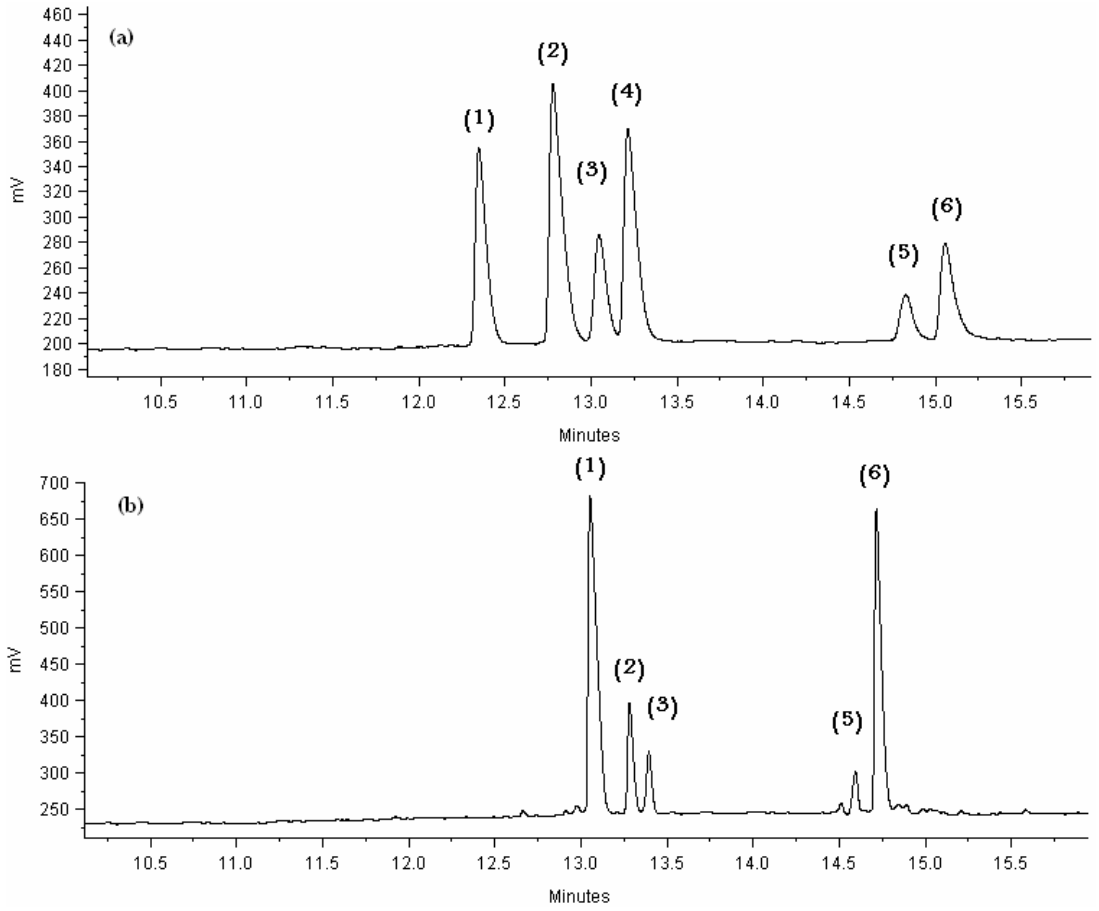


Figure 5-5: Typical electropherogram of (a) 500 ng/mL mixture of (1) morphine; (2) codeine; (3) thebaine; (4) 1000 ng/mL nalorphine; (5) papaverine and (6) noscapine; (b) prepared opium sample. Conditions as in Figure 5-3.

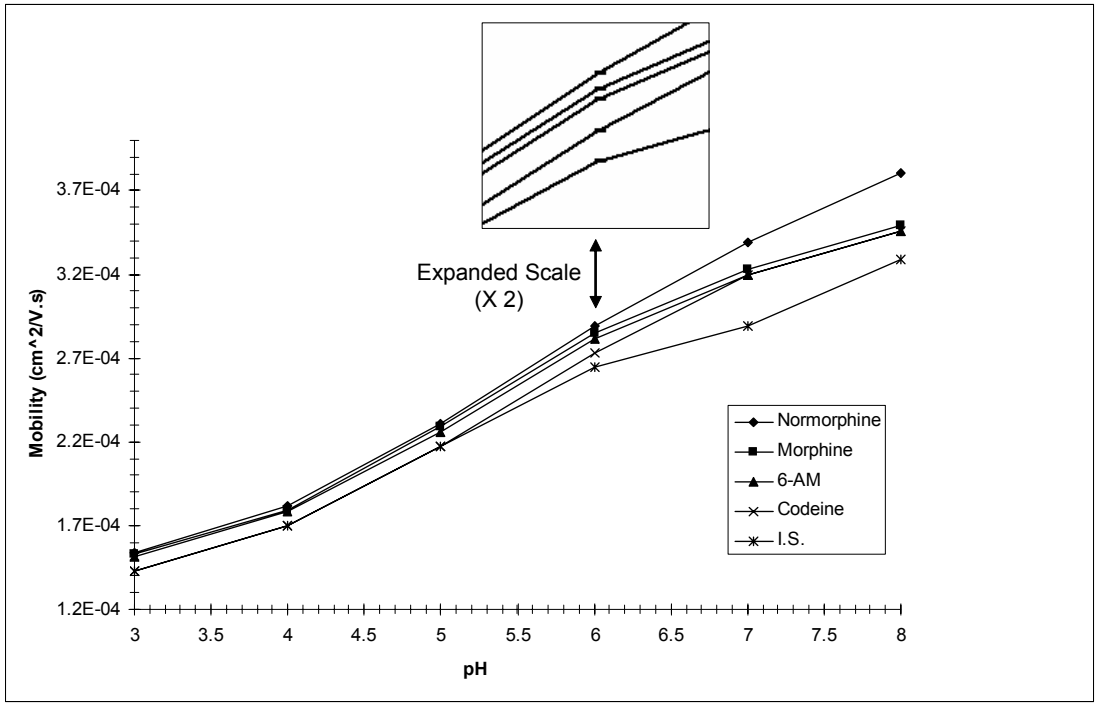


Figure 5-6: Plots of mobility versus pH of mixture of heroin metabolites; conditions as in Figure 5-3.

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

5.1.3 Effect of FAI

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

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

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

The technique of separately introducing a water plug[136] before electrokinetic injection from the aqueous opiate mixture was not found to offer any advantages in increased sensitivity of detection. In addition, the use of organic solvent such

as methanol or acetonitrile, which has been shown to increase sample stacking for a set of antimalarials,[137] was also not found to be effective in the present system. No stacking effect was obtained when the sample was dissolved in the running buffer and injected by either electrokinetic (5 kV, for 10 s) or hydrodynamic mode (0.5 psi for 10 s). However, when FAI conditions (Section 4.1.3) were used, only electrokinetic injection gave low detection limits (Figure 5-7).

The injection time was also investigated for further optimization of the FAI conditions. Injection for longer time (99 s) allowed a large number of opiate drug molecules to enter the capillary at a high velocity and stack at the interface between the high and low conductivity zones. However, as injection time increases, band broadening begins to occur (Figure 5-8). Thus, a 10 s injection time was chosen as the optimum.

5.2 Analytical characterization

To evaluate the linearity of this method, standard curves were prepared by analyzing six different concentrations of a mixture of four opiates in the range of 100-500 ng/mL with a constant amount of nalorphine (1000 ng/mL). Linear regression analyses were performed using the ratios of the peak areas of drugs to the internal standard (nalorphine) against the respective drugs concentrations (Figure 5-9). The linear regression equations for the normorphine, morphine, 6-AM, and codeine standard curves were $y = 0.0011x + 0.3511$ ($r = 0.9960$),

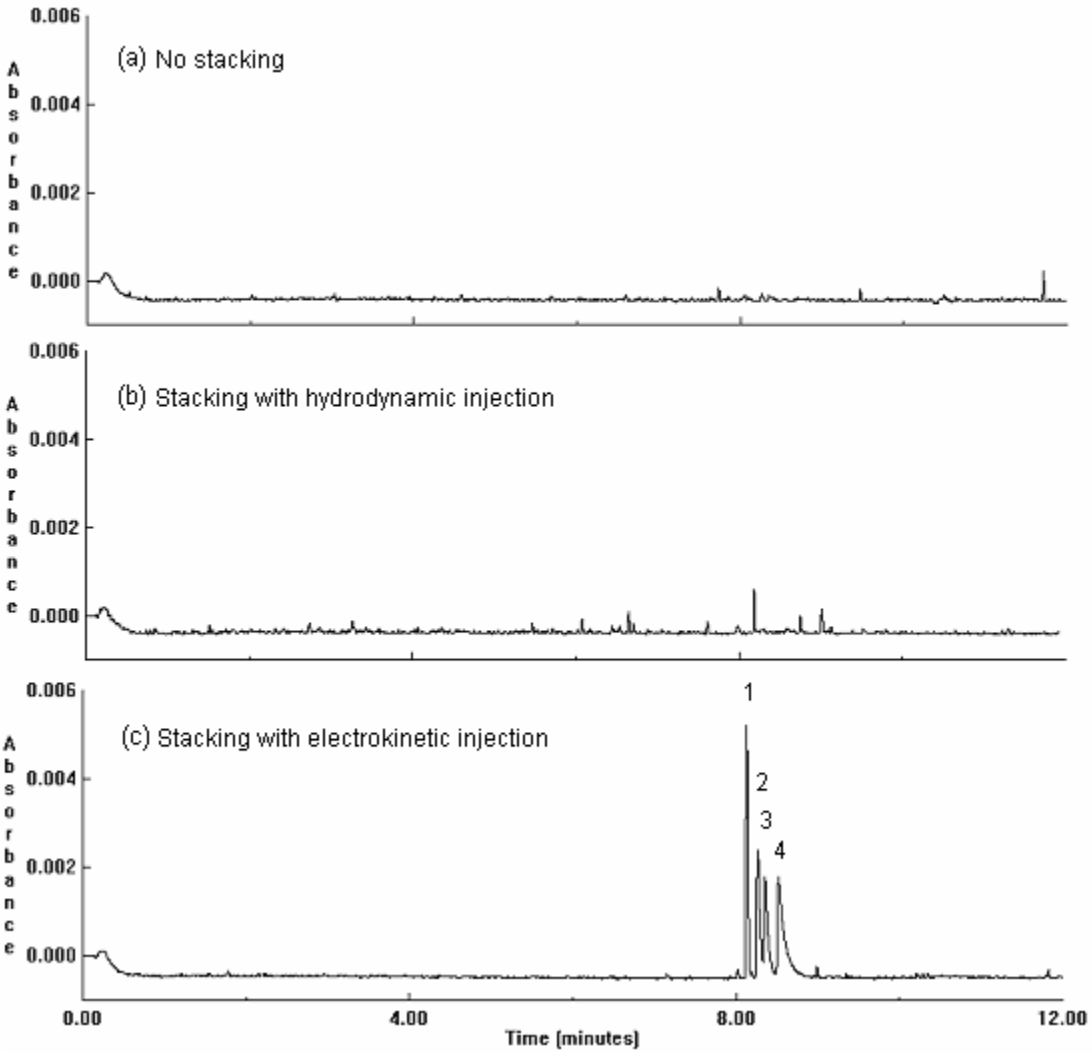


Figure 5-7: Electropherograms show the effect of sample stacking and the injection methods employed on the sensitivity of the CE system for drugs analysis: (a) no stacking (samples dissolved in the running buffer and electrically injected); (b) stacking with hydrodynamic injection (samples dissolved in water); and (c) stacking with electrokinetic injection (FAI conditions, Section 4.1.3). Other conditions and peak identification as in Figure 5-3.

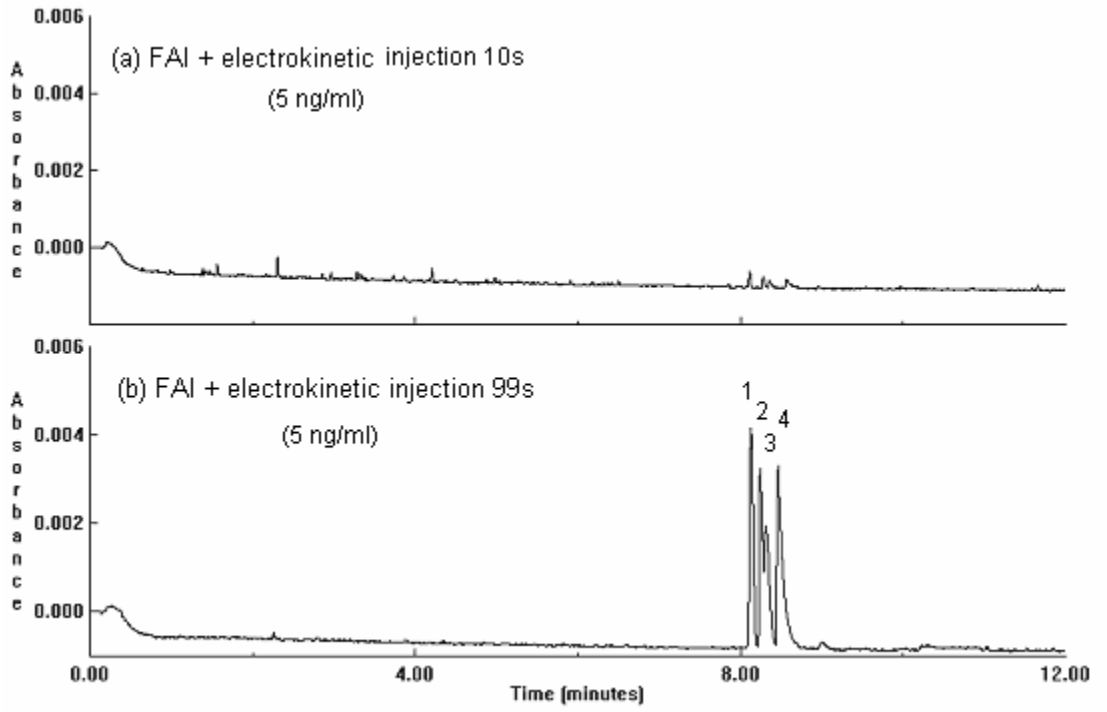


Figure 5-8: Electropherograms show the effect of injection time on stacking: (a) FAI with electrokinetic injection for 10 s; and (b) FAI with electrokinetic injection for 99 s. FAI conditions as in Section 4.1.3. Other conditions and peak identification as in Figure 5-3. Diluted concentration of drug 5 ng/mL.

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

The intra-day and overall accuracy and precision of the calibration curves were determined by analyzing three different concentrations of a mixture of standards containing normorphine, morphine, 6-AM, codeine, and a constant amount of nalorphine on three separate days. Three replicate determinations were made at each concentration level. The results of the reproducibility study are displayed in Table 5-1. The intra-day relative standard deviations (RSDs) for migration times were <0.22%, and the overall precision was <0.14%. The RSDs for peak area were <4.7%. The internal standard (nalorphine) was introduced to minimize variation resulting from the electrokinetic injection used.

The detection limits (signal-to-noise ratio of 3) shown in Table 5-2 are in range from 30-40 ng/mL using the conditions specified in the optimized assay method. This method gave a 100 fold improvement in the sensitivity as compared to the conventional CE methods and at least a 10 fold improvement over the CE methods that utilized sample stacking with hydrodynamic injection (500-700 ng/mL).[93] These low detection limits are more than adequate for the usual analytical requirements for controlled drugs analysis in forensic laboratories (the concentration of 6-AM results from heroin metabolites range from 10-5000 ng/mL).[22, 32]

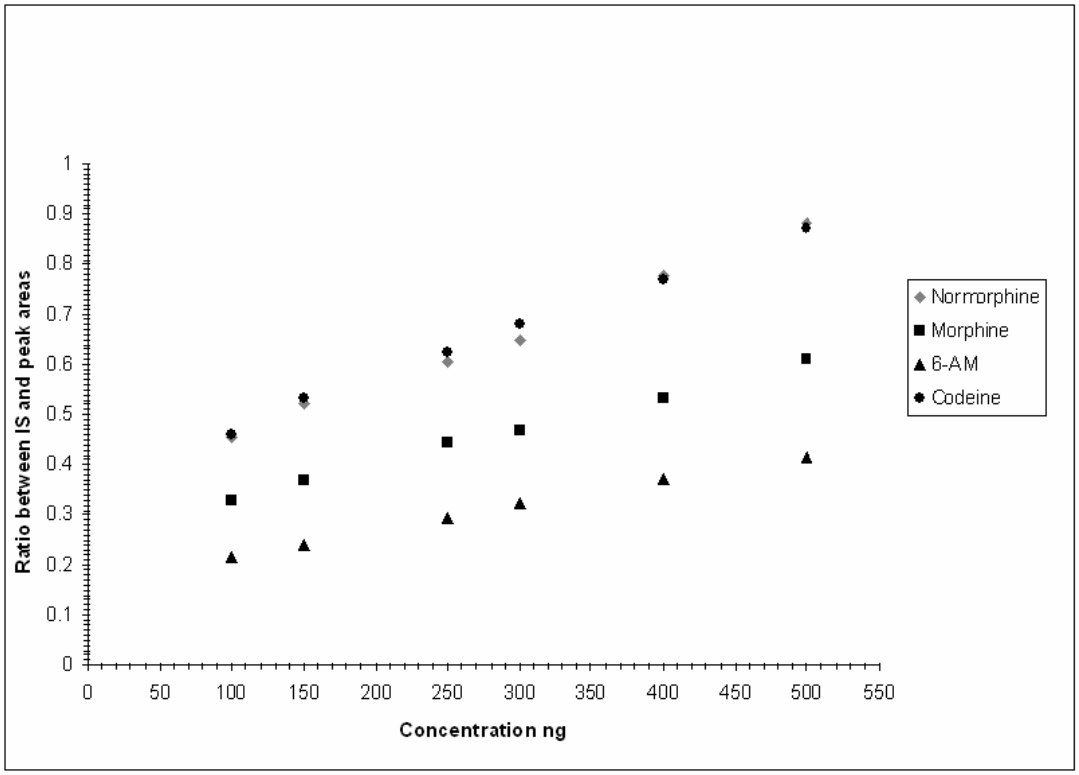


Figure 5-9: Calibration curves of a mixture of normorphine, morphine, 6-AM and codeine with 1000 ng/mL nalorphine (IS_1) obtained from UV absorbance measurements at 214 nm.

Table 5-1: Analytical precision expressed as intra-day (n=3) and overall (n=9) RSD% of relative migration times and peak areas.

Concentration (ng/mL)	RSD (%), migration time				RSD (%), peak area			
	Day1	Day2	Day3	Overall	Day1	Day2	Day3	Overall
Normorphine								
400	0.010	0.009	0.220	0.14	1.4	1.3	1.6	1.5
250	0.008	0.005	0.044	0.13	1.2	1.5	1.1	1.2
100	0.005	0.043	0.015	0.11	0.1	0.5	1.2	1.0
Morphine								
400	0.011	0.047	0.197	0.11	0.4	2.2	0.7	4.7
250	0.009	0.002	0.037	0.11	1.4	1.2	2.1	1.4
100	0.008	0.048	0.009	0.089	1.8	1.5	3.0	2.0
6-AM								
400	0.007	0.014	0.171	0.096	2.1	0.8	1.8	1.5
250	0.018	0.004	0.041	0.077	1.0	1.2	1.6	2.8
100	0.013	0.041	0.026	0.058	0.9	1.6	0.8	1.3
Codeine								
400	0.012	0.017	0.078	0.049	1.6	0.6	0.6	1.4
250	0.006	0.005	0.015	0.009	0.4	0.2	0.6	0.8
100	0.011	0.006	0.005	0.009	1.2	2.1	0.5	1.3

Table 5-2: Detection limits for a mixture of pure standards of normorphine, morphine, 6-AM, and codeine using electrokinetic injection and a UV-visible absorbance detector.

Analyte	LOD (ng/mL)
Normorphine	30
Morphine	30
6-AM	40
Codeine	40

5.3 Applications

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

Figure 5-10a shows a representative electropherogram of urine spiked with heroin metabolites and levallorphan (IS_2) and including nalorphine (IS_1) after mixed-mode SPE and electrophoresis with detection at 210 nm. The electropherogram obtained by subjecting a blank urine sample to the same treatment is shown in Figure 5-10b. It is evident that very few endogenous compounds in the urine are being extracted and applied to the capillary under the electrokinetic conditions used. The endogenous species detected have migration times shorter than 6-AM.

The other major metabolites of heroin are normorphine, morphine, and codeine. These metabolites were assayed for any possible interferences, and the chromatograms are shown in Figure 5-11. There are no interferences present at

any of the respective retention times. Moreover, the results from urine samples showed RSDs <2.4% (Table 5-3).

5.4 Conclusions

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

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

The combination of off-line SPE with FAI was found to be a powerful method to achieve extremely high enrichment of target compounds in real samples. The use of the mixed-mode column resulted in an excellent extraction and separation of opiate drugs from human urine samples as well as good peak

shape. There were no interferences with analytes from the extracted endogenous compounds.

In conclusion, CZE coupled to field amplified sample stacking offers a sensitive, precise, low cost, and rugged method for drug analysis. It can easily be used as a complementary technique for confirmation of results obtained with traditional methodologies.

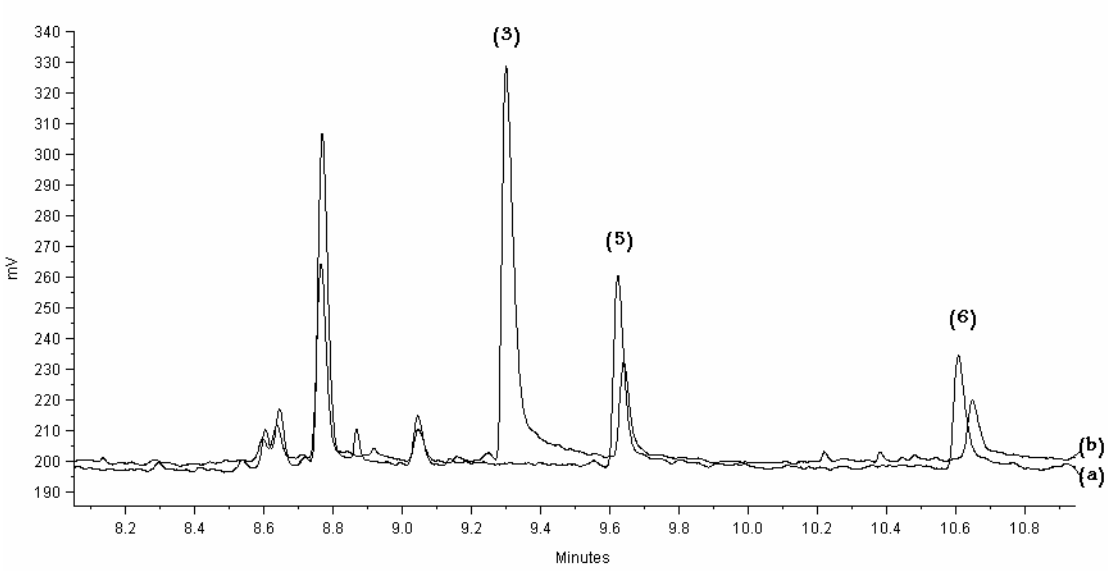


Figure 5-10: Typical electropherogram of (a) human urine spiked with 500 ng/mL 6-AM and 1000 ng/mL levallorphan (second internal standard used for SPE); and (b) blank urine sample spiked with levallorphan. The nalorphine (internal standard for calibration) was added after extraction. Manual SPE conditions as in Section 4.1.4 and Section 4.1.5. Other conditions and peak identification as in Figure 5-3.

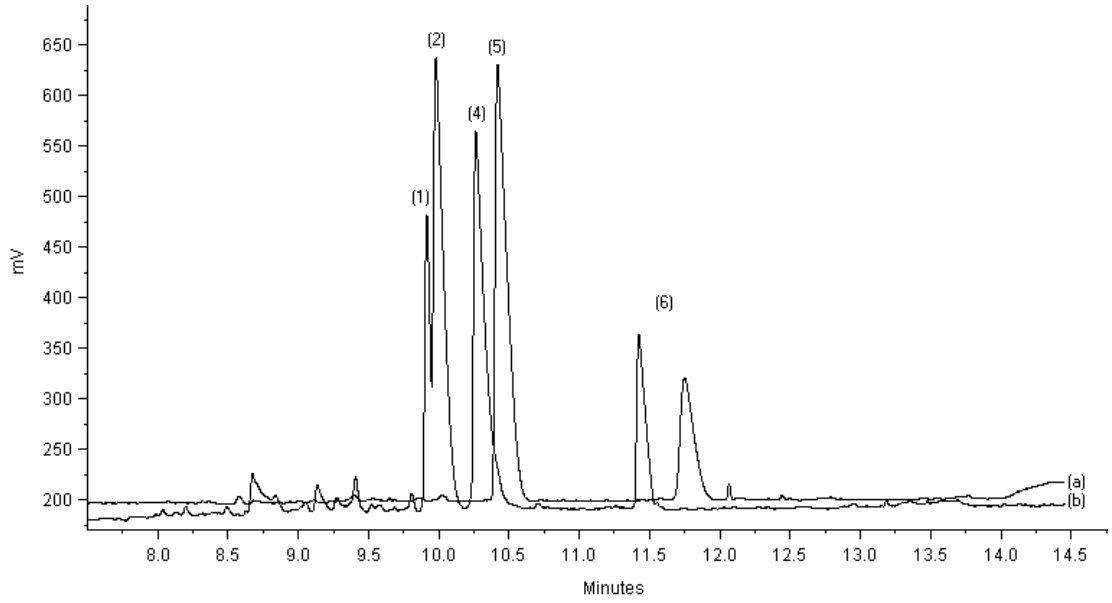


Figure 5-11: Typical electropherogram of (a) human urine spiked with 500 ng/mL normorphine, morphine and codeine and 1000 ng/mL levallorphan; (b) blank urine sample spiked with levallorphan. Normorphine was added after extraction. Manual SPE conditions as in Section 4.1.4 and Section 4.1.5. Other conditions and peak identification as in Figure 5-3.

Table 5-3: Reproducibility of analysis for urine samples spiked with normorphine, morphine, 6-AM, and codeine, *(n=3).*

Actual concentration (ng/mL)	Calculated concentration (ng/mL) mean ± S.D.		R.S.D. (%)	Recovery (%)
Normorphine				
500	490	±5.23	1.06	98
400	380	±3.21	0.84	95
250	240	±2.08	0.87	96
Morphine				
500	512	±8.59	1.67	102
400	394	±6.42	1.63	99
250	240	±2.36	0.98	96
6-AM				
500	480	±10.88	2.27	96
400	394	±8.54	2.17	99
250	235	±5.55	2.36	94
Codeine				
500	456	±9.65	2.11	91
400	393	±5.33	1.36	98
250	220	±4.43	2.01	88

Chapter 6 Determination of Multiple Drugs of Abuse in Human Urine Using Capillary Electrophoresis with Fluorescence Detection

In this chapter the potential of CE coupled with fluorescence detection for the determination of multiple drugs of abuse (mainly heroin metabolites: normorphine, morphine, 6-AM, and codeine) in biological fluids are examined. Both native fluorescence and fluorescence derivatization were examined and a comparison of the results was made with respect to the separation, sensitivity, precision, and simplicity. The applicability of these methods to practical samples was also evaluated using extracted urine samples.

6.1 Native fluorescence

Fluorometric detection has been widely used in many fields of science and is particularly useful in forensic assays because of its sensitivity and selectivity. A number of drugs of abuse can be determined in aqueous solution by means of their native fluorescence[97-101]. The advantage of detecting native fluorescence is the selectivity improvements over the more common UV detection.

The procedure for using native fluorescence was based on our previously published method.[57] A typical electropherogram from a mixture of heroin metabolites is shown in Figure 6-1. In this case, separation was accomplished with a running buffer composed of 50 mM sodium phosphate (pH 6) and 0.015 M ß-CDs. Under these conditions, analytes were separated in the following order: normorphine, morphine, 6-AM, codeine, and nalorphine (IS).

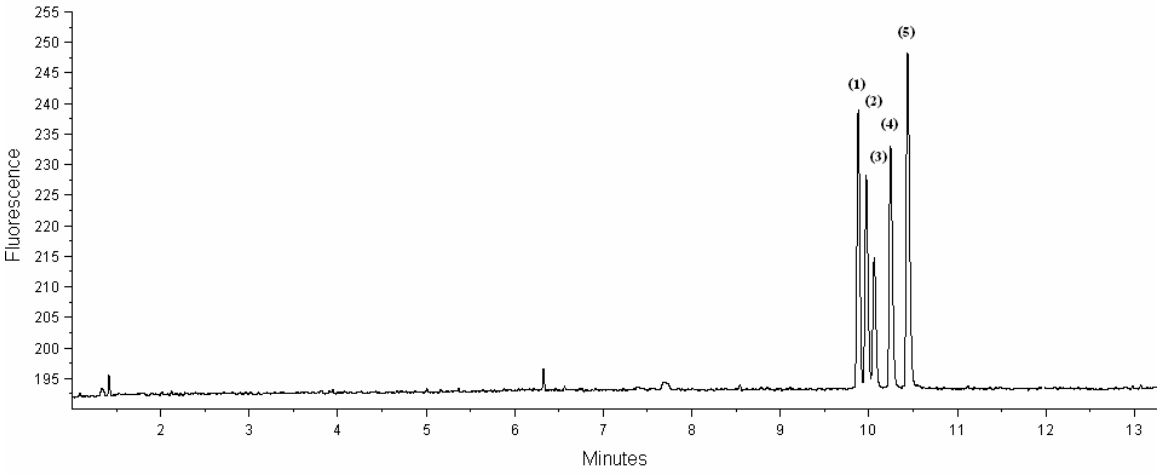


Figure 6-1: Typical electropherogram of 1 µg/mL mixture of heroin metabolites using native fluorescence detection. Conditions: electrokinetic injection of 5 kV for 10 s; uncoated fused-silica capillary (77 cm × 50 µm I.D.); buffer, (pH 6) containing 50 mM sodium phosphate and 0.015 M ß-cyclodextrins; separation voltage of 25 kV; detection using fluorescence, excitation using a mercury-xenon lamp at 245 nm and emission using a 320 nm cut-off filter. Peaks: (1) normorphine; (2) morphine; (3) 6-AM; (4) codeine; (5) 2 µg/mL nalorphine (IS).

Calibration curves were prepared by using known concentrations of the test drugs with a constant amount of nalorphine (IS). Table 6-1 summarizes the results and gives the detection limits obtained by CZE and native fluorescence. The detection limits (signal-to-noise ratio of 3) are in a range of 200-300 ng/mL using the conditions specified in the assay method. This detection limit is about ten times higher than the value obtained for CE-UV detection. This unexpected result may be due, at least in part, to the excessive detector noise resulting from the use of the homemade CE-native fluorescence system. In this system, the capillary is threaded through the detector and generally passes close to sensitive electronics, where the high electric field frequently causes electrical disturbances due to inadequate grounding and shielding.[93] This problem has been solved in commercial instrumentation. Furthermore, the decrease in the fluorescence intensity of these drugs could be attributed to their lower quantum yields at low excitation wavelengths.

The intra-day and overall accuracy and precision of the calibration curves were determined in the same way as the CE-UV detection method described in Chapter 5. RSDs for migration times were <0.25%, and the overall precision was <0.75%. As for peak area reproducibility, RSDs were <1.53%.

However, native fluorescence detection proved to have better specificity than UV detection. Results from extracted urine samples spiked with heroin metabolites and analyzed by CE-native fluorescence detection do not show

Table 6-1: Linearity equations, correlation coefficients and detection limits for normorphine, morphine, 6-AM, and codeine with CZE-native fluorescence detection (the slope of the calibration curves was obtained in the range of 500-1000 ng/mL).

Compounds	Equation	R	Detection limit (μg/mL)
Normorphine	y = 0.0009x + 0.3357	0.9920	200
Morphine	y = 0.0008x + 0.1511	0.9887	250
6-AM	y = 0.0006x + 0.0570	0.9787	300
Codeine	y = 0.0010x + 0.2132	0.9852	250

any interferences present at the same migration times. Moreover, the data was also quantitatively reproducible (RSDs < 2.36%).

6.2 Fluorescence derivatization

Chemical derivatizations are necessary when the drug itself is non-fluorescent or exhibits fluorescence of insufficient intensity. In this study, where native fluorescence was insufficient for the detection of trace levels of 6-AM (used for distinguishing between the presence of morphine in biological samples due to poppy seed ingestion versus heroin abuse), more sensitive measurements were made possible using simple derivatization reaction.

Abused drugs with tertiary amine groups can be easily converted to secondary amines in a high yield by reaction of amino groups with α-chloroethyl chloroformate, followed by warming the intermediate carbamate in methanol (Figure 3-5). The reaction worked with most of the illicit drug candidates in our laboratory including codeine, hydrocodone, morphine, hydromorphone, 6-AM, and cocaine. The *N*-demethylated reactions gave a single product for each drug when analysed by CE-UV detection (Figure 6-2). The yields of the demethylated reactions are listed in Table 6-2.

The most obvious advantages of the ACE-Cl method are its simplicity and the moderate reaction conditions. ACE-Cl reacts with drugs containing tertiary amines by forming a carbamate that is easily hydrolysed to the desired product simply by heating with methanol. Other chloroformate reactions have been utilized in the dealkylation of opiate and alkaloid drugs,[125, 138] but these

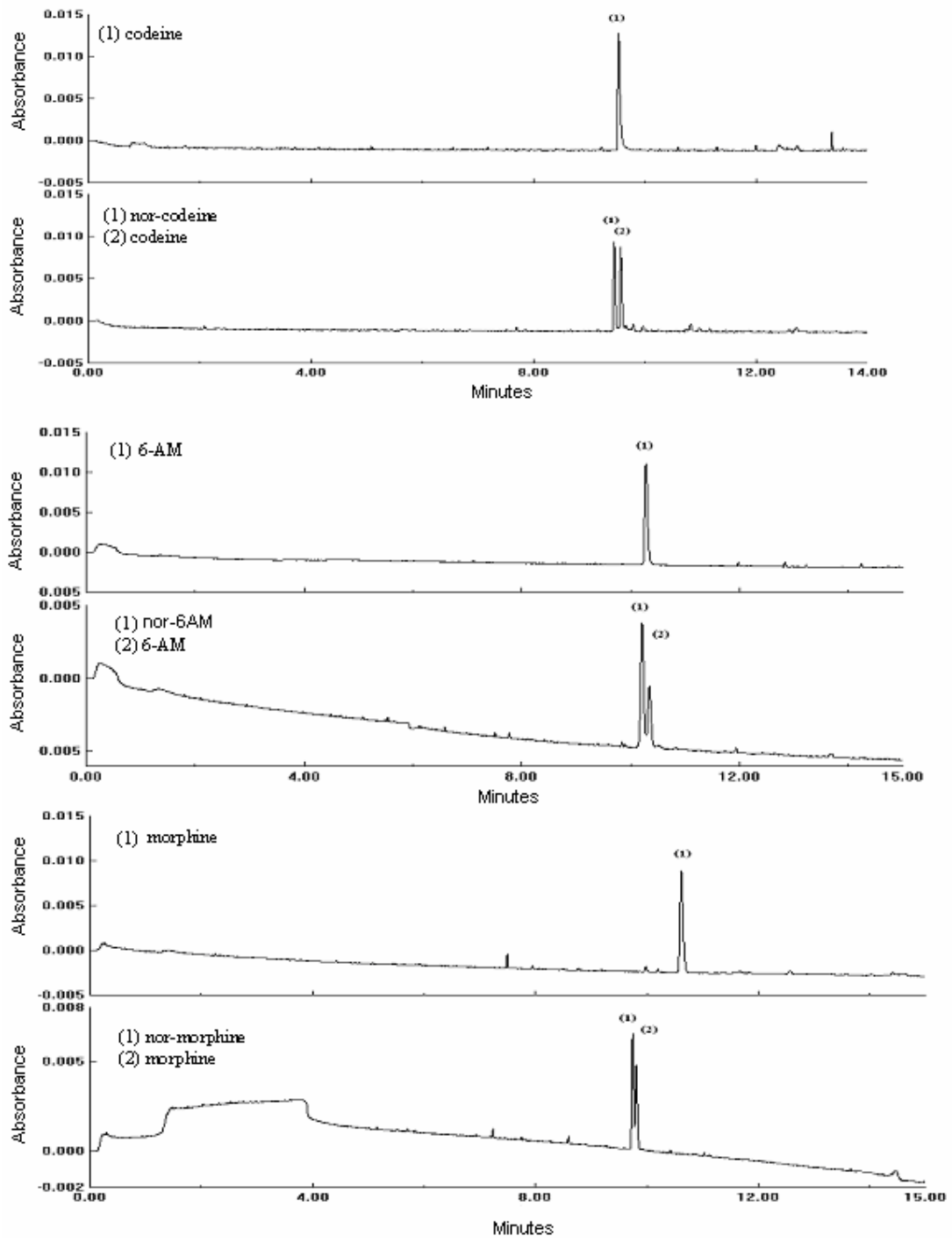


Figure 6-2: Electropherograms of the starting materials and the products of the *N*-demethylation reaction using CE-UV detection. Conditions as in Figure 6-1.

Table 6-2: Yield of the *N*-demethylation reaction of some drugs of abuse.

Parent drug	Yield of nor-metabolite (%)
1. Codeine	94
2. Hydrocodone	98
3. Morphine	95
4. Hydromorphone	98
5. 6-AM	99
6. Cocaine	90

reactions often require a long hydrolysis under harsh conditions. In addition, the demethylated products do not require a purification step, reducing the total derivatization time.

6.2.1 Optimization of the demethylation reaction

The reaction was first performed without organic solvents. In this experiment, a variety of tertiary amine drugs were dissolved directly in ACE-Cl. Using this method, a response for nor-drugs was not observed. Three different solvents were then tested, including 1, 2-dichloroethane, 1, 2-dichloromethane, and acetonitrile. The only one of these solvents that worked was 1, 2-dichloroethane (excess ACE-Cl was used in the later experiments). The yield of demethylated reactions was similar when the reaction solution contained 1-4 mL dichloroethane. Therefore, 2 mL dry dichloroethane was selected for further experiments.

Both reaction time and temperature play an important role in the demethylation reactions. As expected, the rate of reaction increased with refluxing time at 85 ºC. Under these conditions, the *N*-demethylation of illicit drugs was completed after 3-4 h. The reaction takes only 2 h at high temperature and high pressure. However, these conditions also produced side reactions lowering overall yield. Thus, a 4 h refluxing time at ambient pressure was selected for the recommended procedure.

6.2.2 Optimization of the FITC reaction

FITC reacts with primary and secondary amines like phenyl isothiocyanate under alkaline conditions to form fluorescein thiocarbamyl derivatives (Figure 3-5). These derivatives exhibit strong fluorescence with an excitation wavelength that matches the 488 nm light provided by an argon laser that is used in many CE system with LIF detection.

The conditions for the FITC reaction were optimized using a standard solution of normorphine (1 μg/mL). Several parameters affecting the FITC reaction were studied, including the FITC concentration, the pH of the buffer, the proportion of organic solvents, the reaction time, and the temperature.

The effect of FITC concentration was examined over a range of 1-10 mM. A constant and maximal peak area for the normorphine derivative was obtained at FITC concentrations higher than 2.0 mM; 2.5 mM was chosen.

The fluorescence of FITC-derivatives is pH dependent. Both the wavelength and the emission intensity are likely to be different for the ionized and nonionized forms of the compound. Therefore, the pH effect of the FITC reaction mixture was examined by using a phosphate buffer (pH 8.5 or 10), a borate buffer (pH 10), and a carbonate buffer (pH 8.5 or 10). The fluorescence intensity of the derivatization reaction was found to be highest when the pH of all of the buffers tested was the lowest. This may be arising from the differing number of resonance species that are associated with the acidic and basic forms of the derivatives. Carbonate buffer at pH 8.5 afforded the highest intensity (Table 6-3). The degree of derivatization was also affected by changing the concentration of

Table 6-3: Effect of pH and buffer system on the fluorescence derivatization.

Buffer	pH	RFI*
Phosphate	8.5	73
Phosphate	10	70
Carbonate	8.5	100
Carbonate	10	96
Borate	10	36

*RFI: relative fluorescence intensity. Normalized to response for morphine-FITC derivative in carbonate buffer (pH 8.5).

the buffer from 20-200 mM. A low buffer concentration was found to lead to an increase in the fluorescence intensity. Therefore, 20 mM carbonate buffer (pH 8.5) was used for the reaction.

Organic solvents are necessary for preparation of the FITC derivative. Since these organic solvents can enhance or decrease the reaction yield, the influence of some of these solvents was investigated. The solvents examined included acetone, ethanol, methanol, acetonitrile, and dimethyl formamide (DMF). Very similar fluorescence intensities (within 6.5%) of FITC-derivatized normorphine were obtained with both acetone and ethanol. A lower intensity (up to 60%) was observed when the reaction solution contained methanol, acetonitrile, and DMF (Table 6-4). It was also discovered that the side reaction products were lower with acetone and DMF. Thus, FITC was dissolved in acetone in the recommended procedure.

The reaction time and temperature are critical parameters for the FITC reaction. Therefore, the reaction was examined at room temperature, 40 ºC, and 80 ºC. The reaction of FITC at room temperature was relatively slow, taking approximately 24 h to complete. On the other hand, by increasing the temperature to 40 ºC, the reaction time was decreased to 2 h. At 80 ºC, the FITC reaction with normorphine was completed after 30 min (Figure 6-3). Therefore, a temperature of 80 ºC was utilized for 30 min for the FITC reaction.

Table 6-4: Effect of organic solvents on fluorescence development.

Solvent	RFI*
Acetonitrile	43
Acetone	100
N,N,-dimethylformamide	80
Ethanol	107
Methanol	85

*Relative fluorescence intensity (peak area) obtained by the reaction of morphine with FITC dissolved in acetone was taken as 100.

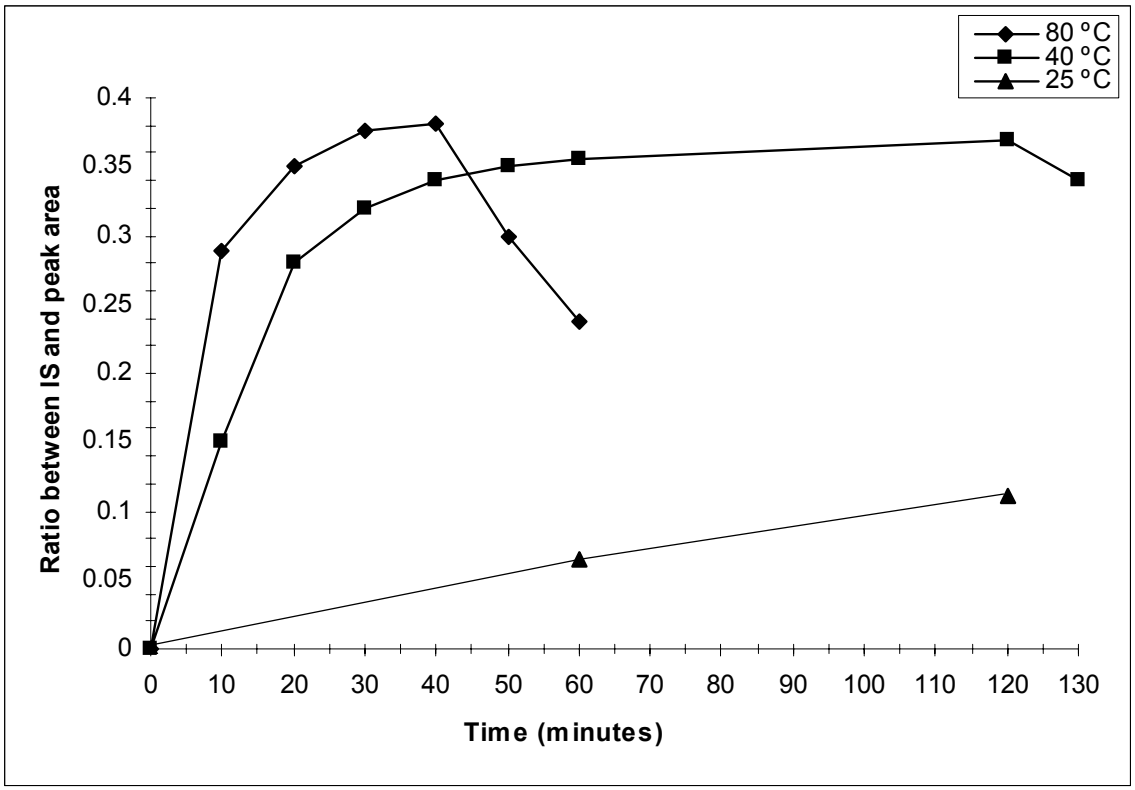


Figure 6-3: Effect of reaction temperature and time on the fluorescence derivatization of morphine with FITC. Conditions as in Figure 6-6.

6.2.3 Stability studies

The stability of normorphine derivatives at room temperature, in the refrigerator (4 °C), and in the freezer (-20 °C) in the dark were studied over a period of time. There was no decrease in response after 3 days of storage in all of the cases (RSDs <3.79%).

6.2.4 Optimization of the separation conditions

An optimization study was undertaken by examining the migration behavior of a derivatized mixture of codeine, 6-AM, morphine, and fluorescein (IS). Various parameters such as buffer concentration, pH, β-CD concentration, and organic solvent content were examined in order to optimize the separation, sensitivity, and analysis time.

In the first stage of our work, we used a buffer containing 20 mM sodium borate at pH 9.5 and an applied voltage of 25 kV. Under these conditions, the analytes were only partially separated. This may be attributed to the fact that all analytes migrate according to their charge-to-size ratio in CE, which in this case are very similar (FITC-derivatives may appear more similar in size and charge than underivatized drugs due to the large structure of the fluorescence tag). Further experiments were performed by changing the pH and the buffer concentration; however, the results did not provide an acceptable separation. Instead, 20 mM β-CDs were added to the electrolyte buffer but the resolution of the FITC-derivatized drugs was still unsatisfactory. However, by adding different organic solvents such as acetonitrile, isopropanol, and acetone to the buffer,

good separations were obtained. The effect of these organic solvents on the separation of FITC-derivatives in the presence of β-CDs is shown in Figure 6-4. Organic solvents affect both electrophoretic and electroosmotic mobility by changing the viscosity of the run buffer (Eq. (1-2) and Eq. (1-3)). In this study, isopropanol decreases both μ_{EOF} and μ_{EP} because it increases the viscosity of the run buffer while acetonitrile does not affect or may slightly increase the overall electrophoretic mobility. We found that a mixture of acetonitrile and isopropanol (10% each) improved the separation and also yielded a shorter run time.

In addition to organic modifier, the concentration of the electrophoretic buffer is an important separation parameter. Higher concentrations of borate led to slightly better resolution, but an increase in the migration time and in the electrophoretic current were also observed. The pH of the running buffer was also optimized. Figure 6-5 shows the influence of buffer pH on the separation of FITC-derivatives. In this method, pH 9.8 was utilized.

Under the above conditions analytes were separated using 20 mM borate (pH 9.8) with 10% isopropanol, 10% acetonitrile, and 20 mM β-CDs (other conditions as shown in Section 4.2.4). As can be seen in Figure 6-6, the separation of heroin metabolite derivatives is obtained within 10 min using a 47 cm capillary (40 cm to detector).

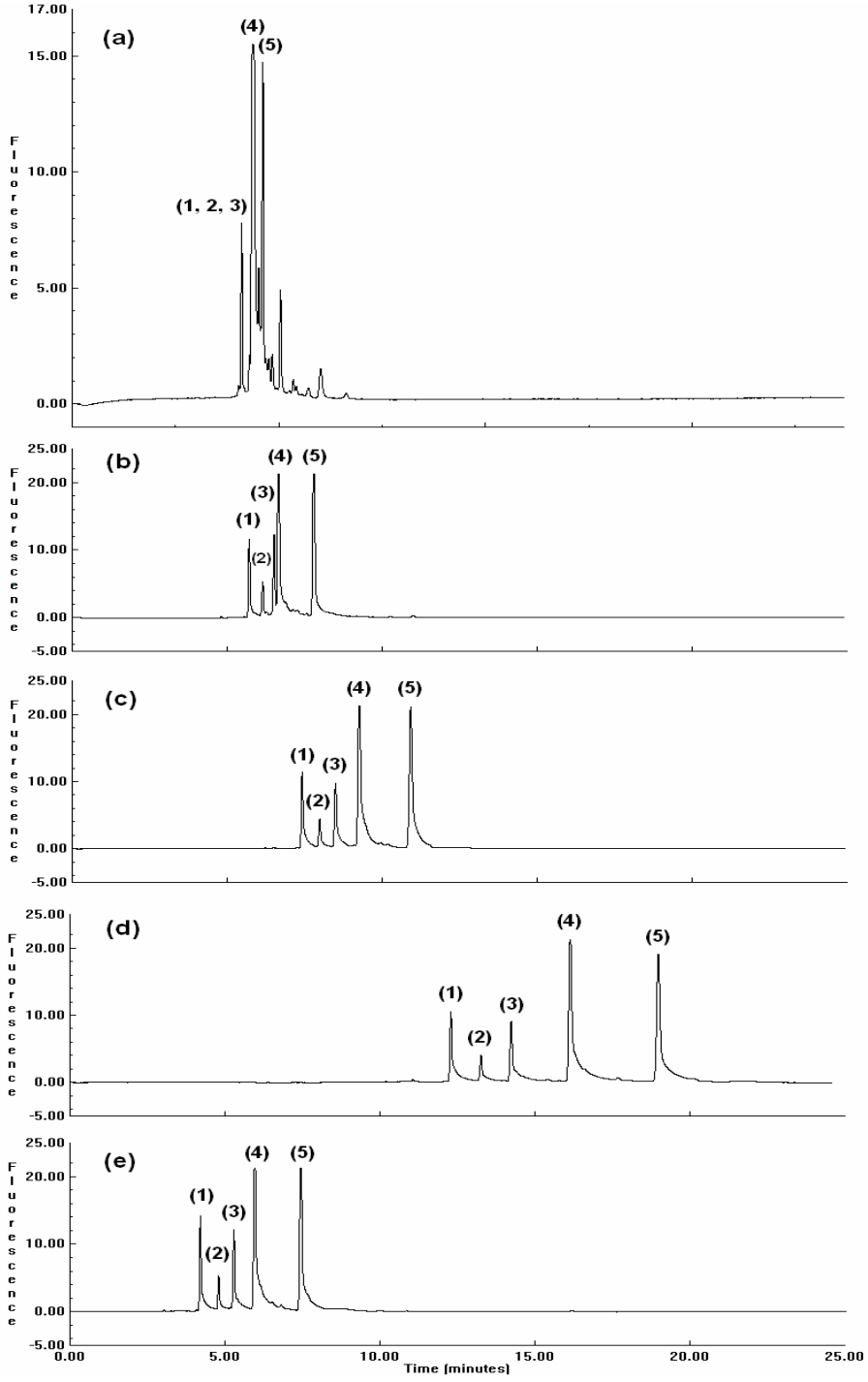


Figure 6-4: Electropherograms of FITC-derivatives in the presence of β-CDs using different organic solvents: (a) 0% organic solvent; (b) 20% acetonitrile, (c) 20% acetone; (d) 20% isopropanol; and (e) 10% isopropanol-10% acetonitrile. Conditions and peak identification as in Figure 6-6.

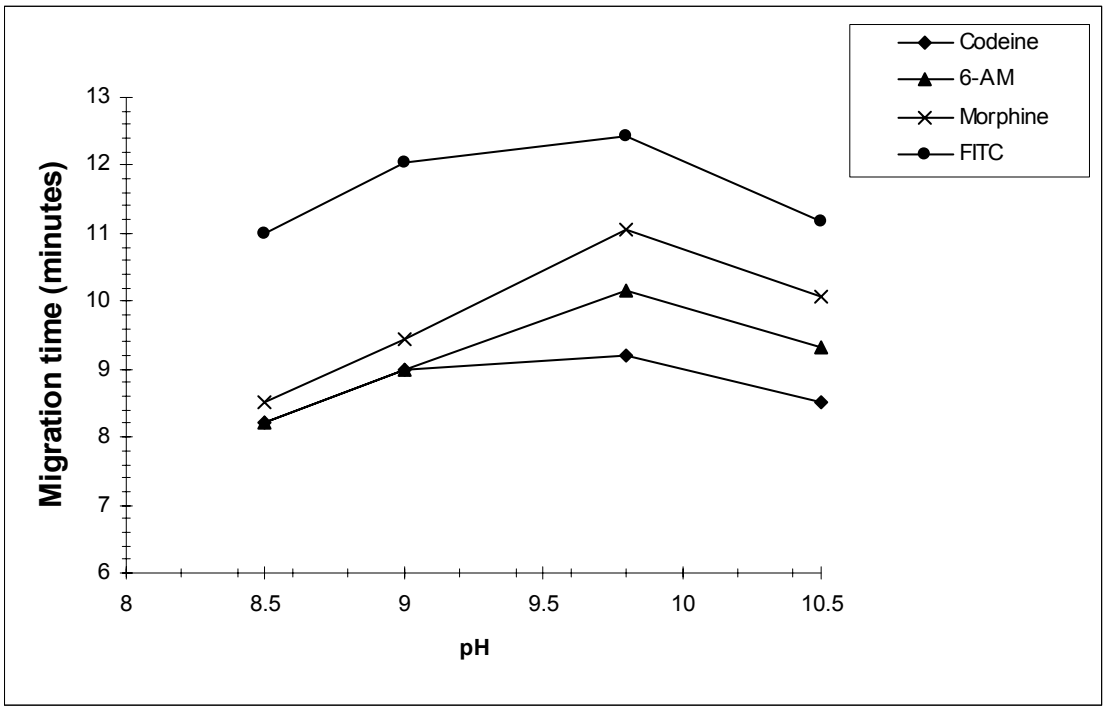


Figure 6-5: Influence of pH on the separation of FITC-derivatives in the presence of an organic modifier and β-CDs. Analytical conditions as in Figure 6-6.

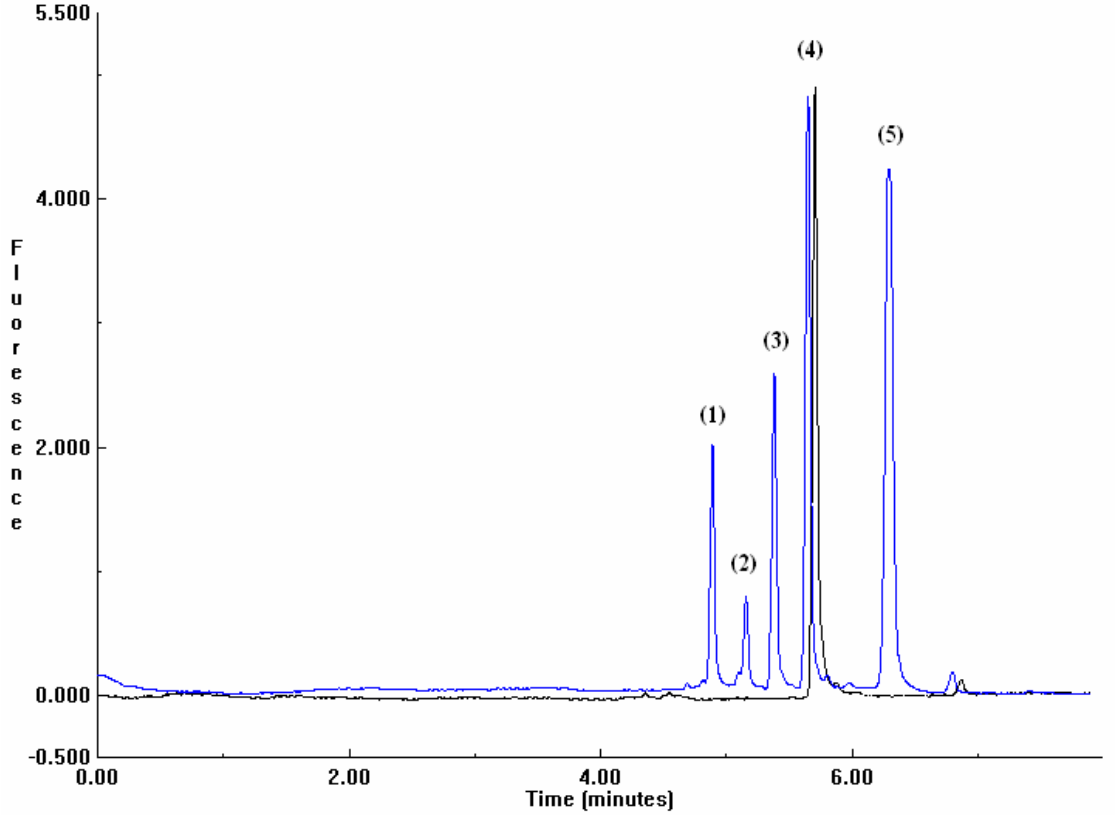


Figure 6-6: LIF electropherograms of a standard of FITC-labelled drugs (upper electropherogram) and reagent blank (lower electropherogram). Conditions: buffer; 20 mM borate-10% isopropanol-10% acetonitrile-20 mM ß-CDs; capillary, 47 cm x 75 µm (40 cm effective length); injection, 2 s, pressure; applied voltage, 25 kV (~65 µA); detection, LIF fluorescence detection operated at 488 nm excitation wavelength and emission wavelength filter of 520 nm. Peaks: (1) codeine, (2) 6-AM, (3) morphine, (4) FITC, (5) fluorescein (IS). Diluted concentration of drug 500 ng/mL.

6.2.5 Analytical characterization

The electropherogram of nor-drug derivatives is presented in Figure 6-6. Under the conditions already mentioned, analytes were separated in the following order: codeine, 6-AM, morphine, and fluorescein (IS) within 10 min. The calibration data and detection limits obtained by CE-LIF are listed in Table 6-5. As can be seen, the LODs (signal-to-noise ratio of 3) for heroin metabolite derivatives are in a range of 50-100 pg/mL using the conditions specified in the assay method. These low detection limits are more than adequate for the usual analytical requirements for controlled drugs analysis in forensic laboratories. The proposed CE method provides equivalent or better detectability than what can be obtained by HPLC or GC (Table 6-6). In addition, by increasing the injection time, the detection limits of the developed procedure can be lowered even further.

The intra-day and day-to-day accuracy and precision of the method were determined by running three replicates of the standard (50 ng/mL). Each FITC-derivatized nor-drug showed high reproducibility in terms of peak areas and migration times. The RSDs of the peak areas were between 0.09% and 0.74%. As for the reproducibility of migration time, RSDs were <0.03%. The day-to-day reproducibility was < 3.1%.

6.2.6 Applications

Several drugs of abuse were examined for potential interferences with 6-AM using the above method. These drugs included codeine, hydrocodone,

amphetamine, methamphetamine, morphine, hydromorphone. No interferences were found (Figure 6-7).

Extracted urine samples spiked with heroin metabolites and derivatized using the above procedure were analyzed by CE with LIF detection. Representative electropherograms of these samples are displayed in Figure 6-8. It is evident that very few endogenous compounds in the urine are being extracted, derivatized, and injected into the capillary under the conditions used. In addition, the endogenous species that are detected have migration times shorter than 6-AM and the other major heroin metabolites (morphine and codeine). Furthermore, results from the extraction of urine samples were found to be very reproducible (RSDs < 2.4).

6.3 Conclusions

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

Table 6-5: Linearity equations, correlation coefficients and detection limits for codeine, 6-AM, and morphine derivatives with CE-LIF detection (the slope of the calibration curves was obtained in the range of 5-50 ng/mL).

Compounds	Equation	R	Detection limit (pg/mL)
Codeine	y = 0.0048x + 0.1959	0.9923	65
6-AM	y = 0.0056x + 0.3175	0.9965	100
Morphine	y = 0.0093x + 0.5044	0.9948	50

Table 6-6: Comparison of the detection limits reported for drugs of abuse.

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

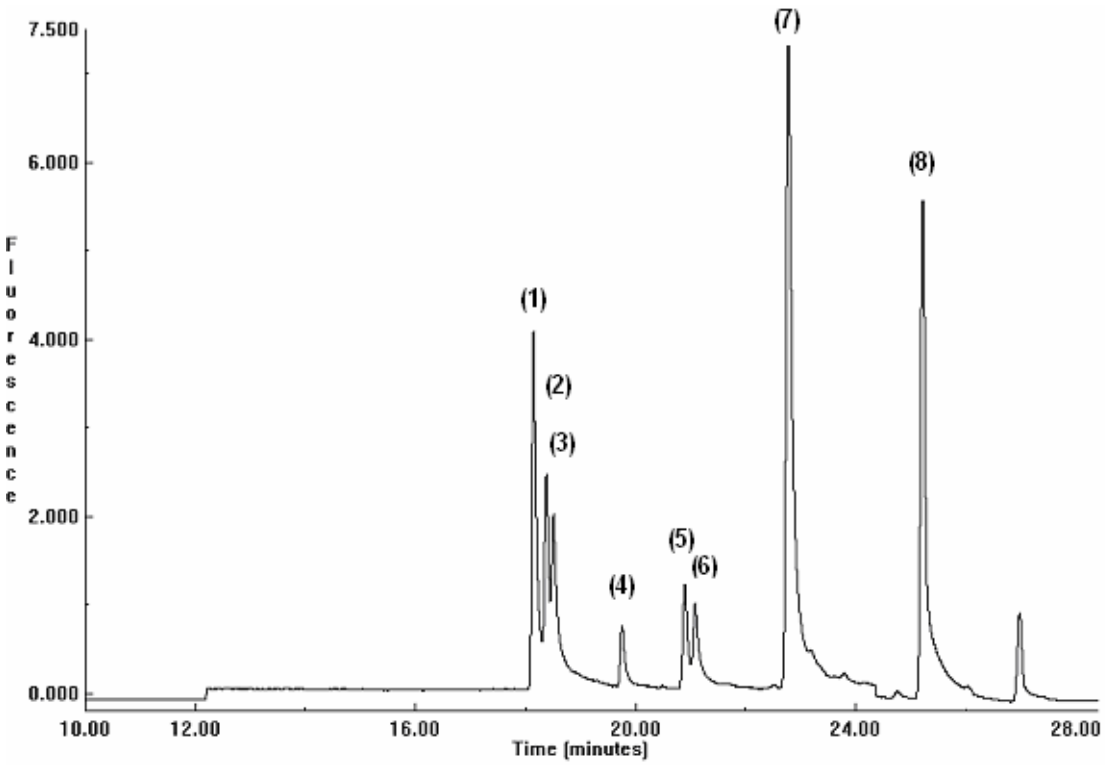


Figure 6-7: Typical electropherogram of multiple drugs of abuse derivatized using FITC reaction and analyzed by CE-LIF detection. Conditions: buffer; 20 mM borate-20% isopropanol-20 mM ß-CD; other condition as in Figure 6-6. Peaks: (1) codeine and hydrocodone, (2) amphetamine (3) methamphetamine (4)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

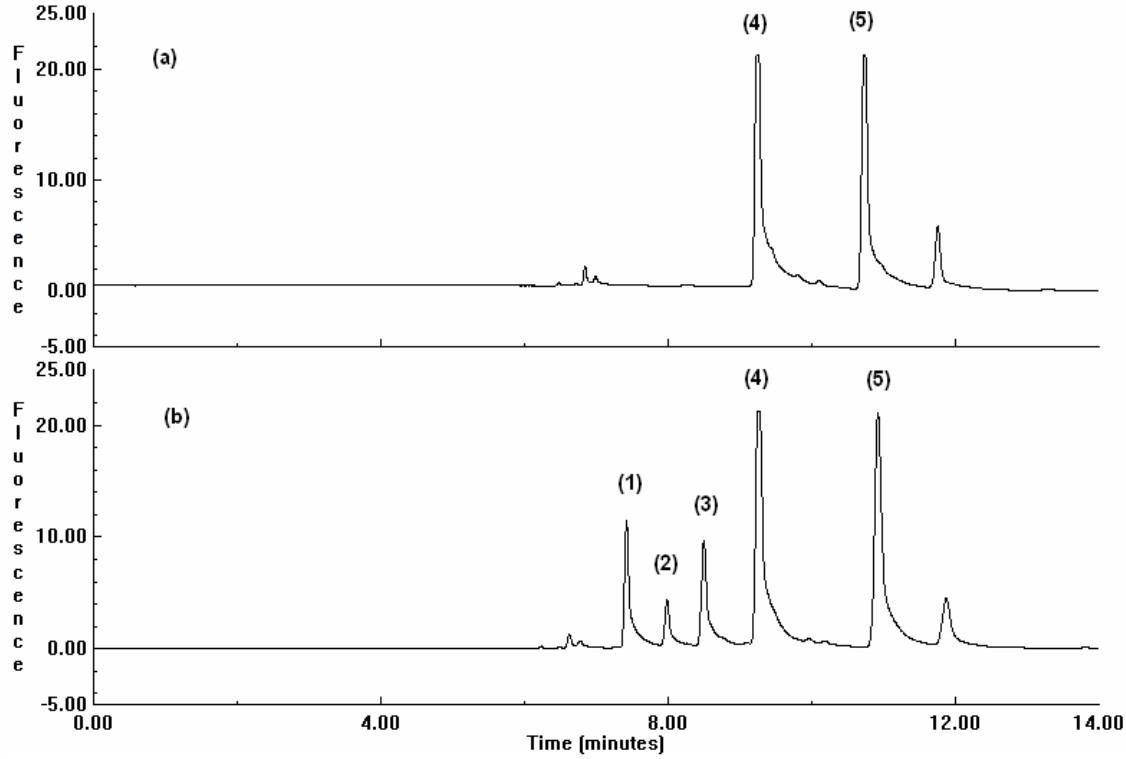


Figure 6-8: Typical electropherogram of (a) blank urine sample; (b) urine sample spiked with 500 ng/mL heroin metabolites derivatized and analyzed by CE-LIF detection. Manual SPE conditions as in Figure 4-1. Other conditions and peak identification as in Figure 6-6.

Chapter 7 Automated Solid-phase Extraction for the Enhancement of Concentration Sensitivity in Capillary Electrophoresis: Application to the Analysis of Abused Drugs in Human Urine

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

7.1 Method development

Initially, our attention was focused on developing an extraction technique that could be performed directly on the CE capillary. Early experiments involved

the use of on-line SPE-CE, as this technique does not require any modification of the CE instrument. Several groups have demonstrated the enhancement of CE sensitivity using on-line SPE.[69, 70] However, it was our experience that CE performance was compromised. In our experiments, the use of on-line SPE resulted in reduced analyte resolution, broader peaks, and substantial component tailing (Figure 7-1). These observations can be attributed, at least in part, to increased analyte-analyte and analyte-wall interactions that can occur in the CE capillary.[60] When analyzing electropherograms for the best resolution and peak shape, the concentration of the injected analytes should be approximately 100 times lower than the concentration of the run buffer[7]. In addition, the increased back pressure induced in the CE capillary by the solid-phase and frit material (used to prevent solid particles from entering and blocking the CE capillary) leads to a reduced hydrodynamic flow. This can cause an anomalous electroosmotic flow and irreproducible analyte migration times. Furthermore, from our studies, it can be concluded that the relatively large volume of organic phase required to efficiently remove analytes from the adsorptive material also tends to reduce electroosmotic flow and compromise CE performance.

The use of the mPC technique slightly improved the CE performance, (Figure 7-2); however, the technique can not be used to analyze large samples due to the limited volume of adsorptive phase (the use of two or three layers of the polymeric phase blocked the CE capillary). Also, the analysis time was longer

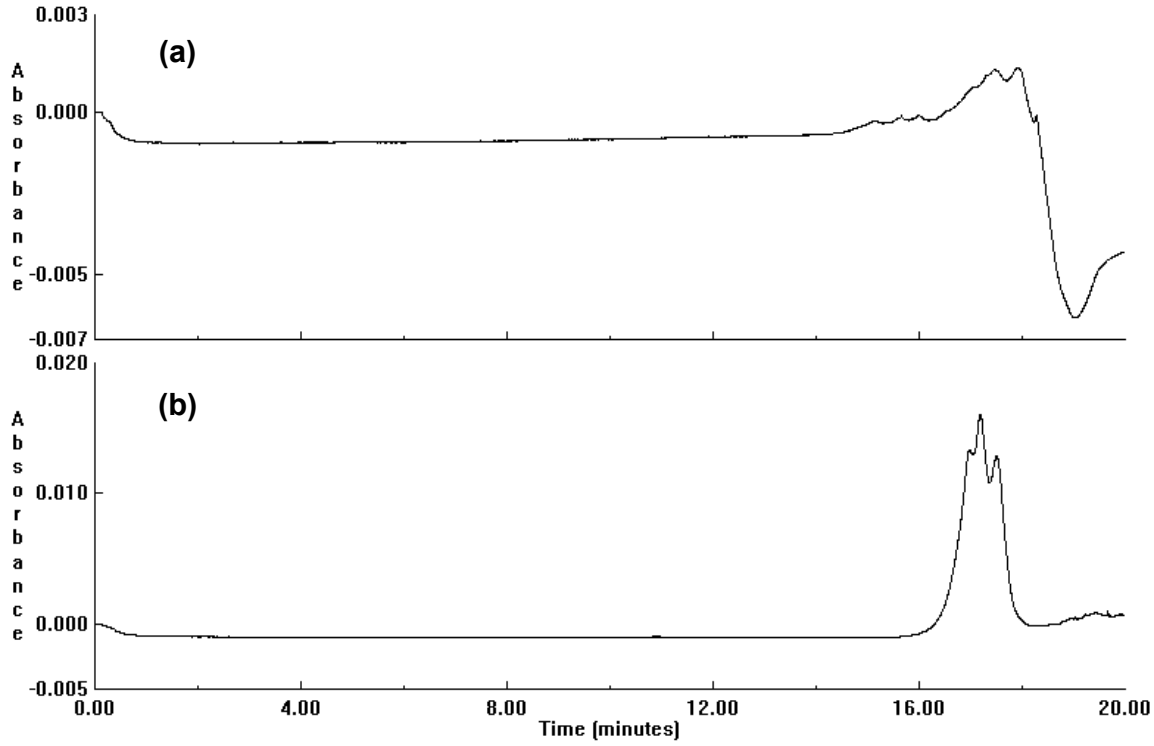


Figure 7-1: Electropherogram of (a) water blank, and (b) water spiked with a mixture of heroin metabolites (normorphine, morphine, 6-AM, and codeine). Analysis was performed using on-line SPE. Extraction and pre-concentration conditions as previously described.[69, 70] Separation conditions as in Figure 5-3.

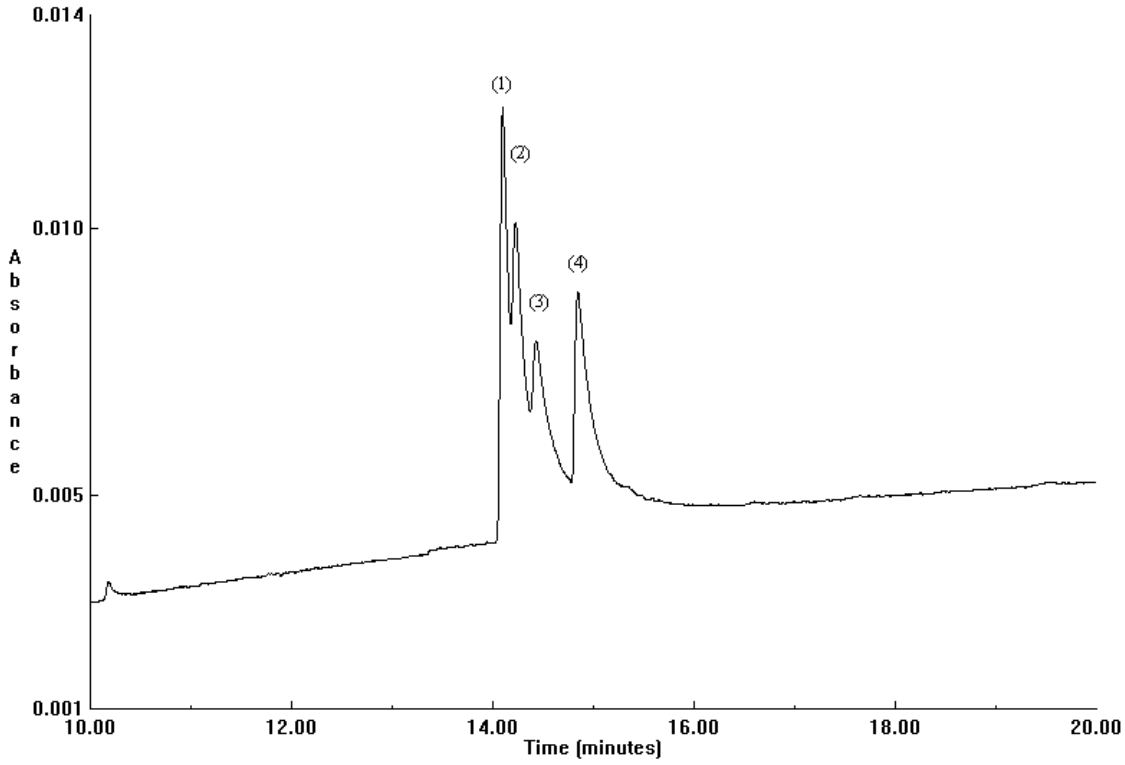


Figure 7-2: Electropherogram of a mixture of heroin metabolites (1) normorphine, (2) morphine, (3) 6-AM, and (4) codeine. Analysis was performed using mPC. Extraction and pre-concentration conditions as previously described.[72, 73] Separation conditions as in Figure 5-3.

and the capillary was prone to clogging, especially when urine samples were analyzed.

One possibility for overcoming the previously mentioned problems is to couple SPE at-line with CE--a method developed in our laboratory for drug analysis. In this procedure, an external C_{18} column was connected to the CE system by means of a switching valve (Figure 2-5). Liquid chromatography pumps were used to deliver the samples and the reagents through the C_{18} column. Once samples were loaded onto the column they could be cleaned with an organic solvent and then the analytes could be eluted and transferred directly to the CE system for analysis. This technique provided a way to automate the extraction and pre-concentration processes without affecting the electrophoresis performance.

7.2 Optimization of the automated SPE-CE conditions

The pre-concentration and clean-up steps were carried out on a C_{18} sorbent phase that was previously flushed with methanol and water. Methanol helps to wet and activate bonded functional groups to ensure consistent interactions between the sorbent and analyte, and water (adjusted to pH 9.5) helps to maximize the reverse-phase retention prior to sample loading. Two min (1.6 mL) was found to be enough time to condition and equilibrate the C_{18} column. Urine samples (or working standards) adjusted to pH 9.5 were introduced into the system and an appropriate volume was passed through the C_{18} column in order to retain adequate amounts of the compounds and, therefore, obtain intense CE

signals. The non-polar groups on the drugs are attached by Van Der Waal's or dispersive forces to the sorbent until a more favorable solvent will carry the analytes off the column and directly into the CE system. A 4 mL urine sample was found to be optimal.

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

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

7.3 Optimization of the separation conditions

Optimization of the electrophoretic separation was achieved by testing the migration behavior of twenty basic drugs (Table 7-1). The effect of various parameters, such as buffer concentration, pH, β-CD concentration, organic additives, applied voltage, and length of the capillary, were examined in order to determine the best separation conditions.

Initially, experiments were performed using a 100 mM phosphate buffer at pH 6 and a constant field strength of 373 V/cm. Under these conditions, the resolution of some basic drugs was unsatisfactory. To enhance this separation, an additional experiment was performed utilizing 20 mM β-CDs in the buffer solution. While the separation was slightly improved, this system also did not provide an acceptable separation (Figure 7-3) (the effect of β-CDs concentration versus drugs mobility was studied as previously shown in Figure 5-4. Also the role of β-CDs in the separation process was given in Section 5.1.1). The complete separation of the twenty basic drugs was achieved only after the addition of an organic modifier to the buffer system in the presence of β-CDs. This is due to a decrease in both the EOF and the μ_{EP}, which, according to Eq. (5-2), increases resolution. EOF decreased because of an increase in the viscosity of the run buffer and a decrease in the zeta potential. The zeta potential decreases due to solvation of the ions by the organic solvent. The μ_{EP} for the basic drugs decreased because of an increase in run buffer viscosity and a decrease in the *p*Ka of the various solutes. The decrease in the *p*Ka at pH 6 led to these compounds being less ionized and, therefore, possessing a lower μ_{EP}.

The type and concentration of organic solvent were found to be very important in obtaining good resolution. Therefore, the effects of different organic solvents, such as methanol, ethanol, acetonitrile, acetone, and isopropanol, in concentrations ranging from 5%-25% were examined. Increasing the organic solvent concentration was found to improve resolution. However, at

Table 7-1: Peak identification for Figure 7-3 to Figure 7-12.

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

*Not reported

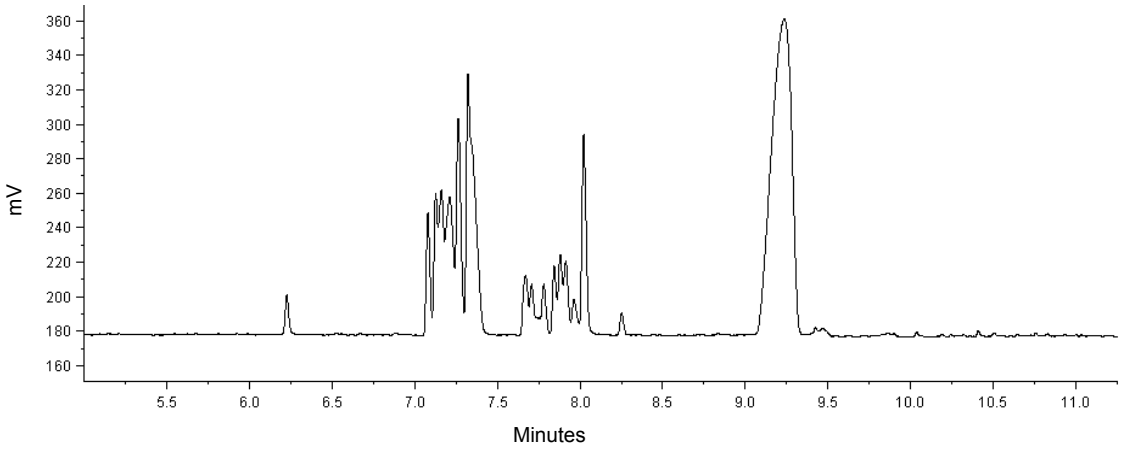


Figure 7-3: Electropherogram of mixture of twenty basic drugs before the addition of an organic modifier. Conditions: buffer: 100 mM phosphate, pH 6 and 20 mM β-CDs; capillary: 51 μm I.D., 60 cm long to the detector; detection: UV 210 nm; injection: electrokinetic, 15 s; temperature: 25 °C; separation voltage: 25 kV; drug concentration: as in Table 7.3.

concentrations above 25% (v/v) the solubility of β-CDs decreased in all of the solvents. As expected, the best results were obtained after the addition of 25% (v/v) isopropanol (Figure 7-4). However, tailing and incomplete resolution between both cocaethylene and methadone and between thebaine and fentanyl citrate were observed. In addition, the migration time was longer than 30 min and the background signal was relatively high. To improve the separation, several experiments were performed using a mixture of two different organic solvents at different concentrations (data is not shown). As a result, the base line separation of twenty basic drugs was achieved using 5% acetonitrile and 20% isopropanol (Figure 7-5).

The effect of buffer concentration on the separation of the twenty basic drugs is shown in Figure 7-6. As expected, increasing the ionic strength increased the resolution because of the concomitant decrease in electroosmotic flow. This was true up to a concentration limit of 100 mM. Above this concentration, the resolution was reduced due to the high current generated. This high current causes Joule heating, which warms the solution and leads to convective diffusion. Therefore, drugs in the warmer center of the tube migrate faster than those near the cooler wall, leading to zone spreading. This effect produces poor drug separations.

Buffer pH was also found to be an important parameter in obtaining selectivity because it modulates the electroosmotic flow. Usually, separations

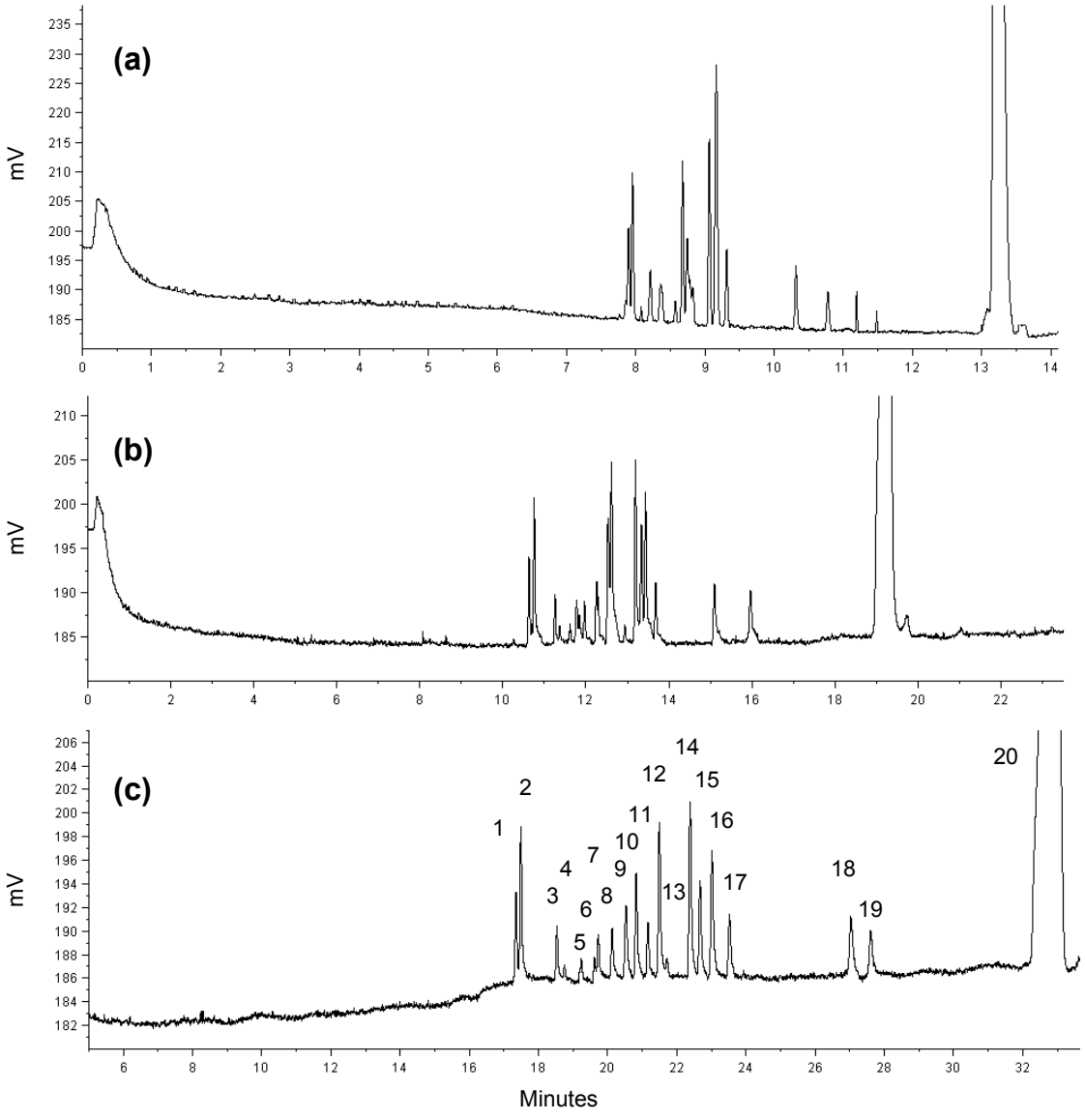


Figure 7-4: Electropherograms showing the effect of the addition of an organic solvent to the buffer on the resolution and migration times: (a) 25% acetonitrile; (b) 25% acetone; (c) 25% isopropanol. Conditions: buffer: 100 mM phosphate, pH 6, 20 mM β-CDs, organic solvent as indicated; other conditions as in Figure 7-3. Peak identification and drug concentration as in Table 7-3.

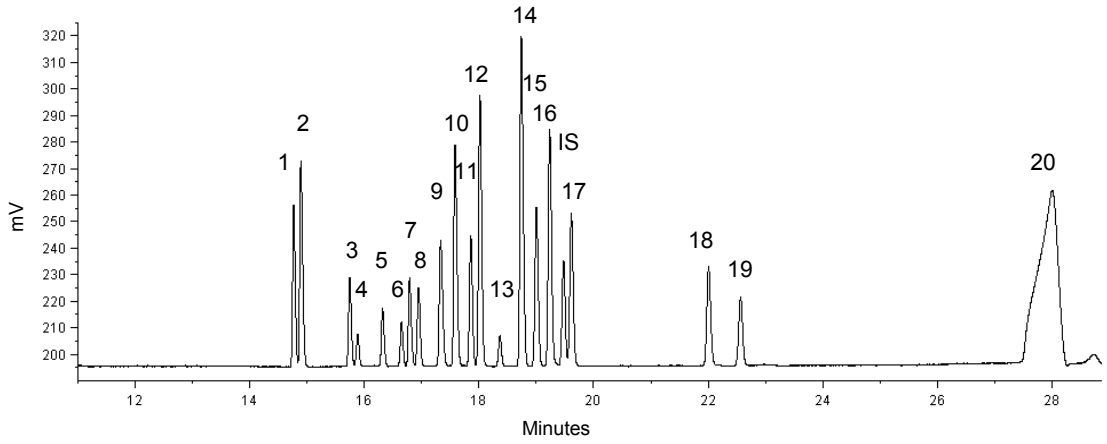


Figure 7-5: Electropherogram for a standard mixture of twenty basic drugs obtained under optimal conditions. Conditions: buffer: 100 mM phosphate, pH 6, 20 mM β-CDs and 5% acetonitrile and 20% isopropanol; capillary: 51 µm I.D., 60 cm long to the detector; detection: UV 210 nm; injection: electrokinetic, 15 s; temperature: 25 °C; separation voltage: 25 kV; drug concentration: as in Table 7.3.

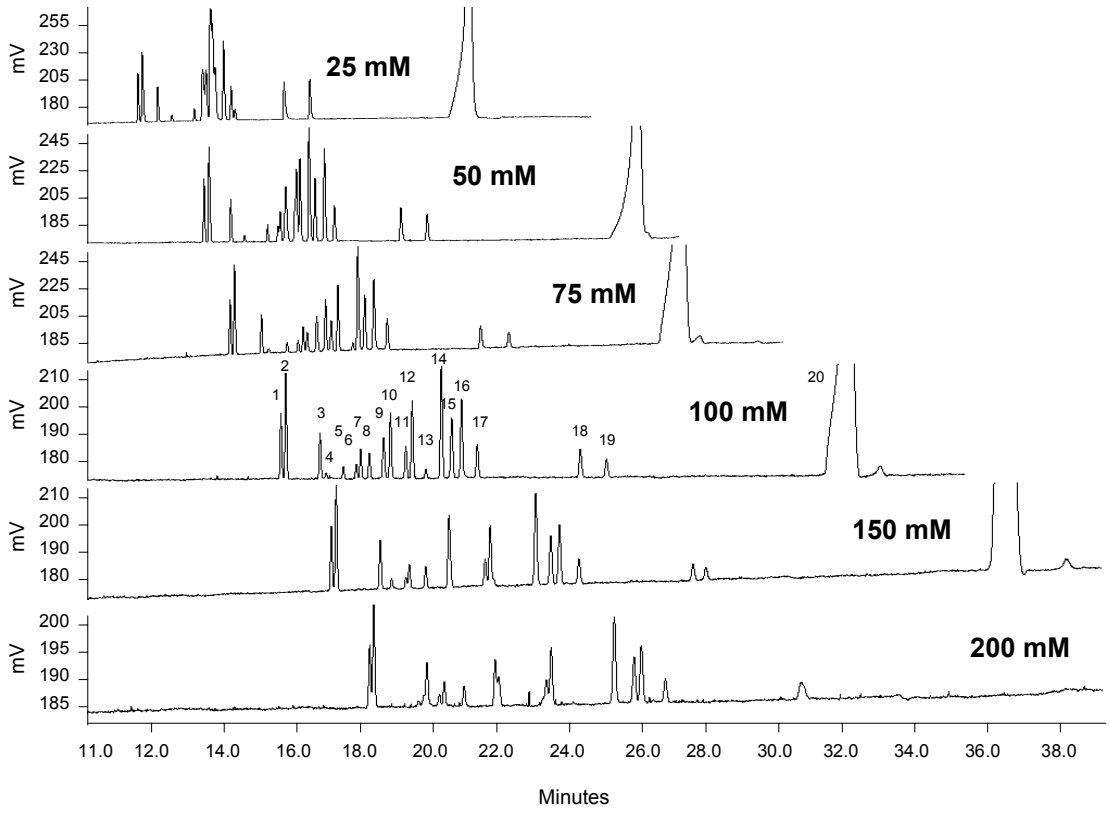


Figure 7-6: Electropherograms showing the effect of buffer concentration on selectivity for the separation of basic drugs. Conditions: buffer: phosphate concentration as indicated, other conditions as in Figure 7-5. Peak identification and drug concentration as in Table 7-3.

using CDs are performed at low pH, at which electroosmotic flow is very low and, therefore, the migration of these neutral compounds toward the cathode is negligible.[143] In this study there was a decrease in the selectivity at low pH values (Figure 7-7). As mentioned earlier, at low pH values, all drugs are protonated and their relative mobilities are constant. On the other hand, as the pH is raised, the buffer approaches the pK_a of the different drugs (4-10) and the selectivity increases. We found that all twenty basic drugs can be resolved at pH 6 and that separation was most reproducible at this pH value.

Changing the voltage is an easy way to modify the electroosmotic flow because it causes a variation in the field strength (obtained by dividing the applied voltage by the length of the capillary). From Eq. (1-4), it can be seen that an increase in the field strength increases the EOF and reduces migration times, leading to shorter analysis times, as illustrated in Figure 7-8. Also, increasing the field strength leads to higher efficiencies, *N*:

$$N = (\mu_{EP} + \mu_{EOF})V / 2D_m \qquad (7\text{-}1)$$

where D_m is the analyte's diffusion coefficient in cm^2/s. The optimum separation voltage was found to be 25 kV (373 V/cm). At this field strength all of the drugs could be separated within the shortest analysis time and with an acceptable maximum current output.

Based on these results, the best separation concentrations were found to be 100 mM phosphate buffer (pH 6), 20 mM ß-CDs, 5% acetonitrile, and 20%

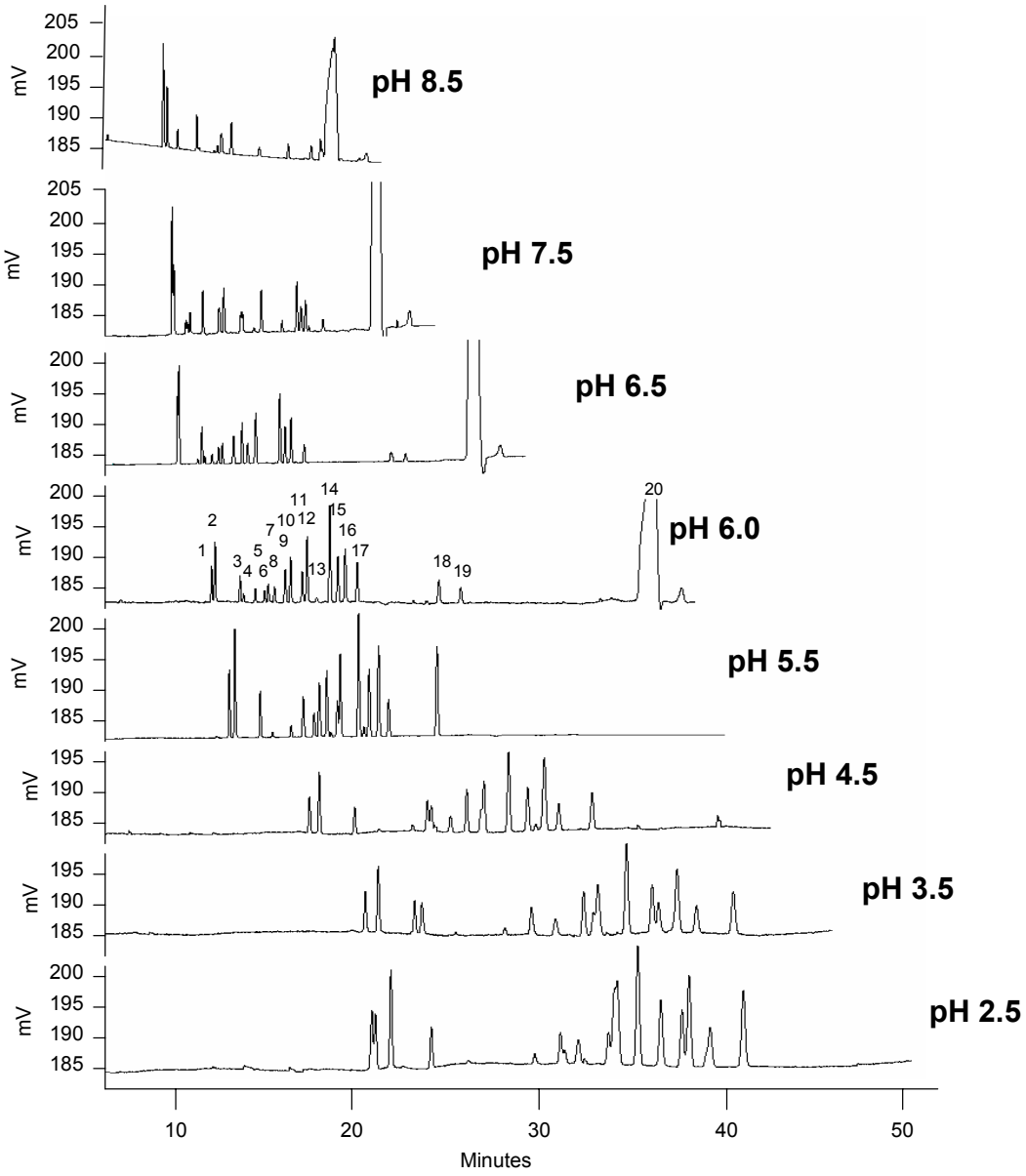


Figure 7-7: Electropherograms showing the effect of pH on selectivity for the separation of basic drugs. Conditions: pH as indicated, other conditions as in Figure 7-5. Peak identification and drugs concentration as in Table 7-3.

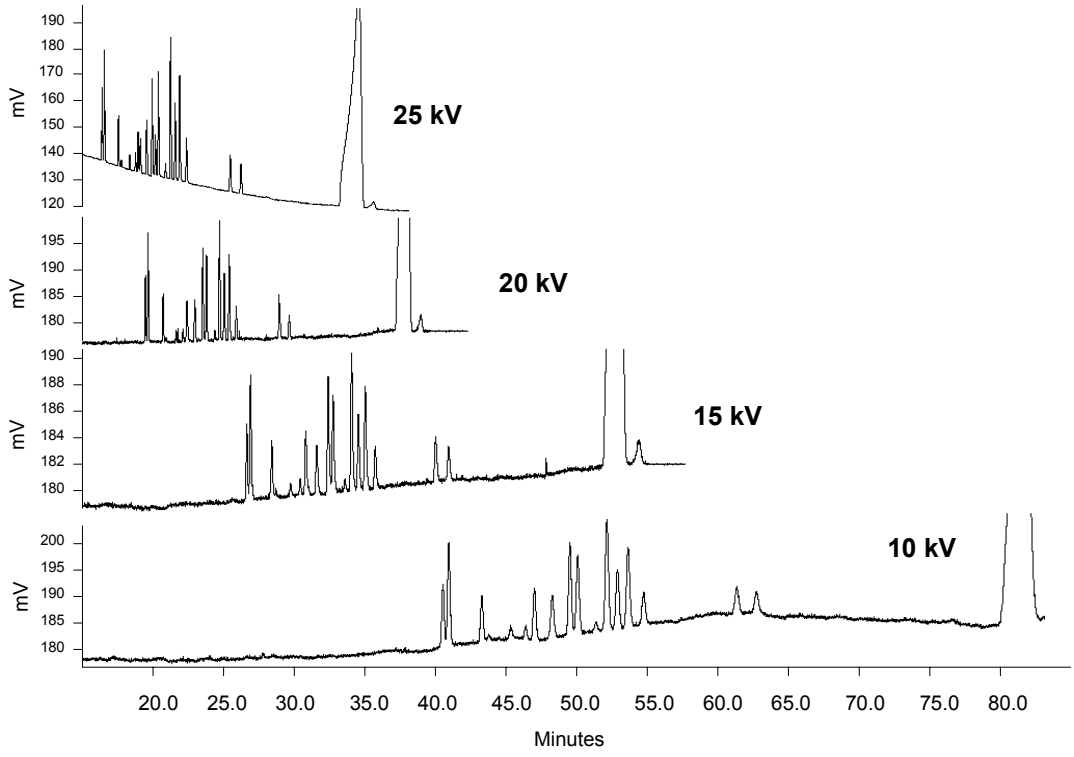


Figure 7-8: Electropherograms showing the effect of applied voltage on migration times. Conditions: voltage: as indicated, other conditions as in Figure 7-5. Peak identification and drug concentration as in Table 7-3.

isopropanol with an applied voltage of 25 kV. As can be seen in Figure 7-5, the complete separation of twenty basic drugs of different classes and different polarities was achieved within 30 min. In this separation, all tested drugs showed migration times of 15 to 23 min except M-3-G, which eluted at 28 min.

7.4 Performance of the standard CE method

Calibration plots were obtained by using standard solutions with drug concentrations ranging from 0.5-6 μg/mL. The response of all tested drugs was linear throughout this range. The linear regression data along with the detection limits are shown in Table 7-2. Run-to-run reproducibility of three consecutive runs is displayed in Table 7-3. The RSD values of the peak areas ranged between 0.77% and 8.40%. Relatively stable migration times (RSDs less than 0.13%) could be obtained when the capillary was rinsed with sodium hydroxide after each run. Therefore, it appears to be better to rely on migration times for peak identification because of their greater reproducibility. The internal standard (quinine) was introduced to minimize variation resulting from the electrokinetic injection used.

7.5 Signal enhancement by automated SPE-CE

Figure 7-9 shows a comparison between a standard CE and an automated SPE-CE for the analysis of twenty basic drugs. Table 7-4 summarizes the characteristics of the proposed method for the pre-concentration and clean-up of standard solutions. As can be seen in the figure, automated SPE-CE provides a

Table 7-2: Linearity equations*, correlation coefficients and limit of detection** for the twenty basic drugs using CE-UV detection.

Peak No.	Compounds	Equation	R	LOD ng/mL
1	Amphetamine	y = 1.726x – 2.771	0.999	26
2	Methamphetamine	y = 2.881x– 4.567	0.998	16
3	Ephedrine	y = 1.069x – 1.650	1.000	42
4	Psilocin	y = 3.452x – 0.513	0.999	13
5	Cocaine	y = 0.375x – 0.852	0.999	120
6	Cocaethylene	y = 0.530x – 0.816	0.999	84
7	Methadone	y = 1.346x – 0.893	0.994	33
8	PCP	y = 0.765x – 1.121	0.997	58
9	Pheniramine	y = 1.283x – 1.595	0.996	35
10	Diphenhydramine	y = 1.853x – 2.546	1.000	24
11	Oxycodone	y = 2.418x – 1.864	0.997	18
12	Thebaine	y = 2.389x – 3.239	1.000	19
13	Fentanyl	y = 0.949x – 0.286	0.996	47
14	Codeine	y = 3.074x – 4.051	0.996	15
15	Morphine	y = 1.586x – 2.176	1.000	28
16	6-AM	y = 2.159x – 2.810	0.993	21
17	Heroine	y = 1.550x – 2.199	1.000	29
18	Noscapine	y = 0.727x – 0.088	0.992	61
19	Papaverine	y = 0.320x – 0.098	1.000	140
20	M-3-G	y = 40.933x – 75.313	0.999	***

*All drugs run at concentrations ranging from 2-4 µg/mL except cocaine which was run from 3–6 µg/mL, methadone and oxycodone from 1–2 µg/mL, and fentanyl citrate from 0.5-1 µg/mL.

**Limit of detection was calculated based on a signal-to-noise ratio equal to 3.

***M-3-G is not quantified because it interferes with the EOF.

Table 7-3: Reproducibility of migration time and peak area.

Drug	Conc.	Migration time		Peak area	
	µg/mL	Mean ± S.D. (min)	RSD (%)	Mean ± S.D. (drug/IS)	RSD (%)
Amphetamine	2.00	15.4 ± 0.008	0.053	0.66 ± 0.028	4.30
Methamphetamine	2.00	15.6 ± 0.010	0.061	1.14 ± 0.048	4.26
Ephedrine	2.00	16.5 ± 0.002	0.013	0.49 ± 0.010	1.97
Psilocin	0.20	16.6 ± 0.005	0.027	0.18 ± 0.015	8.40
Cocaine	3.00	17.1 ± 0.009	0.051	0.27 ±0.003	1.15
Cocaethylene	2.00	17.5 ± 0.011	0.060	0.23 ± 0.005	2.07
Methadone	1.00	17.7 ± 0.016	0.088	0.41 ±0.002	0.54
PCP	2.00	17.8 ± 0.005	0.028	0.37 ± 0.007	1.97
Pheniramine	2.00	18.2 ± 0.010	0.052	0.92 ± 0.009	0.97
Diphenhydramine	2.00	18.6 ± 0.024	0.127	1.14 ± 0.021	1.82
Oxycodone	1.00	18.8 ± 0.002	0.011	0.53 ± 0.015	2.94
Thebaine	2.00	19.0 ± 0.003	0.014	1.52 ± 0.053	3.48
Fentanyl	0.50	19.4 ± 0.006	0.028	0.18 ± 0.001	0.77
Codeine	2.00	19.8 ± 0.001	0.005	2.01 ± 0.119	5.93
Morphine	2.00	20.1 ± 0.002	0.009	0.99 ± 0.066	6.68
6-AM	2.00	20.3 ± 0.001	0.005	1.35 ± 0.087	6.39
Heroine	2.00	20.7 ± 0.001	0.003	0.91 ± 0.065	7.17
Noscapine	2.00	23.4 ± 0.005	0.021	1.31 ± 0.028	2.16
Papaverine	2.00	24.1 ± 0.011	0.046	0.53 ± 0.039	7.34
M-3-G	2.00	30.2 ± 0.004	0.014	-	-

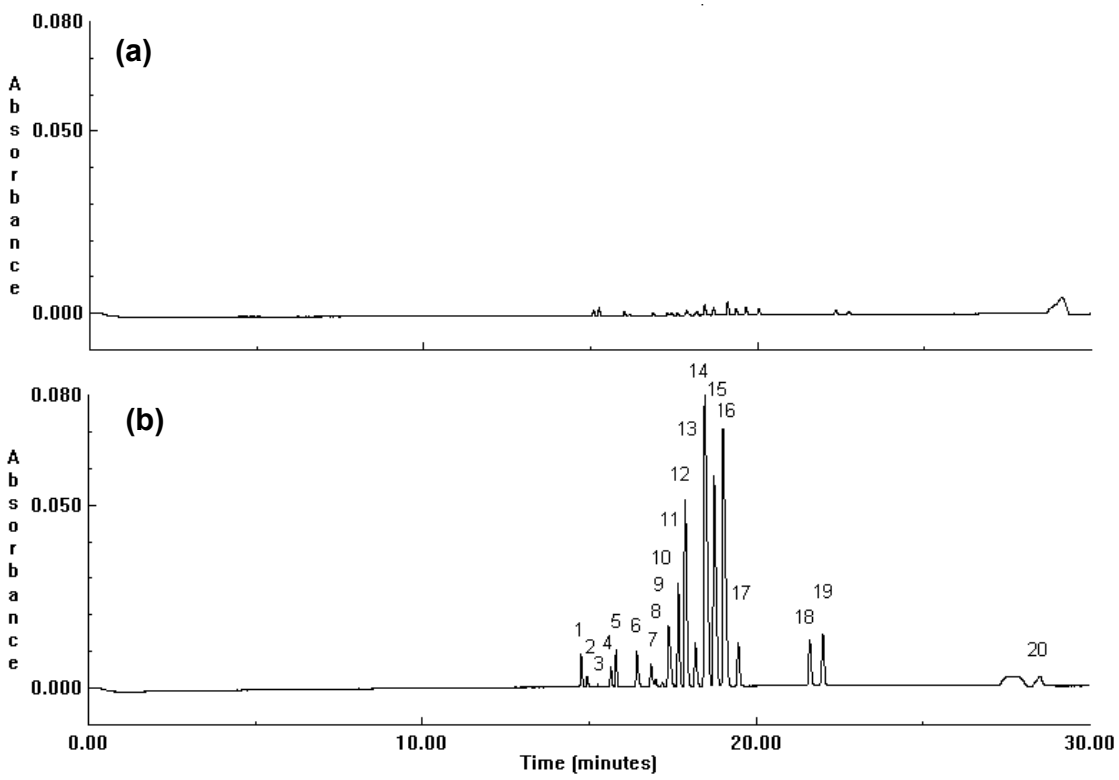


Figure 7-9: Comparison of (a) standard CE and (b) automated SPE-CE for the analysis of basic drugs. Conditions as in Figure 7-5. Peak identification and drug concentration as in Table 7-3.

Table 7-4: Characteristic of the automated SPE-CE with standard solutions.

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

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

7.6 Applications

Figure 7-10 shows the electropherogram for a blank urine sample and a urine sample spiked with the twenty basic drugs which were then extracted, pre-concentrated and analyzed using automated SPE-CE. It is evident that few endogenous compounds in the urine are being extracted and applied to the capillary under the optimized SPE conditions.

Since the off-line mixed-mode SPE method is commonly used for the routine analysis of abused drugs, its accuracy and precision were also determined and compared to that of the proposed automated method. We found that the off-line SPE has slightly better recovery for all tested drugs (ranging from 88% to 102%). This may be due to the use of mixed-mode technology which allows a strong wash solvent to be utilized without the problem of losing analytes. In automated SPE, in which a C_{18} column was used, 20% methanol was found to be important to minimize the interferences resulting from the urine matrix. However, this solvent can also elute basic compounds of interest prematurely. On the other hand, the precision of the off-line SPE was found to be lower than that of the automated procedure (the RSDs were between 1.1% and 7.6%). This can be attributed to the minimal sample and reagent handling that occurs when using the automated method. Figure 7-11 shows electropherograms for a blank urine sample and a urine sample spiked with the twenty basic drugs which were then extracted, pre-concentrated, and analyzed using off-line SPE-CE.

Solid-phase microextraction (SPME) was also tested for the analysis of drugs of abuse. The extraction procedure was as previously published.[144, 145] The electropherogram resulting from the spiked water sample which was extracted using SPME and analyzed by CE-UV detection is shown in Figure 7-12. As can be seen, several drugs of abuse were not detected, including ephedrine, psilocin, cocaine, cocaethylene, methadone, 6-AM, and heroin. The extraction efficiency and accuracy for most detected drugs were also greatly affected. Therefore,

further examinations should be carried out with different fiber materials to optimize this extraction method for drug analysis.

7.7 Conclusions

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

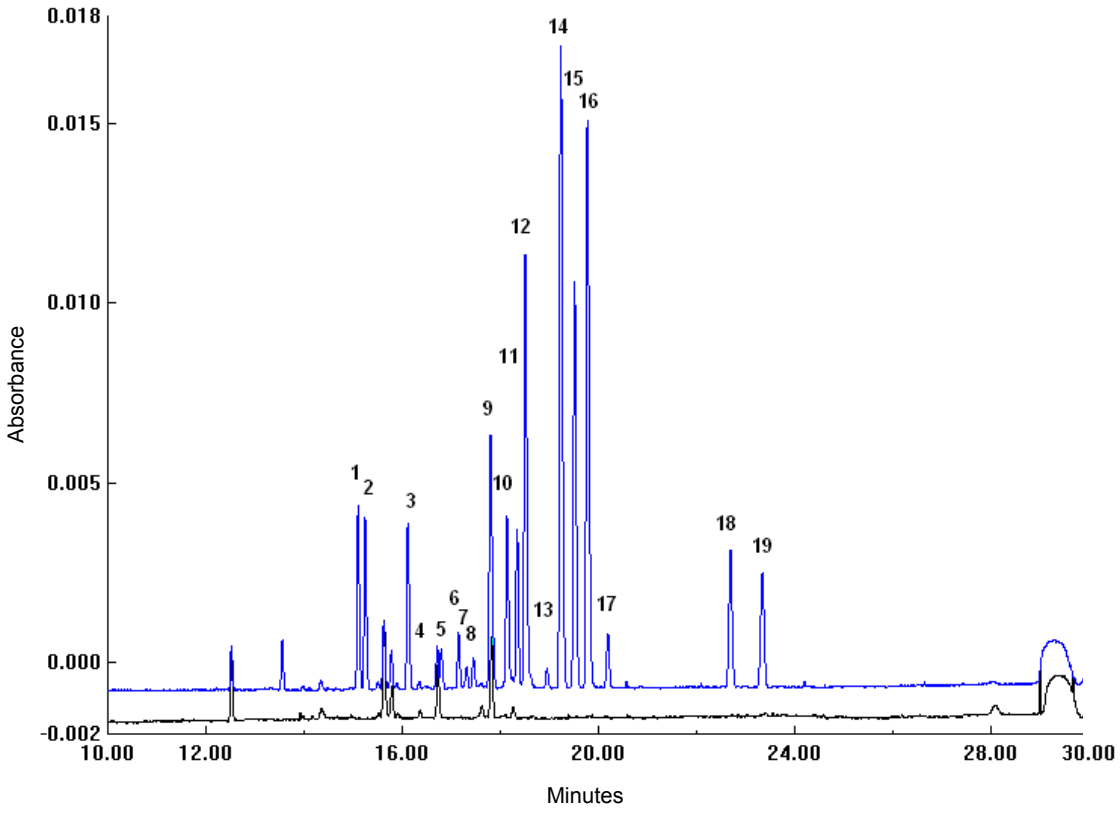


Figure 7-10: Electropherogram of automated SPE-CE of urine spiked with twenty basic drugs (upper electropherogram) and blank urine sample (lower electropherogram). Extraction procedures and conditions as in Section 4.3.4. Separation conditions as in Figure 7-5. Peak identification and drug concentration as in Table 7-3.

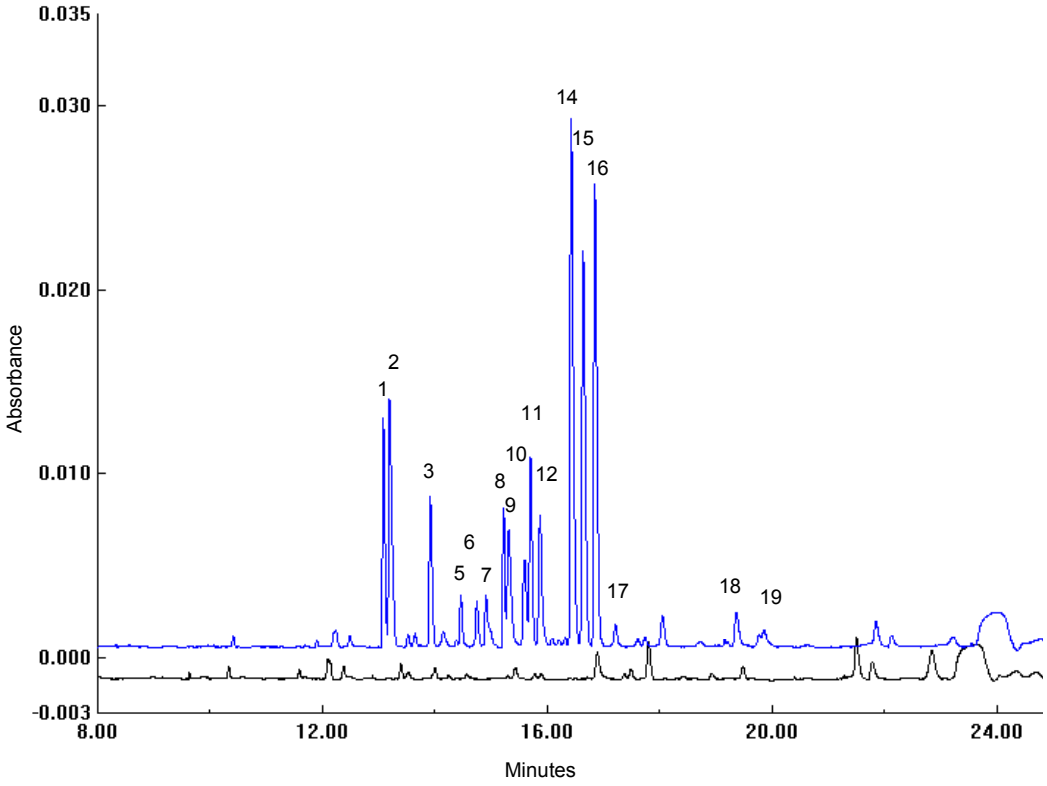


Figure 7-11: Electropherogram of off-line SPE of urine spiked with twenty basic drugs (upper electropherogram) and blank urine sample (lower electropherogram). Extraction procedures and conditions as in Figure 4-1. Separation conditions as in Figure 7-5. Peak identification and drug concentration as in Table 7-3.

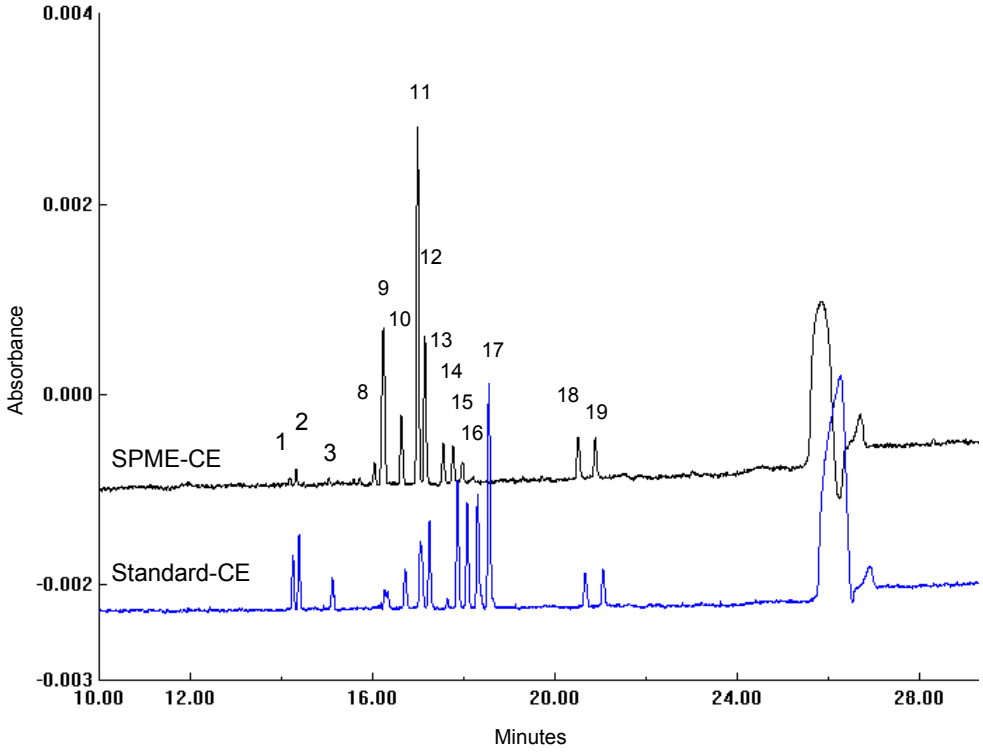


Figure 7-12: Electropherogram of SPME of water spiked with twenty basic drugs. The extraction procedure was as previously published.[144, 145] Extraction was performed by immersion of the fiber material (fused silica coated with a 100 µm polydimethylsiloxane stationary phase) directly into the 5 mL spiked water sample. 500 mg of a mixture of sodium carbonate and sodium bicarbonate was added to the sample and the pH was adjusted to 9.0 using ammonium hydroxide. The drugs were desorbed from the fiber with 500 µL of methanol by stirring for 30 min. Other conditions as in Figure 7-5. Peak identification and drug concentration as in Table 7-3.

Chapter 8 Conclusions and Future Research

8.1 Conclusions

This research project demonstrates the role that pre-concentration techniques can have on improving sensitivity in capillary electrophoresis. Both chromatography and electrophoresis-based pre-concentration techniques were used and improvement of up to 100 orders-of-magnitude was obtained under optimal conditions.

The studies completed also demonstrate the importance of fluorescence derivatization in combination with laser induced fluorescence detection in obtaining sensitive analysis method. Using ACE-Cl and FITC derivatives, the detection limit of 50 pg/mL is superior to most published procedures.

Furthermore, this work demonstrates the importance of ß-cyclodextrins as a complexing agent for improving the separation selectivity of CE. The role of buffers in the separation process of CE was discussed in detail, with emphasis on buffer concentration, buffer type, pH, and ß-CDs concentration. The effect of organic solvents on separation and migration behavior in the presence of CDs was also investigated. Optimization of such parameters can greatly enhance selectivity in capillary electrophoresis.

8.2 Suggestion for future research

On-line sample pre-concentration in conjunction with on-line chemical derivatization (UV or fluorescence) represents a promising method to enhance

CE sensitivity. Recently, sample stacking with on-line derivatization of amino acids with 1, 2-naphthoquinone-4-sulphonate was reported to improve sensitivity by over 1000 fold with respect to conventional pre-capillary derivatization.[146]

On-line pre-concentration techniques are also critical to the relatively new types of detectors such as mass spectrometry[49] and nuclear magnetic resonance,[147] which provide a high information content for the qualitative identification of unknown metabolites, but suffer from very poor detection sensitivity.

Recent interest in the adaptation of microchip electrophoresis systems for rapid separations also benefit from the on-line focusing and fluorescence derivatization methods because of the poor concentration sensitivity and low column efficiency of these techniques. Recently, Suzuki and co-workers[148] reported the rapid and sensitive analysis of amino sugars by microchip electrophoresis with LIF detection using 5-carboxyfluorescein succinimidyl ester. The analysis time, including the derivatization of amino sugars, was less than 1 min. This method is also applicable to the analysis of abused drugs such as amphetamine, methamphetamine, and ephedrine using a microchip electrophoresis system. Furthermore, this method could be applied to opiate analysis after a simple *N*-demethylation reaction.[126, 149]

N-demethylation using enzymatic reactions is an alternative way to speed up the derivatization process. Ladona et al.[150] used an in vitro study with human fetal tissue to demonstrate that the *N*-demethylation of codeine and other opiate

drugs is possible using CYP3A enzymes. These enzymatic reactions take less than 30 min to complete.[151]

A new direction in sample stacking involves the analysis of samples in high ionic strength matrices. Hadwiger and colleagues[152] have shown that pH-mediated sample stacking can be used for the on-column concentration of isoproterenol in dialysates. Double-capillary pH-mediated stacking is also an important new area which can be used to perform a 300-fold sample concentration.[153] While the high ionic strength of the sample buffer is detrimental to normal CE separations, some sample stacking methods are actually improved when a high ionic strength matrix is used instead of water. For example, Shihabi[137] has shown that field amplified sample stacking can be enhanced by diluting samples with acetonitrile, if the sample is initially in 1% saline. Also, electrokinetic focusing strategies that can be applied to new modes of separation in CE, such as capillary electrochromatography using monolithic silica columns,[154] can also be useful for enhancing detector sensitivity.

Certain drugs of abuse, such as LSD and opiates, can produce native fluorescence at low excitation wavelengths.[97, 149] Therefore, with the advent of lower wavelength diode lasers, such compounds should be accessible to direct analysis on microfluidic systems without the need for derivatization. Another possibility for direct detection is UV absorbance. However, special techniques must be applied for there to be sufficient detection sensitivity to use these

techniques in microfluidic devices. These techniques include the use of wave guides bubble cells and special detection windows.

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