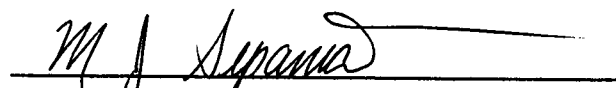
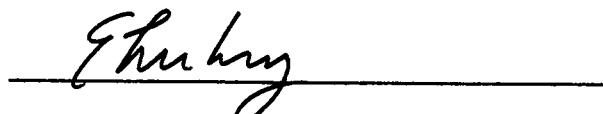


To the Graduate Council:

I am submitting herewith a thesis written by Craig Alan Chwojdak entitled "Evaluation of Extended Light Path Capillaries for use in Capillary Electrophoresis with Laser Induced Fluorescence Detection." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Chemistry.


Micheal J. Sepaniak, Major Professor

We have read this thesis
and recommend its acceptance:




Accepted for the Council:


Associate Vice Chancellor and
Dean of The Graduate School

**Evaluation of Extended Light Path Capillaries for
use in Capillary Electrophoresis with Laser
Induced Fluorescence Detection**

**A Thesis Presented
for the Master of Science Degree
The University of Tennessee, Knoxville**

**Craig Alan Chwojdak
December 1995**

Dedication

To my wife Megan
For her undying love and support

Acknowledgements

This thesis is dedicated to my wife Megan, whose love and constant support have made this journey bearable. Thank-you for being there for all the highlights and especially all the downfalls.

I would like to express my appreciation to Dr. Michael J. Sepaniak, my research advisor, for giving me the opportunity to pursue my dreams and aspirations. His patience and guidance were invaluable in my development of this work. Thanks for the extra length of rope.....many lessons learned.

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Finally, I would like to thank my committee members, E. L. Wehry and D. C. Kleinfelter.

Abstract

An interesting development in improved Capillary Electrophoresis (CE) detection sensitivity is the Hewlett-Packard Extended Light Path Capillary (ELP). These capillaries have a "bubble" in the flow path at the detection zone which provides increased optical path length. While extensively characterized for use in absorbance detection, they have not been adequately assessed for applications of laser-induced fluorescence (LIF) detection. Similar advantages (*e.g.* improved detectability) are expected with LIF, in addition to simplifying optical alignment. Thus, experiments were conducted to make comparisons between conventional and ELP capillary formats. In the first experiment, detection sensitivity was compared. The larger dimensions of the ELP capillary allowed efficient, far field spatial rejection of scattered light with an adjustable, precision slit. The greater optical path length permitted wider slit settings, thereby improving signal transmission by several fold. This experiment also revealed that alignment of the ELP capillary with the excitation beam and collection optics was significantly simplified. Clean transmission of the excitation beam was easily accomplished with both 50 and 25 μm i. d. ELP capillaries, representing a significant improvement in ease of alignment over the conventional case. To further illustrate this feature, profiles of capillary wall scatter/fluorescence were generated by linear

translation of the photomultiplier assembly in the far field while maintaining a constant slit width. These profiles clearly illustrate the improved spatial characteristics of the ELP capillary relative to conventional capillaries. The rapid, high sensitivity separation of the metabolic enzyme markers 7-ethoxycoumarin and its metabolites in a liver slice incubate is shown as a practical application. A second pharmacokinetic application of ELP capillary was also investigated. This preliminary study probed the applicability of the ELP capillary for increased sensitivity of the antipsychotic drug CP-88,059 and a related metabolite.

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Chapter 1

Introduction

Motivation for development of a Laser Induced Fluorescence (LIF) detection system incorporating the use of Extended Light Path Capillaries.

Laser induced fluorescence (LIF) has been employed in high sensitivity separation science applications since the late seventies (1). This approach to detection combines the classical advantages of conventional fluorescence with the laser-based features of high excitation beam intensity and excellent focusing characteristics. These characteristics permit ultra trace analysis of complex mixtures with absolute detection limits at the attomole level and below (2,3). As a result, LIF has been extensively utilized for both High Performance Liquid Chromatography (HPLC) (1,4-8) and capillary electrophoresis (CE) (2,3,9-14) applications. Our interest in the technique centers on the high sensitivity afforded by laser excitation for the analysis of xenobiotic materials and related metabolites in biological matrices.

Existing Methodology/Statement of Problem

While LIF has significant potential for application to trace bioanalysis, it remains a technique practiced nearly exclusively in the academic laboratory. One vendor (Beckman, Inc.) offers an

instrument designed for CE/LIF, but it is limited to excitation in the visible region of the spectrum. Two primary bottlenecks exist which are responsible for the slow evolution of LIF instrumentation. First, practical application of the technique requires excitation in the UV spectral region to allow quantitation of a reasonable number of analytes. This restriction is somewhat diminished by fluorescence derivatization, but not without the problems associated with such chemical treatments (15). Access to laser lines in this region has classically been difficult, requiring complex instrumentation and dedicated facilities. The recent commercial availability of a relatively simple UV tunable laser system (*e. g.* Coherent Inc. offers the FReD system, with lines spanning 229 - 264 nm) greatly broadens the range of potential applications. Second, a key challenge in the development of LIF instrumentation suitable for routine analysis is the design of a rugged procedure for optical alignment of the detection flow cell with the focusing and collection optics. In general, a significant amount of experience is required to achieve rapid alignment and more importantly, optimal system performance. Application of this technology to ultra-sensitive analysis in an industrial setting requires instrumentation which is simple, easy to use, and highly precise in terms of intra-day performance. Thus, improvements in LIF flow cell and instrumentation design are required to make this approach more feasible.

A key source of difficulty in operation of LIF instrumentation is the diminutive size of the flow cell. For instance, in CE the operator

must precisely probe the interior of 50 μm i. d. (or smaller) capillary. Misalignment of the laser beam often leads to degraded detectability due to the collection of scattered light and the generation of fluorescence from silica wall impurities (2).

As a first approach to alleviating these difficulties, an alternate capillary geometry was assessed, namely the Extended Light Path (ELP) capillary (Hewlett-Packard). ELP capillaries show promise as an advancement in detection technology because they are constructed with a 3-5X expansion (bubble) in the detection zone (Figure 1). This bubble has been used to improve absorbance detection sensitivity by increasing optical path length (16,17). The same path length dependence is also present in fluorescence. Due to the increased detection zone dimensions, it was postulated that the ELP design could potentially be used to improve sensitivity in LIF and ease optical alignment constraints.

This report outlines experiments aimed at the quantitative assessment of this postulate. The ELP capillary experiments were applied to two pharmacological systems of importance. The first of these encompassed the determination of 7-ethoxycoumarin and metabolites from in-vitro preparations of liver slice incubates. The second involved a preliminary study of the antipsychotic agent CP-88,059, structurally similar to tiosperone (18).

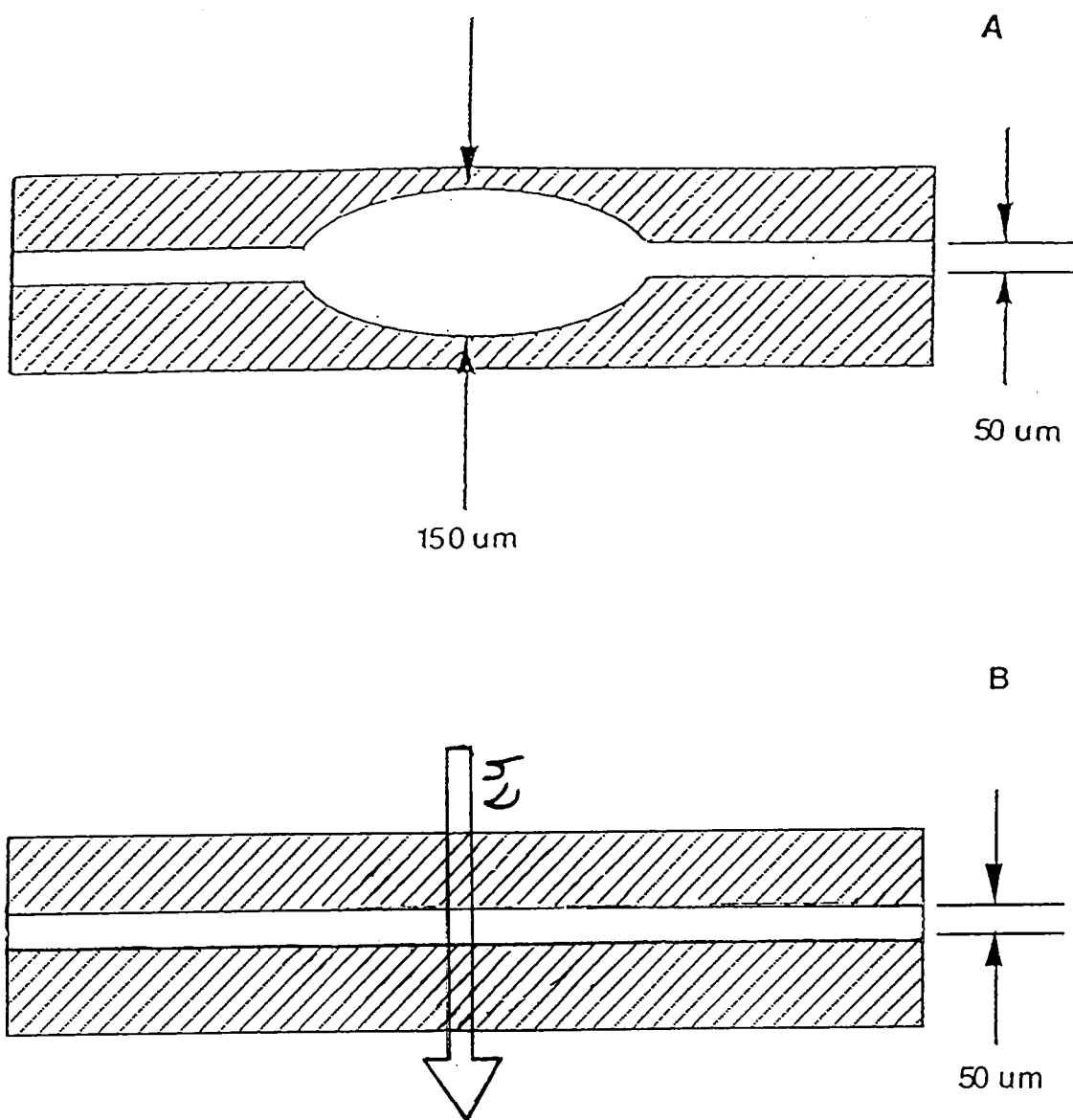


Figure 1. Illustrations of (A) ELP and (B) Conventional Capillaries

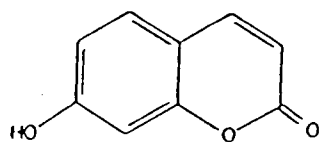
7-Ethoxycoumarin Experiment

CE/LIF, discussed in detail later, is well suited for the analysis of *in-vitro* generated samples due to the high sensitivity and speed of separation that are possible. Most conventional *in-vitro* chromatographic assays are based on HPLC and require gradient elution due to the presence of endogenous material, the complex mixtures generated, and/or the wide disparity in polarity between parent compound and metabolite(s). The high resolving power of CE and the selective nature of fluorescence detection may alleviate many of these difficulties. (see below).

Liver slices are useful in the study of drug metabolism in that they provide insight to both Phase I (*e.g.*, simple hydroxylation) and Phase II (conjugation) metabolism (18). Since the cellular architecture is preserved in these systems, few endogenous interferences are observed. However, multiple metabolites are produced, requiring resolution from one another for accurate analysis. In the case of 7-ethoxycoumarin (7-EC), three metabolites are produced: 7-hydroxycoumarin (7-HC), the 7-glucuronide, and the 7-sulfate. Structures of the above analytes can be seen in Figure 2.

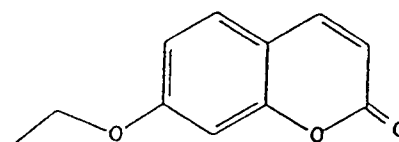
Conventionally, these analytes are analyzed via ion-pair gradient HPLC with UV absorbance detection (19). This approach requires approximately 30 minutes total analysis time. While improvements employing short HPLC columns have recently been made (20), the total analysis time remains at some 10 minutes.

A



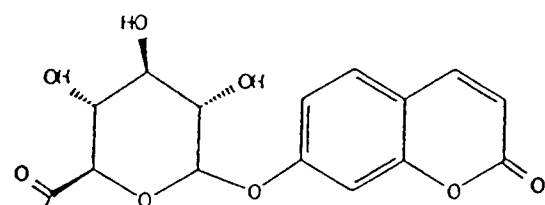
7-hydroxycoumarin

B



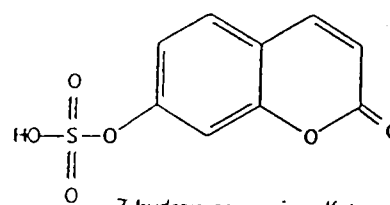
7-ethoxycoumarin

C



7-hydroxycoumarin glucuronide

D



7-hydroxycoumarin sulfate

Figure 2.

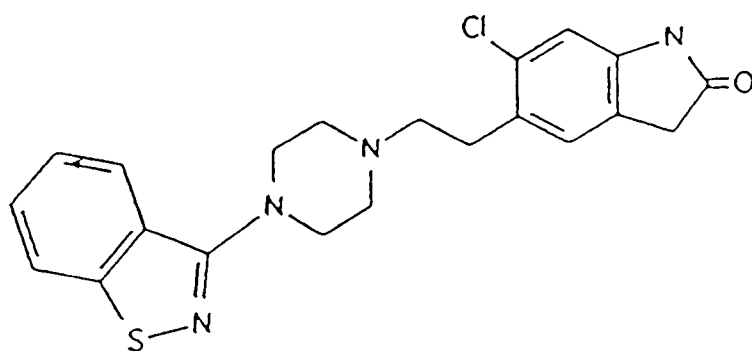
Chemical Structures of Coumarin Metabolites: (A) 7-Hydroxycoumarin
 (B) 7-Ethoxycoumarin (C) 7-Glucuronide (D) 7-Sulfate

Utilizing Micellar Electrokinetic Capillary Chromatography (MECC), the separation of solutes with wide variations in polarity can often be addressed without the use of gradient elution or lengthy runs.

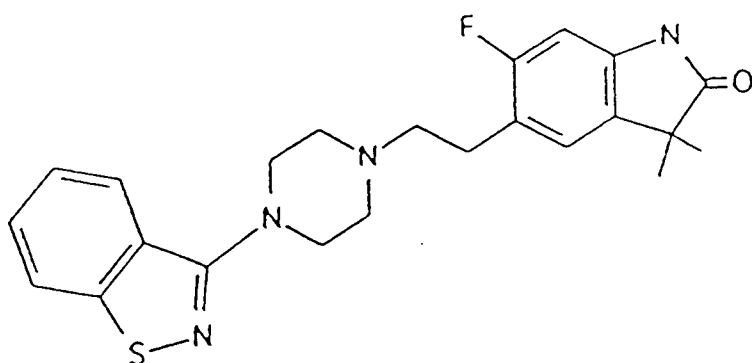
CP-88,059 Antipsychotic drug Experiment

CP-88,059, 5-[2-(4-benzo[d]isothiazol-3-yl-piperazin-1-yl)ethyl]-6-chloro-1,3-dihydroindol-2-one (CP, see Fig. 3), is an antipsychotic agent that has previously been determined by HPLC-Mass Spectrometry (HPLC/MS) (18). This method met the required limit of detection of at least 1 ng/ml in serum, but due to high cost and limited availability for routine sample analysis the method found little practical application. From this came the work of Cole, Fouda, and Janiszewski (18) in which narrow bore HPLC was utilized. Although this method was shown to increase detector response four fold, losses in chromatographic performance, reduced efficiency and peak asymmetry, were observed. These losses, presumably due to band broadening, were assumed to be from the coupling of conventional instrumentation with narrow-bore columns (18). Cole, Fouda, and Janiszewski further stated that changes in flow cell design and attention to extra column volume effects should result in improved chromatographic performance.

As mentioned above, the instrumental shortcomings and poor chromatographic performance of HPLC/MS limited the method in practical application. A hypothesis to alleviate such problems involved the combination of ELP capillary with MECC/LIF. This



A CP-88,059



B Internal Standard

Figure 3. Chemical Structures of: (A) CP-88,059 and (B) Internal Standard

hypothesis is based on the same attributes of CE/LIF discussed previously in the 7-EC section. To determine whether the combination of ELP capillary with MECC/LIF was beneficial, a preliminary study was devised.

CHAPTER 2

Basic Theory

Advantages of Capillary Electrophoresis

The fundamental goal behind an analytical separation system is to be able to separate a multicomponent sample for identification. This feat has been established and perfected to varying degrees on such techniques as Gas Chromatography (GC), HPLC and Slab Gel Electrophoresis. Over the past few decades, other techniques were discovered and have vastly advanced the separation science. Two such notable advancements are Hjerten's pioneering work with free solution electrophoresis (22) and Jorgenson and Lukacs' research with fused silica capillaries (23), developed CE into a powerful analytical method of separation. CE differs from conventional forms of electrophoresis in that no anti-convective stabilizing medium such as gel or paper is used and detection is achieved "on-column". Separations are carried out in free solution within narrow-bore capillaries (internal diameters (i.d.) $\leq 75 \mu\text{m}$). Capillaries of these dimensions are able to dissipate the heat generated by the passage of electric current, thereby eliminating band dispersion due to thermal gradients and convection (24). Some of the advantages of CE are the following:

- (1) Simple apparatus: Only column, power supply and detector are required
- (2) Small sample amounts: 5.0 nl injection volume typically required
- (3) On-line detection: No lengthy post-separation procedures are necessary
- (4) Rapid separations: Sub minute separations are possible
- (5) High efficiency: Plate numbers typically in excess of 250,000
- (6) Wide variety of selectivities possible via running buffer additives

A typical CE system (see Figure 4) is composed of a capillary, two buffer reservoirs, two electrodes, a power supply and some form of detector. The two most common detectors used in CE are based on absorbance and fluorescence. In order to understand fully the manner in which a typical CE system works, a basic understanding of the theory involved is needed.

Electroosmosis

At the beginning of an analytical run, one end of the capillary contains a segment of the unresolved mixture, occupying a very small volume (≤ 10 nanoliters). This segment or "sample plug" transverses (migrates) down the entire column length to the detection zone. This process occurs when an electric field is applied (10 - 30 kV) across the column. The above phenomenon is a result

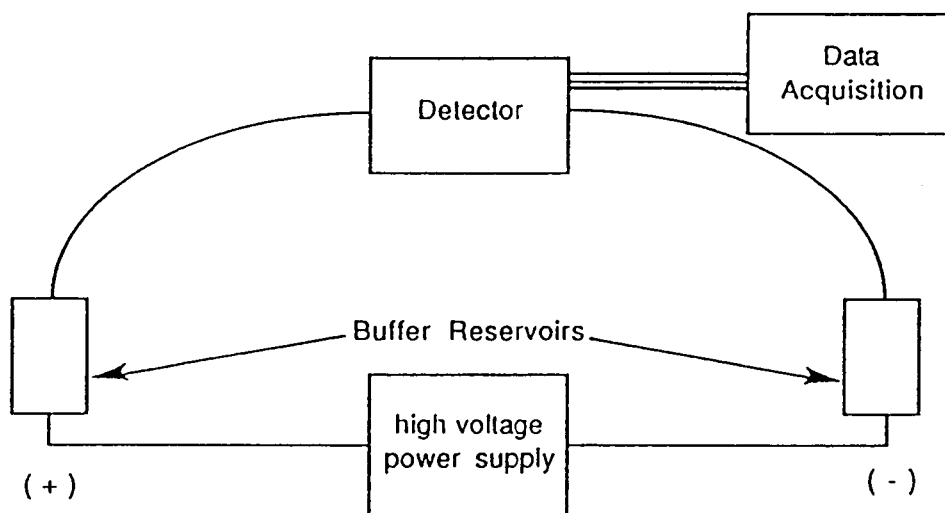


Figure 4. Capillary Electrophoresis System

of electromigration and electroosmotic flow (EOF). These migration processes are described by Equation 1

$$V_b = (\mu_e + \mu_{eof}) E \quad (1)$$

in which V_b (velocity of the solute band) is directly related to E (applied field), μ_e (electrophoretic mobility) and μ_{eof} (the electroosmotic mobility) (to be described later). One comment, regarding solute velocities, is that if the solute is a neutral analyte, μ_e equals zero.

EOF is the bulk flow of liquid due to the effect of the electric field on the counterions adjacent to the negatively charged capillary wall (Figure 5). This stems from the build up of positive counterions on the negatively charged capillary walls, thereby creating an electric double layer potential (zeta potential). Once an external potential is applied, the solvated, positive counterions migrate down the column toward the negative electrode. The bulk flow of the liquid inside the column that accompanies this process is termed electroosmotic flow. The velocity of the EOF is determined by Equation 2 where ξ , ϵ and η , are the zeta potential, electrical permittivity and viscosity of the running buffer respectively.

$$v_{eof} = \frac{E\xi\epsilon}{\eta} \quad (2)$$

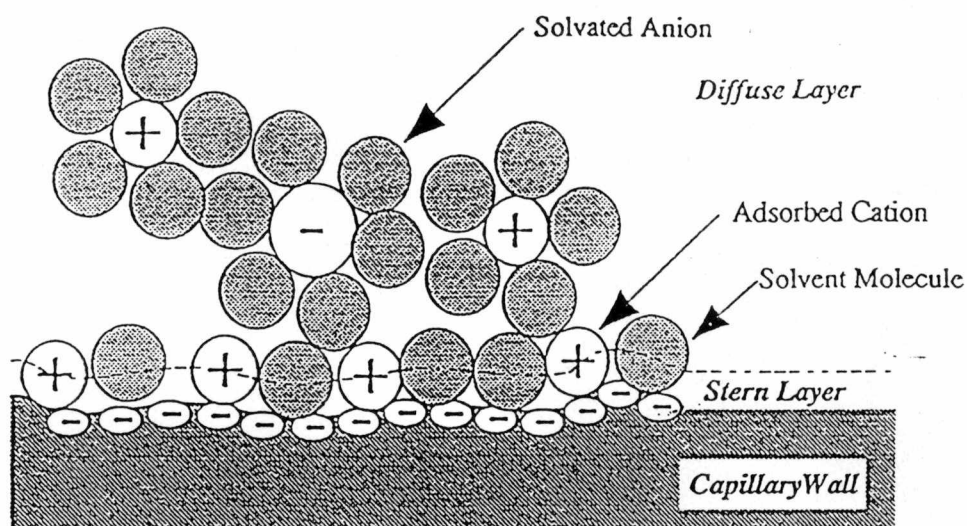


Figure 5. Electroosmotic Flow Diagram

Observed Mobility

In terms of electroosmotic mobility, this equation becomes

$$\mu_{eof} = \frac{v_{eof}}{E} = \frac{\xi \epsilon}{\eta} \quad (3)$$

An important feature of Equation 3 is the absence of a radial dependence in μ_{eof} . This suggests that the velocity profile of EOF is relatively flat across the capillary bore, unlike hydrostatic flow profiles which are parabolic due to pressure displacement. Parabolic flow profiles introduce band dispersion due to resistance to mass transfer in the mobile phase (running buffer). To reduce this resistance to mass transfer, a solute must quickly diffuse across the

capillary diameter to average out the velocity differences encountered at different radial positions. Consequently, slow radial diffusion results in band dispersion. EOF minimizes band dispersion by having a constant velocity profile. Figure 6 shows a representation of a hydrostatically pumped flow profile (such as in HPLC) and an EOF profile.

At the end of the capillary, the separated solute bands are detected on column and an electropherogram is recorded. Resolution (R_s) of these bands in CE can be described by

$$R_s = \frac{\sqrt{N}}{4} \left[\frac{\mu_1 - \mu_2}{\mu_{ave} - \mu_{eof}} \right] \quad (4)$$

where N is the number of theoretical plates, μ_1 and μ_2 are the electrophoretic mobilities of the solutes of interest and μ_{ave} the average of μ_1 and μ_2 .

From the above equations it becomes apparent that if the solutes of interest are neutral, thereby lacking an electrophoretic mobility, they can not be resolved by CE. Therefore, the issue of how to differentiate between neutral components in a mixture must be addressed.

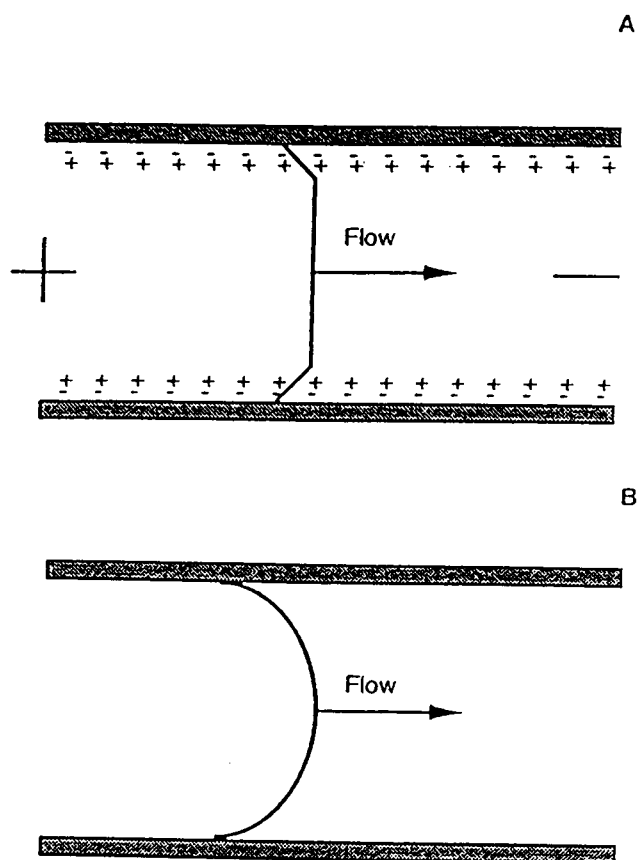


Figure 6. Illustration of flow profiles: (A) EOF and (B) hydrostatic flow

Basic Theory of Micellar Electrokinetic Capillary Chromatography (MECC)

The primary limitation of CE is its inability to separate neutral components. In 1984, Terabe and coworkers (26) added a surfactant (sodium dodecyl sulfate) to the CE running buffer and successfully separated neutral solutes. Named micellar electrokinetic capillary chromatography (MECC), this approach has formidable features (discussed later).

Separation Mechanism

The instrumental design in MECC is the same as in CE. Surfactants are amphiphilic species comprised of hydrophobic and hydrophilic regions. They can be anionic, cationic, zwitterions, or even polar neutral species (25). In certain solvents, surfactants can aggregate to form colloidal-sized assemblies referred to as micelles (26). This aggregation process occurs when the concentration of surfactant has reached what is referred to as the critical micellar concentration (CMC). Normal micelles, (hydrophobic tails inward and polar head groups defining the surface of the micelle) rapidly form when the concentration is above the CMC (see Figure 7). CMC values and the physical characteristics of micelles are dependent on experimental conditions such as temperature, solvent type, and the presence of additives (25). The CMC and aggregation values of the two surfactants used in this work are 8.1 mM and 62 for sodium dodecylsulfate (SDS), respectively, and 12.5 mM and 3 for sodium

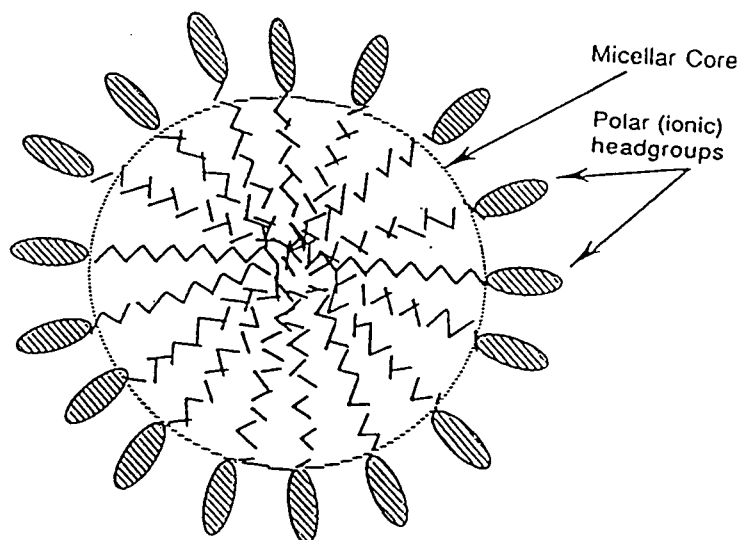


Figure 7. Illustration of micellar structure

cholate (NaC) respectively. Micelles have a charged surface and possess an electrophoretic velocity $v_{e,m}$ that generally opposes the electroosmotic velocity v_{eof} . The net velocities of the micelles ($v_m = v_{eof} + v_{e,m}$), and the velocity of the solute that partitions between the phases can be seen in Figure 8. Differential partitioning between the micelle and solute provides a retention mechanism by which solutes can be separated. In contrast to conventional chromatography, however, the secondary phase (micelles) elutes from the column. Therefore, solutes that completely associate with the micellar phase define a limited elution range for MECC. This elution range is often described by t_o/t_m , which is the ratio of the elution time of an unretained component to the elution time of the micellar phase (see

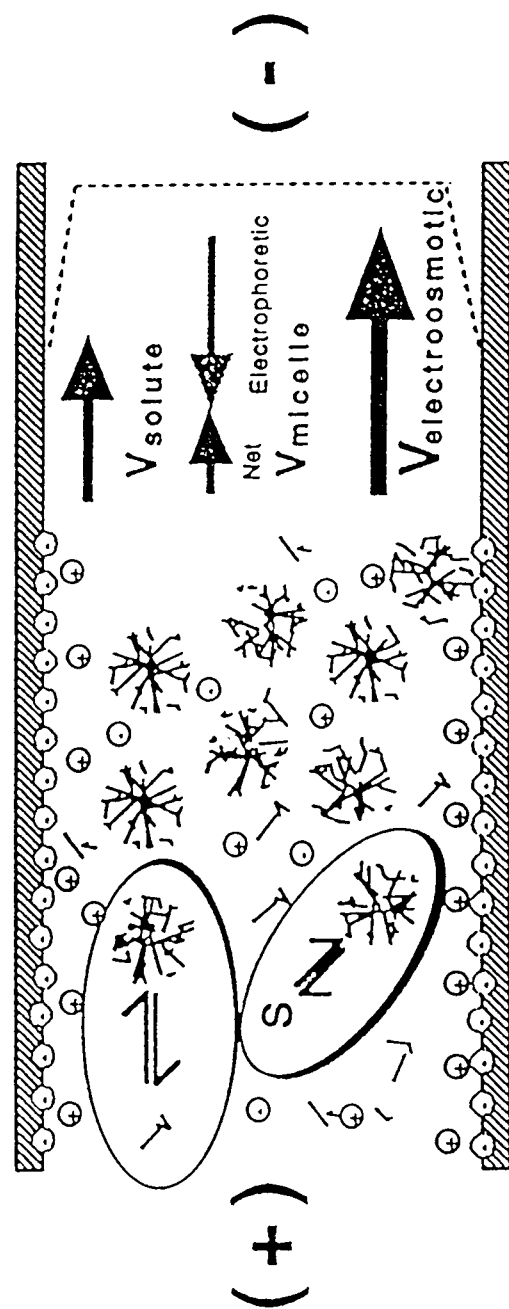


Figure 8. Depiction of flow dynamics in MECC showing velocity vectors for electroosmotic flow ($v_{\text{electroosmotic}}$ or v_{eof}), micellar velocity [both its electrophoretic ($v_{\text{e,m}}$) and net (v_{M}) vectors], and solute velocity (v_{solute}). Also depicted are solute-micelle association and surfactant monomer-N-mer dynamics.

Figure 9). This limited elution range results in reduced peak capacity and places constraints on optimal retention characteristics.

Peak capacity in MECC is given by

$$n = 1 + \frac{\sqrt{N}}{4} \left(\ln \frac{t_0}{t_m} \right) \quad (5)$$

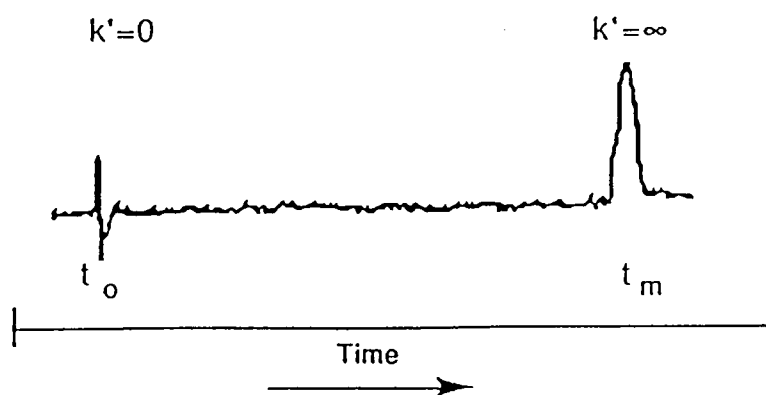


Figure 9. Illustration of elution window of MECC

where n is peak capacity, and N is the number of theoretical plates. Peak capacity (n) can be defined as the number of peaks resolved at any specified resolution level in a given separation time. Peak capacity thus increases with increases in either efficiency or elution window size. Capacity factors (k' 's) can be determined in MECC by using equation 6.

$$k' = \frac{t_R - t_0}{t_0 \left(1 - \frac{t_R}{t_m} \right)} \quad (6)$$

where t_R is the solute retention time. In this case,

$$k' = \frac{\text{amount of solute in running buffer}}{\text{amount of solute in micellar phase}}$$

Note that as t_m approaches infinity, equation 6 reduces to the conventional chromatographic expression for the capacity factor k' .

$$k' = \frac{(t_R - t_0)}{t_0} \quad (7)$$

Resolution between adjacent peaks in MECC is given by

$$R_s = \frac{N^{1/2}}{4} \left(\frac{\alpha - 1}{\alpha} \right) \left(\frac{k'_2}{1 + k'_2} \right) \left(\frac{1 - t_0/t_m}{1 + (t_0/t_m) k'_1} \right) \quad (8)$$

where selectivity (α) is given by k'_2/k'_1 . It is important to note that the last two terms in Equation 8 show a direct correlation between R_s and the size of the elution range. Terabe and coworkers showed that plots of R_s versus k' exhibit a maximum at an optimal value; optimum k' values depend on the size of the elution range and generally fall between 1 and 5 (26). Solutes with k' values greater than 5 begin to suffer losses in resolution. This is generally observed for very hydrophobic compounds, which tend to completely associate with the micelle and elute at or near the micelle migration time (25). The problem of coelution of hydrophobic solutes has been partially

alleviated by the addition of organic modifiers (27), mixed micellar compositions (28) and the addition of cyclodextrins (29).

Modification of Electroosmotic Flow

Organic modifiers have two important effects on the elution range of the MECC separation. First, organic modifiers (such as 2-propanol) extend the elution window, thereby increasing peak capacity. This observation is presumably due to the decrease in EOF which stems from increased interactions between the solvent and SiOH groups on the capillary walls. The decreased EOF allows the micelles to migrate or "swim upstream" more effectively, thereby increasing the elution window. Second, k' values are lowered due to the decreased polarity of the mixed aqueous-organic mobile phase. (In comparison to the pure water case, partition coefficients are altered, and solutes therefore spend more time in the mobile phase.) Since resolution is maximized for solutes possessing k 's of 1-5 (26), this approach can be very effective in separating hydrophobic analytes. Large amounts (>15%) of organic modifier, however, can increase CMC values and, under these conditions, micelle polydispersity can result in poor efficiencies. (See Reference 25 for a detailed discussion of efficiencies in MECC.) Thus, the adverse effects of micelle polydispersity set a limit to which organic modifiers may be effectively employed in MECC. In addition to degraded efficiency, analysis time can also become excessive (30,31,32).

In situations where resolution and efficiency are not pronounced problems, parameters such as overall column length and applied field can be manipulated to achieve rapid separations. The work presented herein focuses on the optimization of such conditions in order to achieve well resolved components and rapid separations.

Theory of Laser Induced Fluorescence (LIF)

The choice of detection for the instrumentation used was Laser Induced Fluorescence (LIF). This method was chosen over conventional methods such as absorption spectrophotometry and mass spectroscopy on three premises: economic considerations, instrumental simplicity, and the inherent advantages associated with fluorescence. In particular, LIF detection is more sensitive than absorbance but costs less than MS.

Fluorescence detection is a widely used detection technique in CE due to the relative simplicity and high sensitivity attainable. Fluorescence intensity, I_f , is approximated by

$$I_f = I_0 \Phi k \epsilon b C \quad (9)$$

where I_0 is the excitation intensity, Φ the fluorescence quantum yield of the analyte, k an instrumental term which depends on such factors as detector efficiency, fluorescence transmission and collection efficiency, ϵ the molar absorptivity, b the path length, and C the concentration of the analyte. Equation 9 is valid for small values ($\leq$

0.05). From Equation 9 a direct relationship of I_o to I_f can be observed. Maximizing I_f can easily be accomplished by using a laser as the excitation source. I_f must be maximized by optimizing I_o , since Φ , ϵ , b and C values are dictated by the analytes of interest and the required limits of detection. Some advantages of using lasers as excitation sources are:

- (1) Lasers are collimated and can be easily focused through small diameter capillaries
- (2) High photon flux / High excitation intensity
- (3) Laser output is monochromatic
- (4) Laser technology has matured to the point of reasonable cost and ease of operation

Unfortunately, a primary limitation of fluorescence detection is that relatively few molecules exhibit native fluorescence. This did not apply, however, since the analytes of interest in this work had fluorescent properties. Fluorescence detection also provided a unique means of selectivity in which the fluorophoric analytes could be distinguished from their corresponding biological matrix. LIF detection was performed on column and the apparatus is depicted in Figure 10.

ELP Capillary Discussion

As mentioned in the introduction, ELP capillaries show promise for increased sensitivity in comparison to conventional uniform bore

capillaries. (A comparison of a conventional capillary column to an ELP capillary can be seen in Figure 1).

Applications of capillaries with unique geometries have been previously reported. In 1990, Yeung examined nonuniform capillary geometries similar to ELP capillaries and determined that sensitivity was enhanced (absorbance) and band broadening mechanisms were minute (33). Yeung further stated that flow perturbations could be minimized by creating a bubble region such that any geometry change would be as gradual as possible (33). The advent of commercially available ELP capillaries (Hewlett-Packard), with advanced cell design, has satisfied this condition.

A second advantage of using ELP capillaries over conventional capillary geometries is a simplification of alignment. Namely, the ELP capillary simplifies procedures to reject capillary wall scatter, which makes possible an increase in the signal/background transmission ratio. A detailed description of the alignment procedure is provided later.

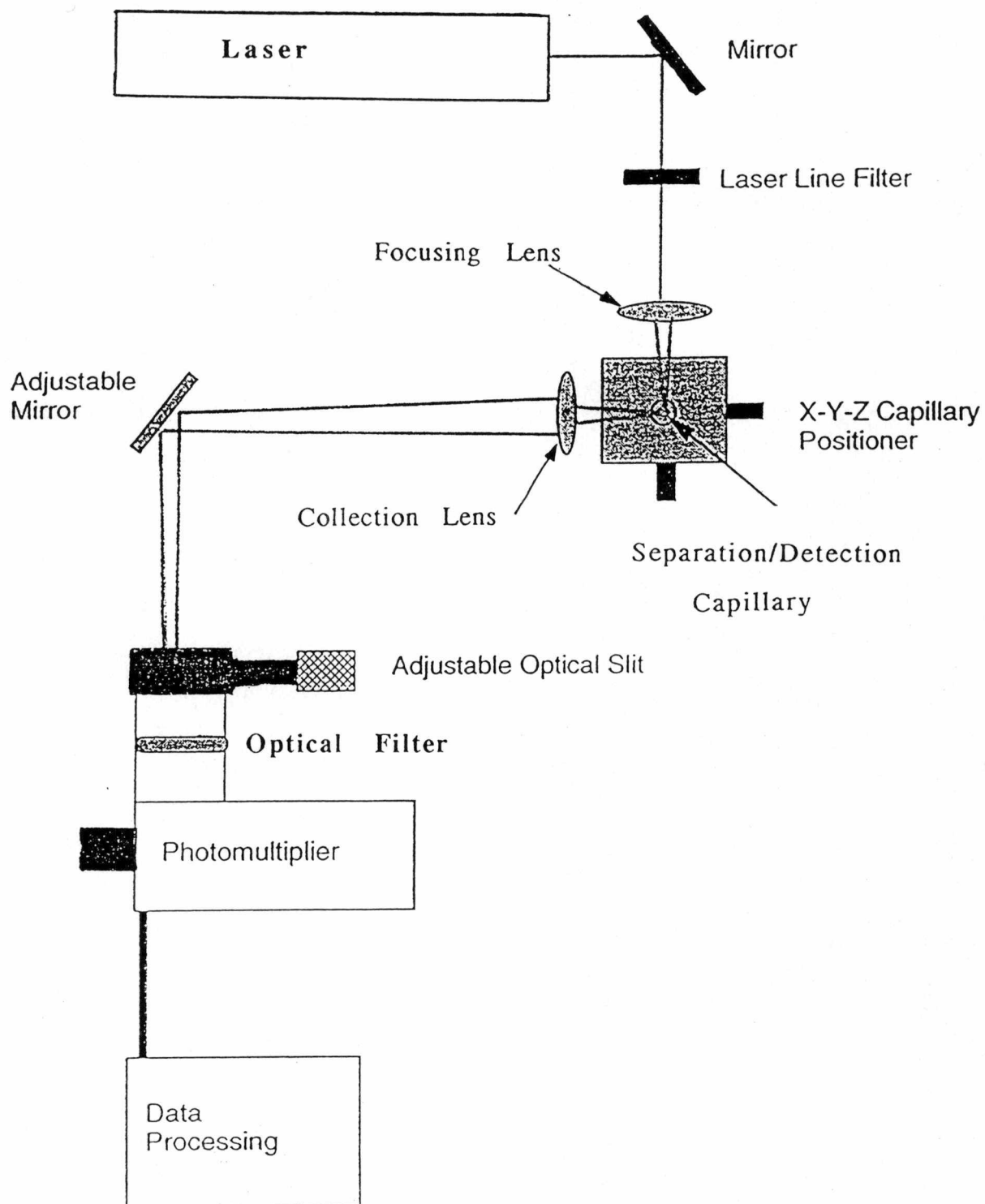


Figure 10. LIF detection system

Chapter 3

Experimental Section

Apparatus

A schematic diagram of the optical layout is shown in Figure 10. Fluorescence excitation was provided by an OmniChrome (Chino, CA) Helium-Cadmium laser, 20 mW, 325 nm, model 3074-20M (used for xenobiotic materials and DNS-Phenylalanine) or a Spectra Physics (Mountain View, CA) Argon ion laser, 15 mW, 488 nm, model 162A (used for sodium fluorescein). A 25 mm diameter, f/1 quartz lens was used to focus the laser radiation through the capillary. Capillaries were immobilized in an aluminum chuck with epoxy resin and were mounted in an X-Y-Z fiber optic positioner (See Figure 11). A similar lens, arranged perpendicularly to the capillary, was used to collect and focus the resulting fluorescence emission. Isolation and spatial filtering of the far-field, fluorescence image was accomplished by varying the opening (100 μm to 3.2 mm) of an adjustable optical slit (Edmund Scientific, Barrington, NJ). The isolated image then passed through appropriate band pass filters to minimize the Rayleigh and Raman scattering contributions to the background. Table 1 contains a listing of the filters used in all the experiments.

The filtered image then impacted on an RP-1 type photomultiplier tube (Hamamatsu Corp., Bridgewater, NJ). The

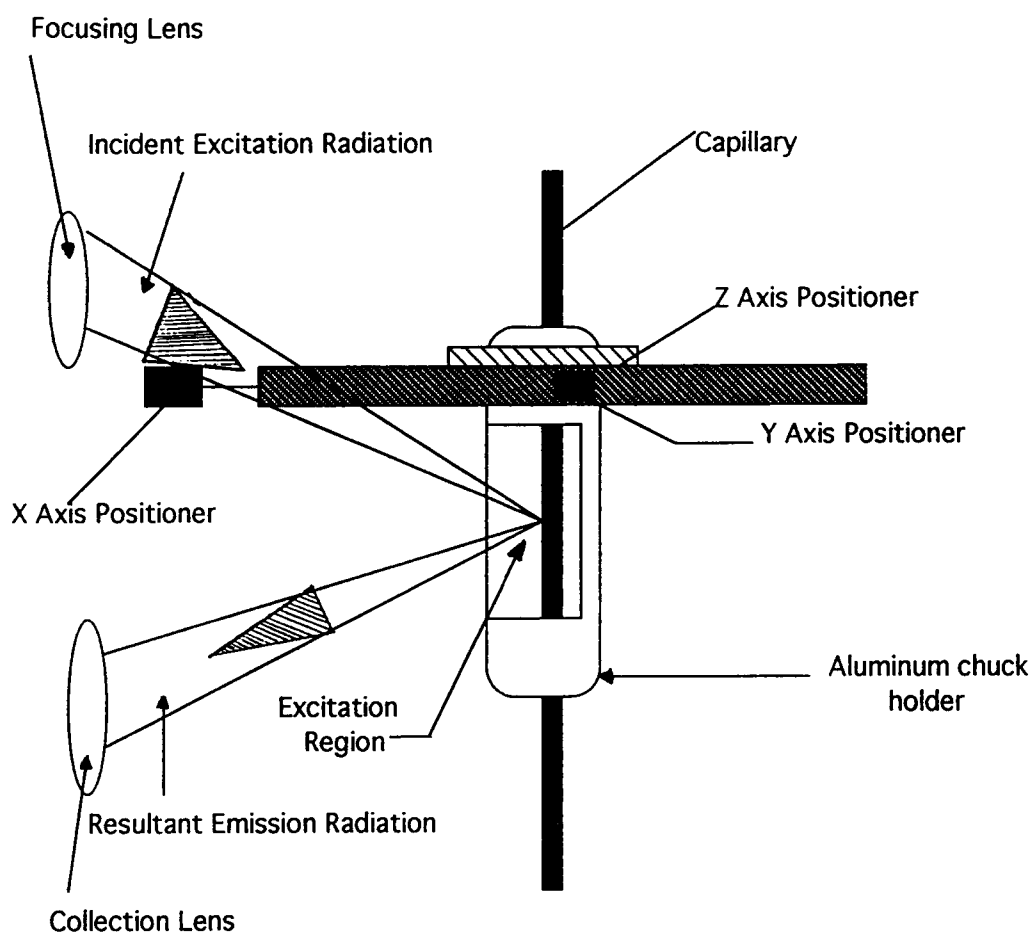


Figure 11. Side view of excitation region of LIF system

Table 1. Summary of emission filters used

Analyte used in experiment	Type of emission filter used	Comments
7-hydroxycoumarin 7-sulfate CP-88,059	Bandpass	FWHM 40 nm @ 460 nm
7-ethoxycoumarin 7-glucuronide	Bandpass	FWHM 40 nm @ 390 nm
Sodium Fluorescein	Cut-on	50% Transmission @ 520 nm
DNS-Phenylalanine	Cut-on	50% Transmission @ 340 nm

adjustable optical slit and PMT assembly was mounted to a translational stage (Oriel, CA) that had a total translation distance of 10 mm. This arrangement was designed to ease alignment procedures (discussed later). The PMT photocurrent was monitored by a Pacific Precision Instruments (Concord, CA) Model 126 Photometer. The photometer was operated with a 0.1 second output time constant. The processed photocurrent was displayed and collected with a PE Nelson model 1020 integrator (Norwalk, Connecticut).

Separation System

A Hipotronics (Brewster, NY) Model 840A high voltage power supply was used to apply electrophoretic fields. Injections were performed hydrostatically by placing the inlet of the capillary into a sample vial at a height difference of +10 cm for 10 seconds. After injecting the sample, the inlet was replaced into the buffer reservoir and an approximate field strength of +370 V/cm was applied across a 40-50 cm long, 30-40 cm effective length, silica ELP or silica conventional capillary. Precautions were taken to prevent cross contamination of the buffer and sample reservoirs.

Separation Capillaries

Conventional 50 μm i.d., as well as 25 μm and 50 μm i. d. ELP capillaries, were obtained from Hewlett-Packard (Wilmington, DE). Conventional 25 μm i.d. capillaries were purchased from Polymicro

Technologies (Phoenix, AZ). Capillaries were cut to length with a sapphire scoring tool and cemented into an aluminum chuck holder. This aluminum chuck holder was 40 mm long and 6 mm in diameter (See Figure 11). A 0.5 mm hole transversed the center of the entire length. A cut was made in the center of the device so that excitation radiation could be focused on exposed capillary surfaces (See Figure 11). Prior to optical alignment or test compound injection, each capillary was rinsed for approximately 10 minutes with 0.1 M NaOH followed by a 5 minute rinse with 0.01 M sodium phosphate buffer (pH 9).

Separation Conditions

Separation runs for the capillary performance studies were carried out via CE for the sodium fluorescein study and MECC mode for CP-88,059, 7-EC and metabolite separations. Running buffer compositions for all CE experiments were 0.01 M sodium phosphate dibasic and 0.006 M sodium borate at pH 9. Running buffers containing either 0.05 M sodium dodecyl sulfate (SDS) or 0.05 M sodium cholate were used for all MECC separations. Exact mobile phase compositions for the CP-88,059 studies are specified later in this thesis.

Chemicals and Procedures

Chemicals

Sodium dodecyl sulfate (SDS), Sodium Cholate (NaC), Sodium Phosphate Dibasic, Sodium Fluorescein and DNS-Phenylalanine were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A). All solvents were HPLC grade and were obtained from Mallinckrodt. Water was purified with a Milli-Q system (Millipore, Marlborough, MA, USA) and had a resistivity of 18.2 ohm/cm at the outlet.

Preparation of Liver Slice Incubates

Biological matrix samples were prepared by Pfizer Pharmaceuticals and required only a dilution of 1:1 with mobile phase before analysis. The detailed procedure that Pfizer Pharmaceuticals, Groton, Connecticut used began with the precision-cut liver slices that were prepared as described by Barr *et al.* (35). Human liver tissue was obtained from the International Institution for the Advancement of Medicine (Exton, PA). Tissue cores were sliced to 10 - 20 mg wet weight with a modified Krumdieck slicer (36). Slices were pre-incubated in Krebs-Henseleit buffer for 90 minutes at 37°C in a roller incubator prior to incubation with 7-EC. Slices were incubated for four to six hours with 100 μ M 7-EC at 37°C prior to reaction termination and analysis. Samples were stored in a conventional freezer at 0°C. Samples were diluted 1:1 with mobile phase before CE analysis.

CP-88,059 Antipsychotic Drug Preparation

Both CP-88,059 and the internal standard were obtained from Pfizer Pharmaceuticals, Groton Connecticut. Biological matrix samples were prepared by Pfizer Pharmaceuticals and required only a dilution of 1:1 with mobile phase before analysis. Standard solutions of both CP-88,059 and the internal standard were made by dissolving in 50:50 v/v % ACN/H₂O. These standard solutions were used in the preliminary spectroscopic and fluorescence work, as well as the experiments to determine optimal separation conditions. Biological matrices containing these analytes were sent directly from Pfizer. These matrices were composed of liver slice incubates, as stated in the above preparation for the 7-EC metabolite study.

Spectroscopic Measurements

Preliminary absorbance and fluorescence data were obtained on standard solutions using a Hewlett-Packard (Palo Alto, CA, USA) Model 8452A Diode Array Spectrophotometer and an Aminco Bowman Series 2 Luminescence Fluorimeter (Urbana, Illinois) interfaced to IBM compatible computers.

Alignment Procedure

Alignment of capillaries was performed with a dual lens detection system, incorporating an X-Y-Z capillary cell holder, two adjustable mirrors, and an adjustable optical slit mounted to a photomultiplier tube on a translational stage (See Figures 10 and 11).

First, the excitation optics were aligned. The laser beam was passed thorough the center of the excitation $f/1$ lens and its position relative to the capillary adjusted to provide a clean focus through the inner channel of the capillary column. Clean focus was determined by placing a white card in the far field and observing a crisp oval-shaped image. The second step in the procedure involves aligning the emission optics. The capillary is filled with a (visible) fluorescent dye and the colored image positioned. In our optical arrangement, the emission lens was adjusted to provide approximately a 20-fold magnification (M) of the image emanating from the dye in the capillary. Image magnification was used to enlarge the far-field image in order simplify the process of rejection of specular scatter. Positioning of the emission lens is determined by using equations 10 and 11.

$$1/f = 1/S_i + 1/S_o \quad (10)$$

$$M = S_i / S_o \quad (11)$$

where f , S_i and S_o are the focal length of the lens, image distance, and object distance, respectively. Object distance describes the position of the collection lens to that of the excitation region of the capillary. Since the value of the focal length of the collection lens is 2.54 cm, only the positioning of that lens (hence S_o) influences S_i . In this work, the distance between the collection lens and the capillary is

slightly greater than f (i.e. 2.55 cm), resulting in a S_i value of approximately 51 cm. Input of the above values of S_i and S_o reveals an approximately 20X magnification of the far field image.

The slit/PMT assembly is positioned in the center of this magnified image. At this point, the capillary is flushed with mobile phase and the alignment fine tuned by observing specular scatter at the capillary walls. This step can be easily understood with respect to the structure of the magnified scatter image. In the case of a 365 μm o. d./50 μm i. d. capillary, the far field image is composed of 4 "spots" distributed over about 7 mm. The "spots" occur as a result of scatter from the inner and outer capillary walls. Proper adjustment of slit position and width results in efficient rejection of specular scatter and transmission of signal. Slit translation experiments followed the above procedure with the exception that the slit/PMT assembly was incrementally translated across the magnified image while maintaining a fixed narrow slit width.

Chapter 4

Results and Discussion

Experiment I. Comparison of Extended Light Path Capillaries (ELP) to Conventional Capillaries

System Alignment

In addition to improving sensitivity, the increased capillary diameter at the detection zone provides a larger "target" when attempting to focus the excitation beam cleanly through the capillary. In this experiment, a Helium-Cadmium laser (325 nm wavelength) and a 350 nm cut-on optical filter were incorporated into the fluorescence detection apparatus shown in Figure 10. Conventional and ELP 50 μm i.d. / 365 μm o.d. capillaries were used. Detector response in this study is largely due to specular scatter that passes through the slit and emission filter. In these investigations, the ELP capillaries were found to be a great deal easier to align than conventional capillaries. The increased size of the ELP flow cell image allows the use of a larger optical slit width while maintaining efficient rejection of optical background. To illustrate this point, background signal levels were mapped relative to detector position (Figure 12).

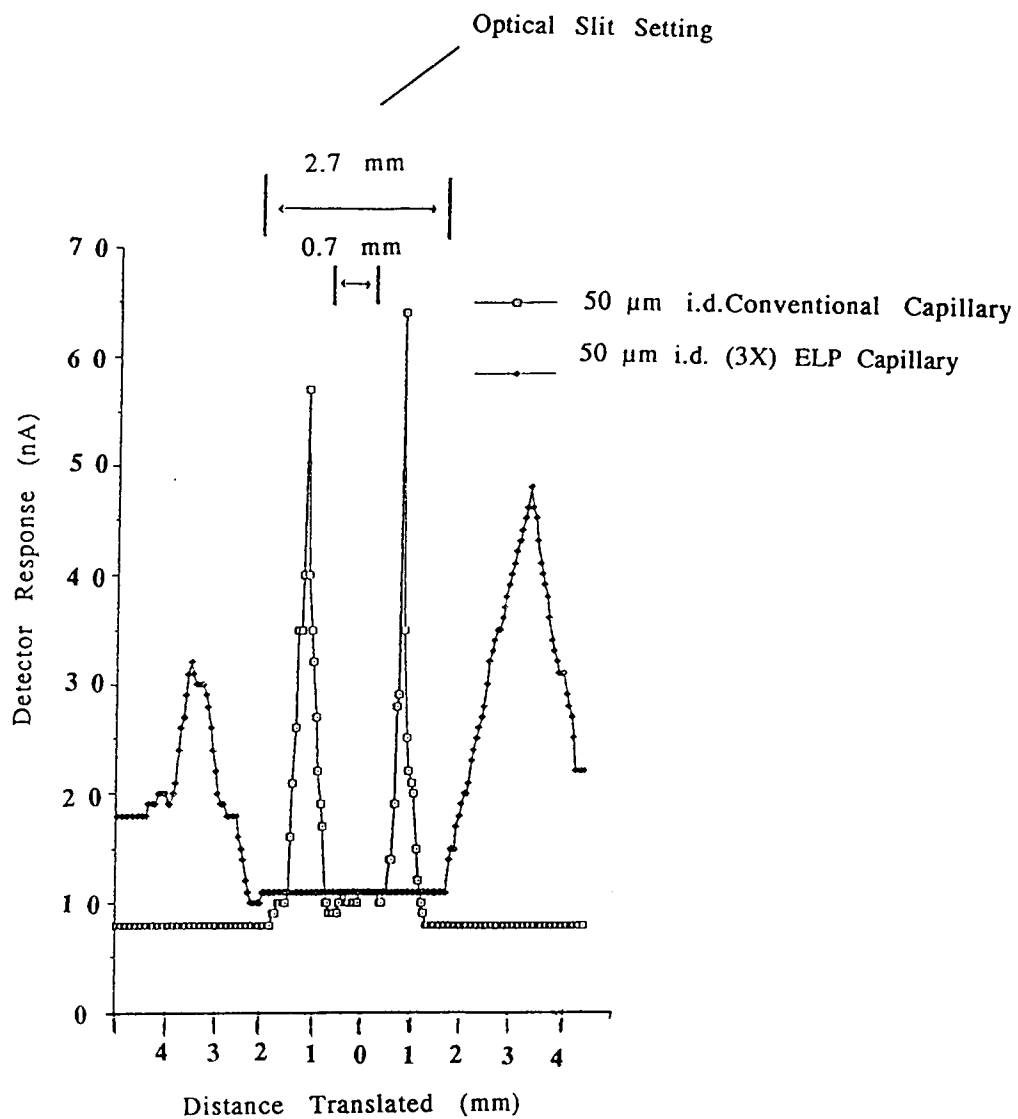


Figure 12. Capillary wall scatter diagram

This plot was obtained by translating the PMT assembly with a narrow slit (150 μm) across the far field image (See Experimental section). As evident from the plot, the conventional capillary scatter profile is 3-fold narrower than the ELP capillary. Vertical lines in the figure mark the slit positions used in the constant background experiment described below. Clearly, a larger percentage of the signal can be collected while maintaining efficient background rejection with the ELP. This observation, however, will be exaggerated at excitation wavelengths in the deeper ultra-violet (UV) region (i.e. $\lambda < 300 \text{ nm}$). This is due to the native fluorescence emitted by the fused-silica capillaries at such excitation wavelengths. Therefore, as more powerful, deeper UV excitation sources become available, ELP capillary applications are expected to increase.

The wider "target" provided by the ELP capillary allows the use of simplified instrumentation. Complex optical configurations consisting of microscope objectives and CCD imaging cameras are not required to achieve clean transmission through the cell or for efficient and selective collection of emission signal. Due to the relaxed instrumentation and alignment constraints, it is expected that use of ELP cells will add a significant degree of ruggedness to this type of LIF configuration. However, care must be exercised in centering the excitation beam on the bubble.

Comparison of Capillary Performance

Optimization of LIF detection sensitivity requires maximum collection of signal coupled with careful rejection of scattered light and background fluorescence. Capillaries of conventional dimensions require relatively sophisticated focusing/collection configurations and efficient rejection of capillary wall fluorescence to achieve high sensitivity (2,14). Since an important goal of these studies was to avoid overly complex instrumentation, ELP and conventional capillary formats were compared using relatively simple optics and an adjustable slit. Use of the adjustable slit was of fundamental importance in these experiments in that it provided variable light rejection and the ability to accurately set slit widths.

A comparison was made of the conventional and ELP capillaries with the optical slit adjusted to positions which yield equivalent background signals (no fluorophore in the detection zone). After alignment of each capillary, the slit width was increased to a distance which gave a background signal value of approximately 44 mV (i.e. 44 nA photocurrent on the 1000 nA scale of the photometer). Figure 13 shows electropherograms of DNS-Phenylalanine obtained for each capillary under these conditions. The ELP signal (peak height) was a factor of approximately seven greater relative to the conventional capillary. The insert in Figure 13 summarizes results for this series of experiments. The difference in slit widths (a factor of 3.8) is close to 3-fold as expected from increased capillary dimensions. However, the observed gain in signal exceeds the expected value (3.8X)

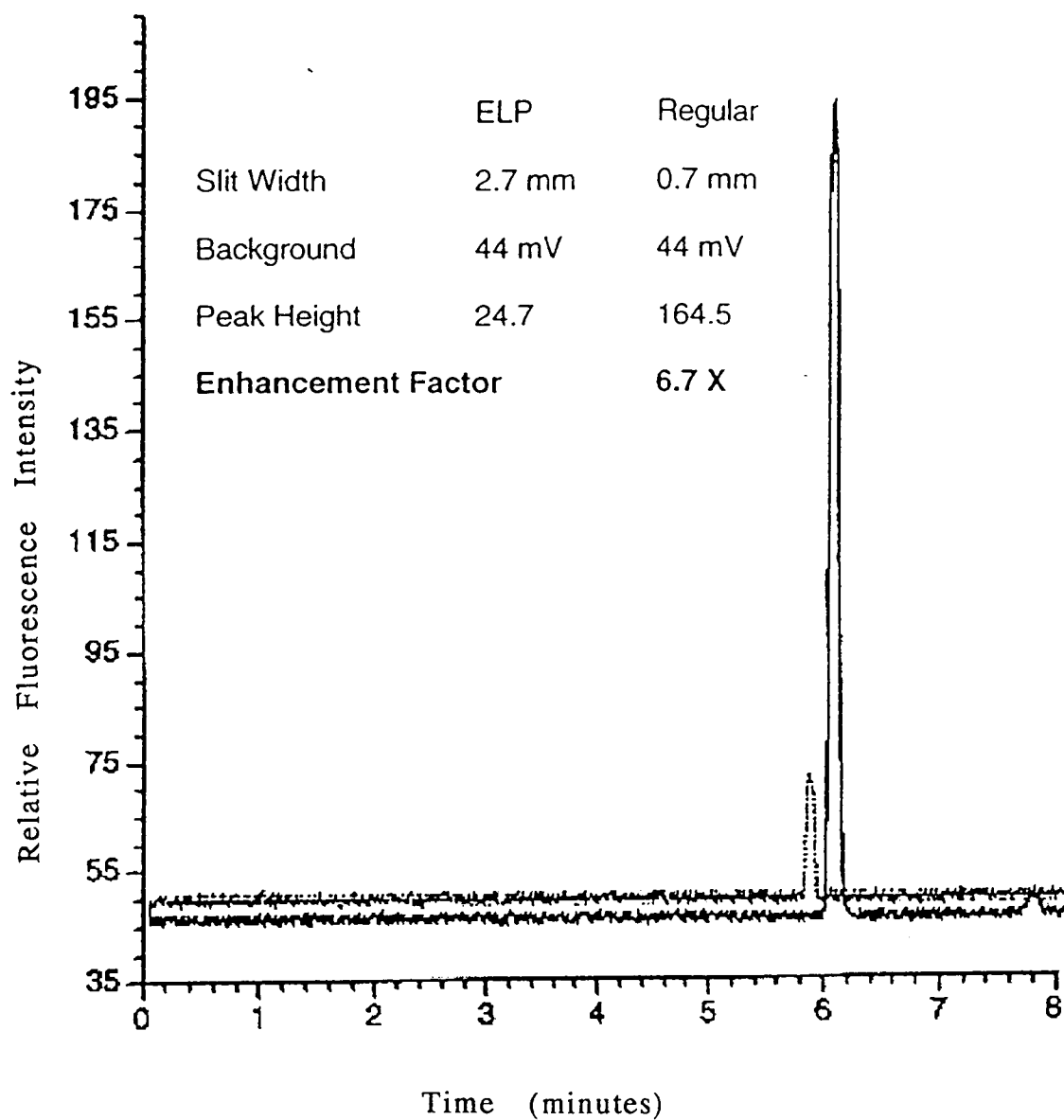


Figure 13. Electropherograms of Capillary Comparison Study of DNS-Phenylalanine

stemming from increased path length. Actually, since the ELP resulted in roughly a factor of 2 reduction in efficiency ($N = 15,000$ versus 30,000 for the conventional capillary), a less than 3.8 fold increase in peak height was expected. The following factors could contribute to the increased enhancement. First, the simple dependence of fluorescence signal on path length assumes a uniform path length for the entire cross section of the excitation beam. In this work, the focused laser beam spot size (estimated to be 30-40 μm) is appreciable relative to the regular capillary diameter, thereby diminishing its effective path length (*i. e.* moving radially from its center, the laser beam intersects the capillary channel over diminishing distances; hence the average path length is less than the capillary diameter). Second, centering of the emission slit relative to the far field signal image is also more difficult to accomplish with the smaller (conventional) capillary so light transmission is less than optimal. Finally, the focusing effects provided by the curvatures of the outer and inner surfaces of the capillaries are expected to influence beam intensity differently due to differences in curvature. In fact, the higher than expected signal enhancement with the ELP capillary underscores the simplicity of alignment.

To assess these effects more carefully, calibration plots were generated for triplicate injections (5 concentrations spanning the limit of detection (LOD) to 5×10^{-7} M) of sodium fluorescein by CE. These plots reflect peak height versus concentration injected. Under carefully optimized conditions, the ELP capillary exhibited a 4.25-

fold improved LOD relative to the conventional case. Table 2 compares the analytical figures of merit for the two capillary formats based on these calibration plots. It is apparent from Table 2 that the slopes of the calibration plots, hence sensitivity, were enhanced approximately 3X in respect to the ELP over conventional column

Table 2. Summary of analytical figures of merit from
sodium fluorescein calibration plots

	50 μm regular	50 μm ELP
Slope	1.056	3.096
LOD (S/N 2)	$2.7 \times 10^{-10} \text{ M}$	$6.4 \times 10^{-11} \text{ M}$
Injected Amount (1 nL)	$2.7 \times 10^{-19} \text{ mole}$	$6.4 \times 10^{-20} \text{ mole}$

Conditions:

Analyte: Sodium Fluorescein, 50 μm i.d. capillaries, 37 cm
total length, 15 kV applied, λ_{ex} : 488 nm, λ_{em} 510 nm
bandpass filter, 10 mM Sodium Phosphate pH 6.0, Slit Width
constant @ 2.7 nm, Dynamic Range approximately 2.5
orders of magnitude in injected concentration

format. This observation is consistent with the increased path length of the ELP.

The use of capillaries with yet smaller dimensions (<50 μm) is attractive for application to rapid CE separations (9,12,19,20).

However, the alignment considerations outlined above become increasingly serious as capillary diameter is reduced. A 25 μm diameter capillary with a 5X expansion is commercially available and it exhibits similar advantages (see below).

Experiment II. Measurement of 7-ethoxycoumarin and Metabolites from in-vitro Preparations

The first pharmaceutical application using ELP capillaries involved using biological samples from a previous pharmacokinetic study of 7-ethoxycoumarin and 3 related metabolites. The goal of this application was to determine if higher sensitivity could be achieved with ELP capillary over conventional capillary.

The experiment involved incorporating a Helium Cadmium laser ($\lambda=325\text{ nm}$) and a 50 nm bandpass filter (65% transmittance centered at 420 nm) into the fluorescence detection apparatus shown in Figure 10. Conventional and ELP 25 μm i.d. / 365 μm o.d. capillaries were used. Liver slice incubate preparation is described in the experimental section. The concentrations of all four analytes in the two samples used were provided by Pfizer Pharmaceuticals.

Adequate resolution and analysis time were first optimized. This was accomplished with a sample containing all 4 analytes at a reported concentration of $5 \times 10^{-5}\text{ M}$. With a complete separation of all 4 metabolites achieved in under 5 minutes (running buffer containing 0.05 M SDS, 0.01 M dibasic sodium phosphate / 0.006 M sodium borate), a more dilute sample was assessed. This sample

was reported to have the following concentration of metabolites: 5.2×10^{-8} M 7-hydroxycoumarin (7-HC), 1.1×10^{-5} M 7-ethoxycoumarin (7-EC), 7-glucuronide (7-G) and 7-sulfate (7-S). Figure 14 shows an overlay of MECC electropherograms of liver slice media obtained by using ELP and conventional 25 μ m i.d./ 365 o.d. capillaries. Here, the ELP expansion is five-fold at the detection region. While all four major metabolites are known to be present in this sample, only 7-HC and 7-EC are detected. In this case, the conjugated metabolites could not be quantitated due to their presence at low levels and the fact that they have significantly different excitation/emission wavelengths. In the 7-HC and 7-EC case, there is a 5.0X and 2.8X increase, respectively, between ELP and conventional capillary formats. The observed 5X response for 7-HC correlates with the 5X theoretical value. However, the observed 2.8X increase for 7-EC does not. The difference in the observed response of the two analytes in the above statement is not understood. Future experiments will probe this phenomenon.

Clearly, the increased response observed in the ELP capillary provides sufficient enhancement to allow quantitation at lower levels, underscoring their utility in industrial bioanalysis. A second noteworthy observation is the absence of interferences in any of the samples.

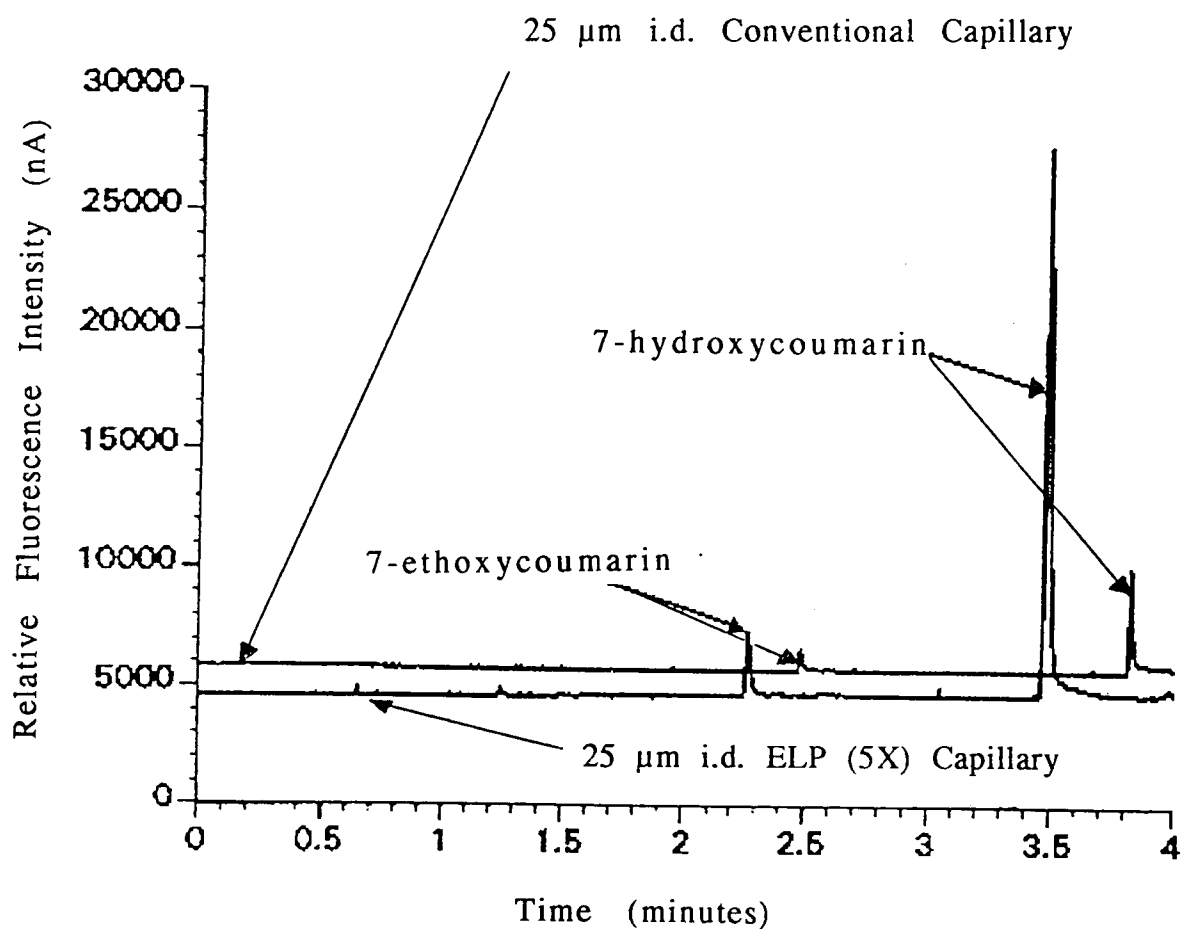


Figure 14. Electropherogram of 7-ethoxycoumarin Study showing sensitivity advantage of ELP capillary

EXPERIMENT III. Application of LIF separation system through the use of ELP capillaries for the measurement of Antipsychotic drug.

Optimization of Separation Conditions

A preliminary investigation of a second application of ELP capillaries involved the antipsychotic drug (CP-88,059) and an internal standard used in assays of the drug. As with the 7-HC metabolite application, the goal was to determine if higher sensitivity was possible with ELP capillaries over conventional capillaries. As mentioned in the introduction, previous limits of detection for CP-88,059 were 2.5×10^{-9} M. However, before the ELP capillary could be assessed, an applicable mobile phase, designed to mimic the HPLC C-18 stationary phase used previously (18), was needed.

The first stage of this experiment incorporated the use of MECC coupled with an absorbance detector to determine the best choice for the mobile phase to separate the drug and internal standard. Preliminary spectroscopic work revealed that the antipsychotic drug and internal standard (See Figure 3) had comparable molar absorptivities at a wavelength of 220 nm and both exhibited fluorescence maxima at approximately 400 nm. Various mobile phase compositions were prepared. Table 3 summarizes the mobile phase compositions that were employed. From the data in the table, the mobile phase consisting of 0.05 M SDS, 15% ACN, and 5% 2-propanol proved to have the shortest overall analysis time with

**Table 3. Summary of mobile phase compositions used
in the CP-88,059 study**

Mobile Phase Composition ***	Elution Range Beginning (t_0) (minutes)	Retention time of CP-80,599 (minutes)	Retention time of Internal Standard (minutes)	Resolution
0.05 M SDS	4.0	11.5	11.5	0.0
0.05 M SDS 15% ACN 10% 2-propanol	7.8	29.0	30.5	>5.0
0.05 M SDS 15% ACN 5% 2-propanol	6.5	23.5	24.3	1.1
0.05 M SDS 15% ACN 5% MeOH	5.0	30.0	30.0	0.0
0.025 M SDS 0.005 M HP γ -CD	3.3	8.4	8.4	0.0
0.025 M SDS 0.005 M HP- γ -CD 5% 2-propanol	3.2	8.3	8.3	0.0
0.025 M SDS 0.005 M γ -CD	3.3	9.0	9.0	0.0
0.045 M SDS 0.001 M γ -CD	3.2	8.8	8.8	0.0
0.045 M SDS 0.005 M γ -CD	3.4	9.0	9.0	0.0
0.045 M SDS 0.007 M γ -CD	3.3	9.0	9.0	0.0
0.025 M SDS 0.007 M β -CD 20% ACN	5.3	18.2	18.2	0.0
0.05 M NaC 5% 2-propanol	4.8	8.6	8.8	0.7
0.05 M NaC 20% 2-propanol	6.1	10.4	10.8	1.1

*** All solutions listed were made in 0.01 M Dibasic Sodium Phosphate / 0.006 M Sodium Borate (pH = 9)

adequate resolution. An argument could be made, however, that the sodium cholate (NaC) phase offers a comparable separation to that of the above and with a shorter total analysis time. The reasoning for not choosing the NaC mobile phase was that its native fluorescence would produce a background that would seriously diminish sensitivity when later applied to the LIF system.

Laser Induced Fluorescence Discussion

With the separation conditions optimized, the next step involved a study to probe the limits of detection of the antipsychotic drug on the established LIF system. A conventional capillary of dimensions 50 μm i.d/ 365 μm o.d was used in the same instrumental setup as mentioned previously. (Conventional capillary was used as a preliminary device to determine if the analytes of interest had a sufficient fluorescence efficiency to permit sensitive detection.) A series of standards of both CP-88,059 and internal standard (ranging from 1×10^{-4} to 1×10^{-9} M) were used to roughly determine the LOD.

The results of this preliminary work were unsatisfactory. An LOD was established for the conventional capillary at approximately 7.5×10^{-6} M (S/N @ 2). This high limit of detection seriously degraded the applicability of this analyte to CE/LIF. The inability to surpass previous limits of detection is believed to be the inability to overload the capillary column. As mentioned in the Introduction, Cole et al. (18) determined an LOD for CP-88,059 of approximately 2.5×10^{-9} M.

However, this determination however was based on sample loading of the analyte on the stationary phase of the C-18 HPLC column. Sample loading was accomplished by injecting a large volume of sample (as large as 500 μ l) whose solvent strength was much less than that of the mobile phase. In the absence of mobile phase, the injected analyte adhered to the bonded stationary phase at the head of the HPLC column. The sample was thereby "focused" or concentrated from occupying a large volume to a smaller one. This "focused" band of analyte was then eluted from the bonded stationary phase once mobile phase was introduced; that is, the concentration of the detection band was considerably greater than that injected. Since there is no such stationary phase in the capillary in CE, sample loading was not possible. An alternate sample concentration technique in CE, referred to as sample stacking (25), may be possible but was not attempted in this preliminary study. Future studies will be conducted to determine if sample stacking can be accomplished.

Although a conventional capillary was used in this preliminary study, extrapolation of the results to the 3X ELP column still pointed to a lack of sensitivity. The diminutive size of the ELP flow cell, (150 μ m) compared to the flow cell Fouda used (10 mm), also explains why sensitivity was diminished. Recent developments of even larger ELP flow cell capillaries, (a 5X "bubble" compared to the current 3X), may also increase sensitivity for this approach.

Clearly, there is more work to be performed if the applicability of CP-88,059 to ELP capillaries in CE/LIF is to be accomplished. Although the preliminary results of this application were not ideal, the above explanations provide avenues of future research to be pursued.

Chapter 5

Conclusions

The use of ELP capillaries for LIF detection in CE results in improved detection characteristics relative to conventional capillaries. Direct comparisons between the conventional and ELP capillaries shows a substantial improvement in sensitivity for the analytes studied in this work. This improvement is attributed to a combination of increased optical path length coupled with improved rejection of specular scatter. In addition to performance characteristics, the increased probe volume in the detector cell eases difficulties with system alignment. Incorporation of ELP capillaries into CE/LIF configurations can be expected to improve system ruggedness and precision. Ongoing improvements, such as larger ELP flow cell dimensions, also indicate the possibility of future research in UV laser based detection.

An application of ELP capillaries was assessed and found to surpass detection limits of previous methodology. Experimental observations were useful in revealing the potential of ELP capillaries for applications such as in pharmacological studies. In addition to applications involving CE, the ELP capillary format may prove useful for HPLC separations. Microbore and microcapillary separations have been interfaced to LIF instrumentation via fused silica capillaries (8), and suffer from similar limitations as outlined above for CE. Use of

ELP capillaries should theoretically improve detection characteristics in these techniques.

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Vita

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