DETECTION OF ENDOGENOUS COMPOUNDS IN BODY FLUIDS

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ABSTRACT

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Increasing efficiency demands on the clinical laboratory have created the need for more rapid and economical testing procedures. The development of sound microanalytical methods that can be readily automated and transferred to high-throughput formats are thus of interest to the clinical community. Advancements in capillary electrophoresis (CE) technology have created avenues for simultaneous analysis of multiple samples through the use of capillary array instrumentation. Although CE is recognized as a rapid and high-resolution separation technique, its use in the clinical setting has been limited due to inherent difficulties associated with real sample analyses (e.g., complex matrix interference, and poor analyte concentration detection sensitivity). As such, two instrumental/assay schemes based on CE and laser-induced fluorescence (LIF) detection for sensitive and specific analysis of compounds within biosamples were designed and characterized.

Ultraviolet (UV) excitation for LIF detection of underivatized compounds, termed laser-induced-native fluorescence (LINF), represents a simple, sensitive and selective method for detecting CE separated compounds that fluoresce appreciably when excited by UV radiation. A major drawback of CE-LINF systems, however, has been the expense and the complexity of the laser required for excitation in the deep UV wavelength range of 200-300 nm. To this end, the performance of a relatively inexpensive, low-power, pulsed KrF laser operating at 248 nm in a pseudo-continuous wave mode was evaluated as an excitation source for native fluorescence detection of tryptophan. On-column LINF detection limits in the low nanomolar range are obtained for tryptophan with the KrF

source, which is similar to that achieved by LINF detectors that use costly large frame frequency-doubled argon ion lasers. The developed CE-LINF instrument is further applied towards the selective profiling of tryptophan-containing proteins and peptides in human serum and saliva, and catecholamines in human urine illustrating the potential use of this system for diagnosing various disease states.

The progressive development of a robust immunoassay for the detection of specific antibodies from crude serum is also presented. Assay characterization is performed in three separate studies involving rabbit antisera to i) fluorescein ii) various neuropeptides and iii) HIV's p24 capsid protein. Off-line immunocapture/immunosubtraction (ICIS) by magnetic particles coupled to subsequent CE-LIF analysis is utilized to detect affinity interactions between solid-phase immobilized host antibodies and selected fluorescent antigens: i) fluorescein ii) fluorescent-labeled neuropeptides and iii) fluorescent-labeled peptides representing antigenic p24 sequences. Moreover, the ICIS-CE assay's capacity to measure relative antibody titer in the sub-nanomolar range and to function under multiplexed conditions is demonstrated. The obtained results suggest that the ICIS-CE assay could be an attractive alternative to the Western blot - an expensive and laborious technique presently being used as a confirmatory assay for the serological diagnosis of viral infections. A notable asset of the ICIS-CE technique that is anticipated to contribute to laboratory efficiency is its adaptability to full automation via the at-line interfacing of microtiter plate robotics and capillary array electrophoresis.

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DEDICATION

To My Mother and Father, Dorothy and Maurice

CONTRIBUTING AUTHORS

The LINF detector described in Chapter 2 was designed by Karen C. Waldron, Donald M. Paquette, Robert Sing and Peter R. Banks. This detector was constructed and characterized exclusively by Donald M. Paquette. Except for the investigation on the relationship between laser excitation pulse rate and fluorescence emission before and after low-pass filter conditioning (performed by Karen C. Waldron), all experiments presented in Chapters 2 were designed and executed by Donald M. Paquette. The manuscript representing Chapter 2 was written in part by Karen C. Waldron (Introduction, Results and Discussion, Conclusion), Peter R. Banks (Introduction), Robert Sing (Results and Discussion) and Donald M. Paquette (Experimental, Results and Discussion). All authors contributed to the editing of the manuscript.

The works presented in Chapters 3 through 5 are from instrumentation (LIF detector) and assay (ICIS-CE) protocols designed, constructed, and characterized by Donald M. Paquette. Manuscripts were written by Donald M. Paquette, and were co edited with Peter R. Banks (Chapters 3 through 5) and Cameron D. Skinner (Chapters 4 and 5).

The review article following Chapter 6 (i.e., Appendix A) was researched and written by both Donald M. Paquette and Michael D. Harvey, and was edited by Peter R. Banks.

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ACE	affinity capillary electrophoresis
AFU	arbitrary fluorescence unit
AGE	agarose gel electrophoresis
APTS	8-aminopyrene-1,3,6-trisulfonic acid
AU	absorbance unit
BODIPY-FL	4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid
CAE	cellulose acetate electrophoresis
CBQCA	3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde
CE	capillary electrophoresis
CGE	capillary gel electrophoresis
CHAPS	3-[(3-cholamidoptopyl)dimethylammono]1-propanesulfonate
CIEF	capillary isoelectric focusing
CITP	capillary isotachophoresis
CSF	cerebrospinal fluid
CW	continuous wave
CZE	capillary zone electrophoresis
DMF	*N,N*-dimethylformamide
ELISA	enzyme-linked immunosorbent assay
EOF	electroosmotic flow
GC	gas chromatography
5HIAA	5-hydroxyindole-3-acetic acid
HIV	human immunodeficiency virus
HPLC	high-performance liquid chromatography
HVA	homovanillic acid
ICIS	immunocapture/immunosubtraction

ID	internal diameter
IgG	immunoglobulin G
3IXS	3-indoxyl sulfate
LIF	laser-induced fluorescence
LINF	laser-induced native fluorescence
LOD	limit of detection
MEKC	micellar electrokinetic chromatography
MS	mass spectrometry
NDA	napthalene-2,3-dicarboxaldehyde
OD	outer diameter
PBS	phosphate buffer saline
PC	phosphate/casein
PMT	photomultiplier tube
SDS	sodium dodecyl sulfate
S/N	signal to noise
SPE	serum protein electrophoresis
UA	uric acid
VMA	vanillylmandelic acid
WB	Western blot

CHAPTER 1

Introduction: Capillary Electrophoresis and Clinical Applications of Capillary Electrophoresis

1.1 Separation Science in the Clinical Laboratory

Separation science is routinely applied in the clinical laboratory for the analysis of biomolecules endogenous to human body fluids. Perhaps the two most common separation techniques utilized by clinical chemists are high-performance liquid chromatography (HPLC) and slab gel electrophoresis. These techniques are used in the identification and quantification of clinically relevant analytes found in complex sample matrices such as serum, urine, cerebrospinal fluid, and amniotic fluid, to name a few. For example, HPLC-based methods are commonly employed to detect and measure urinary catecholamines, porphyrins, hydroxyproline and cortisol [1, 2]. Recent applications of HPLC for hemoglobin variant, vitamin, lipoprotein, glycoprotein, nitric oxide metabolite, DNA and RNA analysis have been reported which demonstrates the ongoing development of this technique as a clinical tool [3]. Separations based on electrophoresis are regularly applied in the clinical laboratory for the analysis of serum, urine, cerebrospinal fluid, hemoglobin, and isoenzymes. An example of the clinical application of electrophoresis is the separation of serum and urine proteins for the diagnosis of multiple myeloma, a malignant disorder distinguished by the presence of monoclonal protein in these fluids [1, 2]. Such analyses have traditionally been performed on porous support media such as cellulose acetate or agarose gel since they provide the appropriate electrophoretic conditions for resolution of the relevant analytes. Although HPLC and conventional electrophoresis methods have proven to be powerful clinical tools these techniques are hampered, in light of increasing laboratory cost-containment pressures, by high reagent and labor expenditures and/or poor turnaround times.

The advent of free-solution microbore electrophoresis in the early 1980's provided chemists with a new means to perform analytical separations [4-6]. Several features of this separation method, called capillary electrophoresis (CE), were responsible for an ensuing decade of explosive development. Perhaps the most important feature was that highly

efficient micro scale electrophoretic separations could be performed with the quantitative precision and instrumental control of HPLC. By the early 1990's several CE instruments were commercially available. These instruments helped to demonstrate the benefits, relative to other separation techniques, of performing analysis by automated CE. In general, these benefits included shortened separation times, improved resolution and reduced labor and reagent costs. As such, CE has since received increasing attention from clinical chemists. Although still in its infancy as a clinical tool, CE has begun to replace less efficient diagnostic methodologies and it is presently being incorporated into many developing diagnostic protocols [7].

The work presented in Chapters 2 to 5 represent research which serves to further demonstrate the practicality of CE for clinical sample analysis. The following three sections provide the reader with a brief introduction to CE and the pertinent modes of operation used in the research. An overview of modern clinical CE is then presented which is intended to help bring the performed research into perspective.

1.2 Capillary Electrophoresis

Electrophoresis in capillary tubes offers rapid and high-resolution separations of ionic and nonionic species. Its broad spectrum of applications includes the separation of inorganic ions, organic acids, chiral compounds, amino acids, proteins and nucleic acids [8]. The separation of such analytes, in capillaries which are typically no longer than 100 cm and with internal diameters of 25 - 75 μm, is normally complete within minutes and provides for the resolution of closely related compounds to be achieved. An impressive example is the separation of an ammonium sample comprised of different nitrogen isotopes (^{14}N and ^{15}N) within an 11 minute time span [9]. Additional assets, which stem from the micro scale dimensions of the capillaries, are small sample volume requirements and

sample volume consumption. In general, sample volumes on the order of μLs are sufficient, of which nanoliters are injected per analysis. Such attributes have proven useful in the realm of high-temporal resolution neurotransmitter monitoring where on-line rat brain microdialysate sample volumes of < 1 μL can be analyzed within a 20 second time spans [10]. For these reasons, CE has become a powerful technique in modern separation science.

A schematic illustration of a typical CE system is presented in Figure 1.1. The instrument consists of two buffer filled reservoirs each having an immersed electrode. One electrode is connected to a high voltage power supply (0 to ±30 kV) while the other is held at ground. Electrical contact between the reservoirs is obtained by placing the ends of a buffer filled fused-silica capillary in each reservoir. Loading of a sample volume into the capillary is initiated by exchanging one of the buffer reservoirs with a reservoir containing the sample solution. An injection is then performed using either a short voltage pulse (electrokinetic injection), gravity flow (hydrodynamic injection), or a controlled applied pressure or suction. Following the injection procedure, the sample reservoir is replaced with a buffer-containing reservoir. Application of a potential via the high voltage electrode produces an electric field across the capillary and drives the separation of sample components. Detection of the separated species is performed at or near the end of the capillary depending on the detection system.

Two decades of CE instrumentation development based on the described configuration has provided analytical systems which offer operations such as automated sample injection from 96 well plates, fraction collection, on-board sample manipulation (e.g., dilution and internal standard spiking) and buffer replenishment. Additional features include multi-technique and specialized data handling software, liquid coolant thermoregulation of capillaries for efficient heat dissipation which provides for better separation efficiencies, sample tray temperature control to enhance analyte and reagent stability, and high pressure fluid delivery capabilities for rapid flushing of viscous buffer

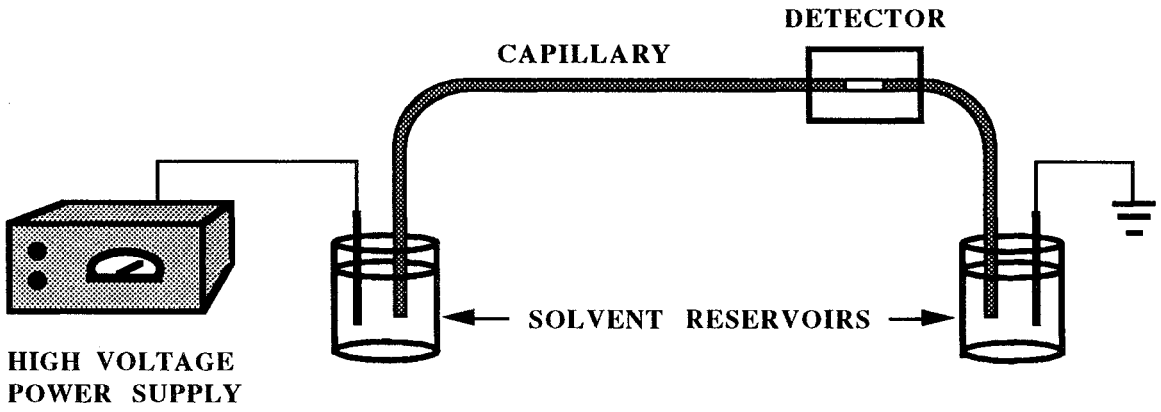

Figure 1.1 A generic capillary electrophoresis system.

systems and gels from the capillary. Instruments possessing arrays of capillaries have also been developed to improve the overall throughput of CE analyses [11-13].

The term "capillary electrophoresis" actually represents a family of techniques which includes capillary isotachophoresis (CITP), capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), micellar electrokinetic chromatography (MEKC), capillary isoelectric focusing (CIEF) and capillary electrochromatography (CEC). The theoretical principles governing all of these techniques have been well characterized and may be found in the literature [14-18]. Table 1.1 provides a reference to the utility of each CE technique with respect to biological sample analysis. The sections that follow focus on the fundamental descriptions of CZE and MEKC as these modes of CE are relevant to the research discussed in Chapters 2 to 5.

1.2.1 Capillary Zone Electrophoresis

Perhaps the most widely used mode of CE, due to its relative simplicity and versatility, is CZE. This utility stems from its capacity to efficiently separate a wide range of ionic species using simple buffer conditions. Zone electrophoresis is similar to most chromatographic techniques in that a small sample plug is introduced into a column and a separation is performed. Application of an electric field across the capillary causes the sample-plug solutes to differentially migrate in the supporting medium (usually aqueous) to produce discrete solute zones. The zones are produced since solute velocity (v_{ep}) at a particular electric field strength (E) is dependent on the solute's electrophoretic mobility (μ_{ep}) in that medium:

$$v_{ep} = \mu_{ep} E \qquad \textit{Equation 1.1}$$

Table 1.1 CE techniques typically used for the analysis of the indicated classes of biomolecules. Adapted in part from [15], with permission.

Peptides	Proteins	Nucleic Acids	Carbohydrates
CZE	CZE	CGE	CZE
CITP	CGE	MEKC	
MEKC	CIEF	CEC	
CIEF	CITP		
CGE			

Thus, CZE separations are based on differences in μ_{ep} values that are in turn determined by the charge (q) to Stokes radius (r) ratio of each solute in a particular medium of viscosity η:

$$\mu_{ep} = \frac{q}{6\pi\eta r} E \qquad \textit{Equation 1.2}$$

An additional factor that influences the migration velocity and direction of sample solutes in CZE is electroosmosis - the flow of solvent in a capillary when a longitudinal electric field is applied. Electroosmotic flow (EOF) is a consequence of counter ion interactions between ionic groups on the interior capillary wall and electrolytes in the solution filling the capillary. The charge on the wall of a capillary, which is typically excessively negative or positive, results from the presence of ionized capillary-wall surface, or surface adsorbed, species. To maintain charge balance, counter ions from the bulk solution stack at the surface of the capillary wall forming a diffuse double layer. Under an applied electric field, counter ions from the fringes of the double layer migrate towards the appropriate electrode. Since these counter ions are solvated, their movement draws along the bulk solution and thus a net flow of solvent towards that electrode occurs. A unique feature of EOF is the flat solvent flow profile, as opposed to the parabolic type flow created from an external pressure pump, which provides for low dispersion of solute zone during migration through the capillary. An additional benefit is that, when sufficient in magnitude, EOF causes all solutes (charged and neutral) to migrate in the same direction.

The EOF velocity (v_{eof}) exhibited by a particular capillary/solvent system is proportional to the strength of the applied electric field and the solvent's electroosmotic mobility (μ_{eof}) in that capillary. The μ_{eof} is in turn a function of the potential difference, called the zeta potential (ζ), which exists at the capillary wall as a result of the double layer, and the separation medium dielectric constant (ε) and viscosity:

$$v_{eof} = \mu_{eof} E = \frac{\varepsilon \zeta}{4\pi\eta} E \qquad \textit{Equation 1.3}$$

The net velocity displayed by sample solutes migrating through a capillary (v_{CZE}) during an electroseparation is therefore proportional to the magnitude of the electric field and the sum of that solute's electrophoretic mobility and existing electroosmotic mobility:

$$v_{CZE} = (\mu_{ep} + \mu_{eo})E \qquad \textit{Equation 1.4}$$

In general, separation selectivity in CZE can be achieved by altering the separation buffer pH and/or through the use of buffer additives such as surfactants or chiral selectors. Such adjustments influence either solute charge or ionic radius which therefore affects the electrophoretic mobility of that solute. Separation buffer parameters (i.e., pH, ionic strength, additives) can also be manipulated to change the magnitude and direction of the electroosmotic mobility. Manipulation of electroosmotic mobility ultimately impacts the migration time and resolution of solutes under investigation.

An additional factor that often needs to be addressed when optimizing CZE separation conditions for a particular type of solute is capillary wall adsorption. Solute wall interactions (i.e., ionic and/or hydrophobic) are deleterious to CZE separations since they can greatly reduce separation efficiency. The most commonly used measures to minimize solute wall adsorption processes include capillary wall surface group modification or operating in a buffer medium which masks effective surface charges (i.e., high ionic strength conditions) or which creates solute/wall repulsion [19, 20].

Relevant to the work presented in this writing is the CZE separation of proteins and peptides. The separation of such compounds is most conveniently, but not always successfully, performed in untreated-fused silica capillaries using high pH buffers (pH >

9). This approach is employed since a marked reduction in analyte wall adsorption is normally observed due to the promotion of electrostatic repulsion: a high pH medium ensures that the silanol groups (pK_a ~ 6.5) at the inner surface of the capillary are fully deprotonated (SiO^-) and that most proteins and peptides will have a net negative charge (given that the pH > pI of the analytes). The effects of working under high pH conditions in a silica capillary with respect to the relative direction and velocity of EOF and analyte migration is displayed in Figure 1.2. Interactions between the negatively charged capillary wall and solvent cations generate a cathodic EOF having a μ_{eof} that is usually superior in magnitude to the μ_{ep} of most analytes in terms of absolute values. As a result, small highly charged cationic analytes display the fastest velocities since they possess large electrophoretic mobilities in the same direction as the EOF. In turn, small highly charged anionic analytes migrate with the lowest velocity since their attraction to the anode significantly opposes, but does not surpass, the EOF. Unlike charged analytes, uncharged analytes are not separated from one another but are nonetheless transported by the EOF through the capillary at a migration velocity that is intermediate to that of cationic and anionic analytes. The application of a positive potential therefore results in the movement of all analytes in the direction of the negative electrode. For this reason, sample injections are done at the anodic end of the capillary with detection being performed at the cathodic end of the capillary.

1.2.2 Micellar Electrokinetic Chromatography

As explained in the previous section, CZE separation of charged analytes in simple buffer systems is made possible due to differences in the electrophoretic mobility of ionic solutes in an electric field. Neutral solutes are not efficiently separated by CZE however, since such species exhibit no electrophoretic mobility. Fortunately, the separation of

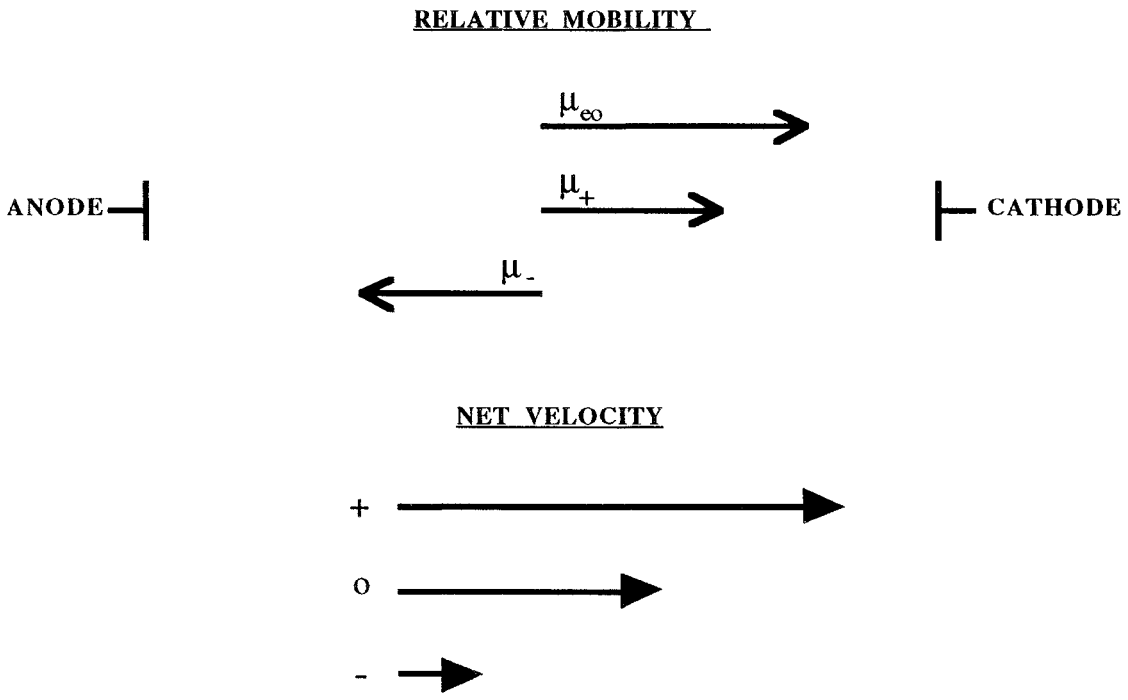

Figure 1.2 Vector illustrations of relative electroosmotic mobility and electrophoretic mobility of similar sized, but oppositely charged, ions in a fused-silica capillary under alkaline buffer conditions. The net migration order for cations (+), neutrals (o), and anions (-) towards the cathode is shown at the bottom of the diagram.

charged and uncharged analytes can be accomplished with the simplicity of a CZE-type approach by incorporating an appropriate amount of ionic surfactant within the operating buffer. The resulting separation mechanism, first described by Terabe *et al.* in 1984 [21], forms the basis of a distinct mode of CE called micellar electrokinetic chromatography, or MEKC.

The separation of neutral solutes exhibited by MEKC stems mainly from the differential partitioning of these compounds between a hydrophobic pseudo-stationary phase and a highly mobile hydrophilic phase (i.e., the aqueous portion of the run buffer). The hydrophobic phase in this technique is represented by the nonpolar microenvironment within spherical-like surfactant aggregates called micelles. Micelles are the result of surfactant hydrophobic tail-in/polar head group-out organization that spontaneously forms in aqueous solutions when the concentration of that surfactant exceeds its critical micelle concentration (CMC). A wide variety of charged and neutral surfactants have been employed for MEKC applications, including: sodium dodecyl sulfate and cholic acid (anionic), dodecyl and cetyltrimethylammonium bromide (cationic), Brij35 and Triton X-100 (nonionic), and CHAPS and *N*-dodecylsultaine (zwitterionic). The most frequently used surfactant in MEKC is sodium dodecyl sulfate, or SDS (CMC $\approx$ 8 mM). The electrophoretic behaviour exhibited by SDS micelles in a buffer-filled capillary (pH > 7) under an applied positive potential is depicted schematically in Figure 1.3. Due to their negatively charged surface, SDS micelles possess considerable electrophoretic mobility towards the anodic end of the capillary. This is however, counteracted by a large EOF mobility in the opposite direction. The net result is a slow migration of SDS micelles towards the cathodic end of the capillary. The migration velocity of a neutral solute away from the anode is dependent on the distribution coefficient of that compound between the micellar and aqueous phase. Thus, a neutral solute's migration time (t_r) generally lies within a window period (i.e., between t_o and t_{mc}) where t_o is the migration time of a hydrophilic solute that does not interact with the micellar and t_{mc} is the migration time of a hydrophobic

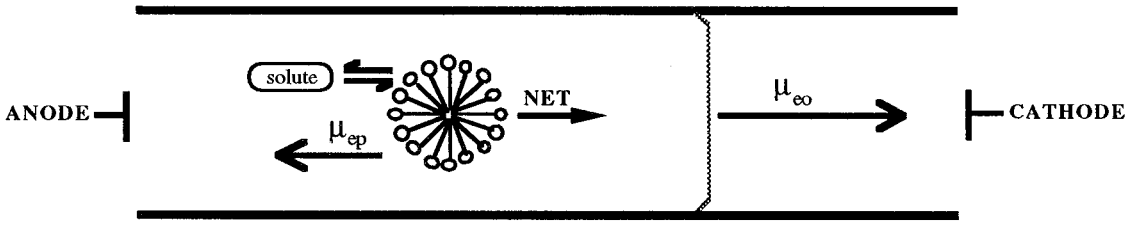

Figure 1.3 Electrophoretic mobility and net migration of SDS micelles in an untreated fused-silica capillary under alkaline buffer conditions. Neutral solute migration is dependent on residency equilibrium between the micellar and non-micellar environments.

solute that is completely retained by the micelles (see Figure 1.4).

Despite the fact that MEKC was originally developed for neutral solutes, this technique can also be applied to samples containing both neutral and charged solutes [14, 22]. In effect, MEKC can even provide enhanced separations of ionic species. The mechanism involved, in this case, is a function of both hydrophobic and ionic interactions. Unless counter balanced by strong hydrophobic interactions, solutes having the same charge as the surfactant head-group experience electrostatic repulsion and thus interact weakly with the micelles. Solutes with a charge opposite to the surfactant head-group exhibit electrostatic association and, in turn, tend to readily interact with the micellar phase. An extensive mathematical description of neutral and ionic solute MEKC separations based conventional chromatography theory exists [22], but is beyond the scope of this text. In light of the work presented in this thesis, a migration order of anions, neutrals and cations (respectively) may be used as a general reference since all MEKC separations were conducted under SDS micellar-phase conditions.

1.2.3 Clinical Capillary Electrophoresis

CE is effective in the analysis of biomolecules found within real sample matrices as attested by the numerous recent reviews on the matter [8, 23-25]. The utility of CE as an analytical tool for disease diagnosis has experienced a progressive, albeit reserved, acceptance in the clinical laboratory. In general, conventional methodologies remain favored by clinicians with respect to assay development or routine diagnostic testing unless such approaches are determined to be sufficiently inferior to a CE-based approach. Indeed, routine clinical CE has been most notably established for applications where distinctive advantages over traditional slab-gel techniques have been demonstrated. Beneficial features such as on-line detection, automated gel replacement, rapid analysis time and capillary array

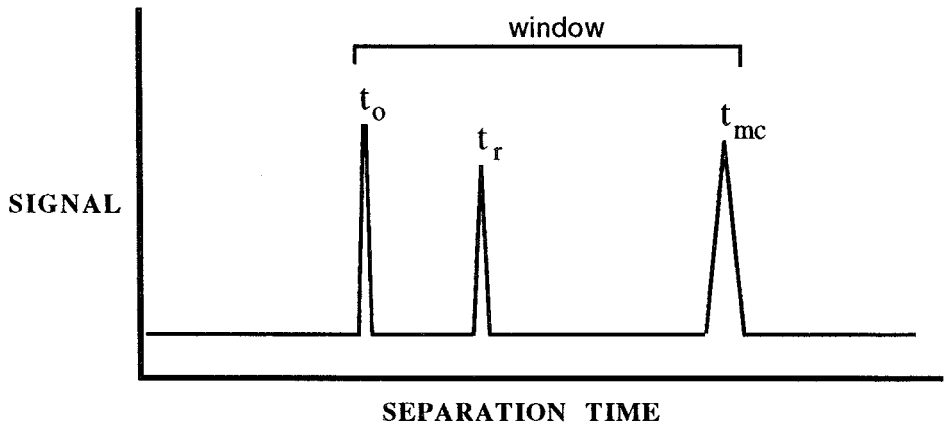

Figure 1.4 Migration time window exhibited by uncharged solutes when analyzed by MEKC. See text for details.

instrumentation have made CGE the technique of choice for genetic analysis [26-29]. Due in part to the dispensing of staining and sample pretreatment procedures (e.g., pre-concentration), protein profiles of cerebrospinal fluid (CSF), urine and serum that have, in the past, been primarily obtained by agarose gel and cellulose acetate electrophoresis (AGE and CAE, respectfully) are now being increasingly acquired by CE [30-33]. CZE has demonstrated superior performance over CAE and AGE with respect to analyzing the γ, β_2, β_1, α_2, α_1 and albumin regions that characterize the typical serum protein electrophoresis (SPE) profile [30, 34, 35]. These advantages have had a pronounced impact on the efficiency of diagnosing multiple myeloma as distinguished by the presence of a monoclonal protein peak observed in serum profiles of affected individuals. Pertinent to the material presented in Chapters 3 to 5 and in relation to SPE by CE is immunosubtraction (IS). IS is a powerful technique now commonly employed for the classification of monoclonal proteins. Because of its influence in the assay design to be described, IS is outlined in Section 1.4.2 of this chapter.

Other notable examples of CE being applied in the clinical setting include the screening of hemoglobin variants for sickle cell and thalassemia conditions by CIEF [36], lipoprotein profiling by CITP for their influence on atherosclerosis [37], and the determination of adrenal gland malfunction, via quantification of serum cortisol concentration, by a CE-based immunoassay methodology [38]. With respect to the latter, various aspects of CE make this technology amenable to immunoassay-type analyses. Indeed, the development of clinically relevant immunoassays by CE has been the focus of much research as recently reviewed by Schmalzing *et al.* [39]. CE-based immunoassays are profusely described in Section 1.4.1 as this topic is of significant relevance to this thesis.

Even though CE has shown to be a practical diagnostic tool for certain applications, the definitive role(s) of this technology in the clinical laboratory have yet to be fully established and ultimately relies on its potential capacity to perform robust assays with high accuracy and precision under high-throughput and/or ultra-rapid analysis conditions. More

fundamentally, CE is challenged by the ability to demonstrate appropriate detection limits for many bioanalytes within real samples while being minimally affected by sample matrix interference. To this end, the research undertaken for this thesis addresses the development of CE-based methods that are both specific and sensitive to particular classes of compounds. Two approaches were investigated: analysis by selective detection and analysis by affinity interactions. An introduction to these subjects in relation to CE is provided in the next four sections of this chapter.

1.3 Analysis by Selective Detection

Numerous on-column and post-column detection systems have been developed for CE [40, 41]. Table 1.2 displays a list of some of the more prominent CE detection methodologies in use today along with their corresponding detection limits. Since on-column detection eliminates contributions to zone broadening owing to the junctions usually associated with post-column connectors, and since the optical properties of fused silica permits the use of radiation-based methods, on-column spectrometric approaches have traditionally been favored. In fact, the most common CE detection device, due to its affordability and near universal nature, is the on-column UV absorbance detector.

Although CE is renowned for its ability to perform rapid and high-resolution separations, these capabilities become confined when dealing with highly complex samples. Needless to say, the number of concomitant species having a similar electrophoretic velocity to an analytes of interest can be expected to augment as the complexity of a sample is increased. As such, the ability to perform specific quantitative and/or qualitative analysis on that analyte begins to rely more and more on the method of detection; that is, minimization of co-migrating species signal interference through selective detection.

Table 1.2 General characteristics of common capillary electrophoresis detectors. Adapted from [14], with permission.

		Approximate Detection Limits	
Detector	Orientation	Moles	Molarity*
UV-Vis Absorbance	on-column	10^{-13} - 10^{-16}	10^{-5} - 10^{-7}
Indirect Absorbance	on-column	10^{-12} - 10^{-15}	10^{-4} - 10^{-6}
Fluorescence	on-column	10^{-15} - 10^{-17}	10^{-7} - 10^{-9}
Indirect-Fluorescence	on-column	10^{-14} - 10^{-16}	10^{-6} - 10^{-8}
Laser-Induced Fluorescence	on-column	10^{-18} - 10^{-21}	10^{-3} - 10^{-12}
Refractive Index	on-column	10^{-14} - 10^{-16}	10^{-6} - 10^{-8}
Radiometric	on-column	10^{-17} - 10^{-19}	10^{-10} - 10^{-12}
Laser-Induced Fluorescence	post-column	10^{-20} - 10^{-24}	10^{-11} - 10^{-15}
Mass Spectrometry	post-column	10^{-16} - 10^{-17}	10^{-8} - 10^{-10}
Amperometric	post-column	10^{-18} - 10^{-19}	10^{-7} - 10^{-10}
Conductivity	post-column	10^{-15} - 10^{-16}	10^{-7} - 10^{-9}

* Dependent upon volume of sample injected.

The analysis of biofluids for specific compounds, or a class of compounds, represents a particularly challenging task for CE. Since CE cannot readily resolve all of the components in such samples, selective detection rather than "nonselective" detection (e.g., absorption at 200 nm) is desired. Absorbance detection at a wavelength that corresponds mainly to the adsorption characteristics of an analyte, relative to adsorption by concomitant species, is perhaps the most practical discriminatory approach. For example, erythrocyte lysate analysis for the determination of hemoglobin variant can be performed using absorbance at 415 nm (heme group absorption) [36]. The utility of absorbance detection for biofluid analysis by CE is unfortunately limited since a balance between degree of detection selectivity and limits of detection (LOD) often exists. Inherently poor LODs are obtained with the on-column absorbance technique because of the direct dependence of absorbance on detection cell pathlength (i.e., the Beer-Lambert Law). Thus, micromolar detection limits for proteins separated in a typical capillary (50 μm internal diameter) are ordinarily obtained. Although up to a 10-fold decrease in LOD can be achieved through the use of extended detection pathlength strategies (e.g., bubble-cell capillary, z-cell detector) [42], such gains often fail to provide the necessary detection limits for most clinically important analytes.

The CE absorbance detection selectivity/limit constraint has led to the development of many hybridized analysis techniques. These include in-line analyte extraction/concentration methods and microreaction CE [43, 44]. A more direct approach to obtaining specific analyte information from biofluids involves the selection of an alternative detector system. For example, CE coupled on-line with mass spectrometry (CE-MS) is applicable for the quantification of amino acids in the serum of children [45]. The utility of CE-MS and CE-electrochemical detection for the analysis of various endogenous metabolites in urine have also been reported [46-48]. While such instrumental combinations provide valued clinical data, a drawback to the overall set-up is that detection is performed in a post-column fashion. As suggested above, post-column detector arrangements have the

propensity to increase the complexity and maintenance requirements of an instrument and can adversely affect the efficiency of CE separations.

Fluorescence represents a particularly attractive mode of detection for CE. Firstly, fluorescence detection offers the instrumental simplicity of absorbance detection since it can be performed on-column. Secondly, an enhancement in detection selectivity is instilled by fluorescence detection because compounds require a more rigorous spectrochemical criterion, relative to absorbance, to produce a detector signal; that is, a compound must both absorb the selected source wavelength(s) and emit radiation at higher wavelengths. Finally, the LOD achieved by fluorescence detection can exceed that obtained by absorbance provided that the compound of interest exhibits a significant quantum yield and that sufficient radiant power at the appropriate wavelength is produced by the excitation source. The utility of fluorescence detectors has been limited in the realm of clinical CE, however, since few analytes of interest possess substantial fluorescence properties. Superimposed is the optical performance restriction brought upon by the conventional excitation sources (e.g., mercury or xenon lamps) in these detectors. In short, the intensity of radiation that can be focused onto a capillary from such excitation sources often cannot compensate for the poor fluorescence quantum yield of weakly fluorescing species. As explained in the next section, this problem has been fittingly addressed through the development of fluorescence detectors that employ lasers as an excitation source.

1.3.1 Laser-Induced Fluorescence Detection

Laser-induced fluorescence (LIF) detection is by far the most sensitive mode of detection for CE. Several features of a laser contribute to its appropriateness as a source for fluorescence detection in CE: the emitted light is coherent, monochromatic, and highly directional. Perhaps the most basic reason for the remarkable LODs associated with

detection system stems mainly from the relationship that exists between excitation power and fluorescence signal magnitude for dilute fluorescent analyte solutions [49]:

$$\Phi_F = 2.3\phi_F \varepsilon c l P \qquad \textit{Equation 1.5}$$

where Φ_F is the fluorescence radiant flux in W, ϕ_F is the quantum yield of fluorescence, ε is the molar absorptivity of the fluorophor at the excitation wavelength in L/(mole•cm), c is the concentration of the fluorophor in mol/L, l is the optical path length in cm, and P is the radiant power of the excitation source in W. The high spectral power produced by a laser source, in concert with the ability to readily focus this radiation onto a capillary, is utilized to make up for the short pathlength associated with on-column CE detection. Although laser power is a variable parameter, excitation intensity cannot be arbitrarily increased. As indicated by Equation 1.6, application of laser irradiance (E), expressed in W/cm^2, that approaches a fluorophor's saturation irradiance (E_s) results in a fluorescence signal nearing the maximum fluorescence flux ($\Phi_{F,max}$) [49, 50]:

$$\Phi_F = \Phi_{F,max} \frac{E}{E + E_s} \qquad \textit{Equation 1.6}$$

However, fluorescence signal saturation is obtained when laser irradiance is significantly greater than the saturation irradiance value. Furthermore, excessive excitation power may contribute to fluorophor photodestruction resulting in an overall reduction in fluorescence emission. Optimal detection sensitivity is thus achieved by providing laser power that balanced maximum fluorescence excitation with minimal photobleaching of the fluorophor.

In addition to providing optimal conditions for fluorophor excitation within a capillary, the coherent nature of a laser beam ensures the production of narrow Raman and Rayleigh scatter bands relative to those produced by incoherent excitation sources (i.e.,

lamps). Spectral filtering of Raman and Rayleigh scatter, which are usually the major sources of background interference, is therefore facilitated.

The first LIF system for CE made use of fiber-optic guides for both detection zone illumination and fluorescence collection [51]. Due to high background signal and poor collection efficiency of the optical fiber, detection limits of only 0.1 μM for dansylated amino acids were obtained (325 nm HeCd laser source). Further improvement to the design of on-column LIF detectors led to the use of high numerical aperture microscope objectives. A schematic diagram of such a detector is illustrated in Figure 1.5. The beam produced from a laser is focused with a microscope objective onto a section of a capillary where the protective polyimide coating has been removed. Analyte fluorescence, Rayleigh scatter, Raman scatter and capillary luminescence are collected at a 90° angle relative to the excitation beam by a second microscope objective at the appropriate working distance. The light gathered by this objective is spatially and spectrally filtered before being detected by a photomultiplier tube (PMT). Spatial filtering is achieved by placing an aperture, having a diameter that matches the size of the detection cell image, in the collection objective's focal plane. The presence of an aperture at this location helps to block out most of the scatter that emanates from laser light and capillary wall-electrolyte interfaces. For spectral filtering, a filter arrangement that allows the transmission of light within a narrow wavelength range is chosen so as to discriminate between the fluorescence and unwanted radiation (i.e., scatter and capillary luminescence). With this detection scheme, on-column detection limits at the picomolar levels are typically attained [52].

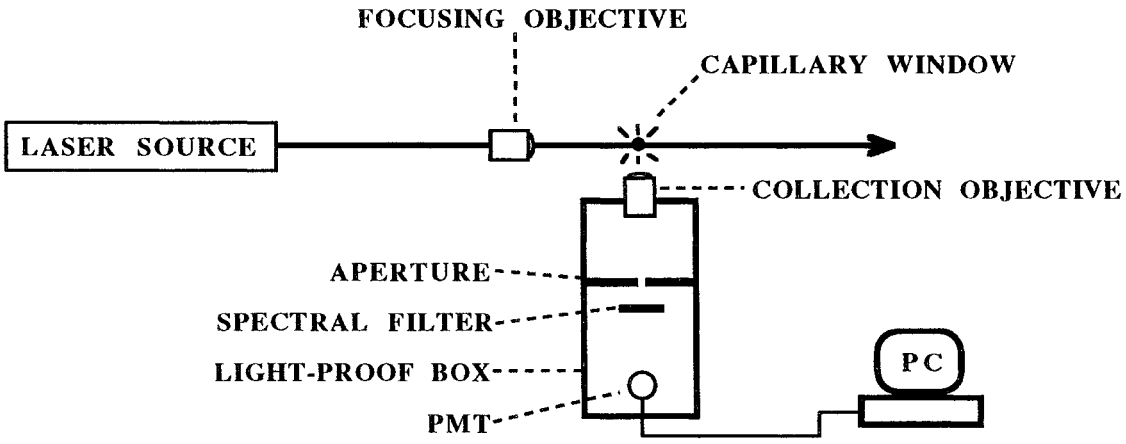

Figure 1.5 Schematic diagram of a typical laser-induced fluorescence on-column detection system for capillary electrophoresis.

1.3.2 Analyte Detectability

The low LODs that can be achieved by on-column LIF detection, as reported in the previous section, require conditions that involve a near perfect match between the laser source wavelength and the maximum absorption wavelength of a highly fluorescent species. Thus, drawbacks to the versatility of LIF detection are that only a limited number of line sources from inexpensive air-cooled lasers are available and that few analytes possess significant fluorescence quantum yield; i.e., the ratio of fluorescence radiant power to absorbed radiant power (Φ_A) [53]:

$$\phi_F = \frac{\Phi_F}{\Phi_A} \qquad \textit{Equation 1.7}$$

The constraints set forth by the requirement for an appropriate fluorophor/laser line combination are usually circumvented by labeling an analyte of interest with the appropriate fluorescent dye. Various labeling methods have been introduced with the most suitable approach depending on the sample type and volume size, and on the physiochemical properties of the analyte and labeling reagent. In brief, a labeling procedure may involve a fluorophoric or fluorogenic reagent, may be covalent or non-covalent, and may be performed, with respect to on-column LIF detection, by implementing an on-column or pre-column labeling protocol [54-57]. In addition, either of two strategies can be applied. In the first, a large molar excess of dye is used thereby ensuring complete and homogeneous labeling of the analyte(s). Although highly practical for certain applications, disadvantages such as the need for relatively harsh reaction conditions, the possibility of product precipitation, the presence of significant side reaction species, the general inability to label a specific compound, and the destruction of analyte biological activity has limited the utility of this approach [54, 58]. The controlled and targeted labeling of a specific

reactive moiety represents the second method in obtaining a highly fluorescent analyte for detection by LIF. This is accomplished by utilizing limited, stoichiometric amounts of labeling reagent, and exploiting reaction conditions that cater to the conjugation of the selected group [59, 60]. Advantages of selective tagging are that analyte biological activity can be retained and that interference from side products is minimized. However, the chemistry of such labeling often requires a "clean" sample (not typical of clinical samples) with analytes at relatively high concentrations (> μM) which negates the need for selective detection and the low LODs offered by LIF. Furthermore, compounds such as proteins and large peptides are usually not homogeneously labeled making electrophoretic analysis of the resulting products difficult [61]. For these reasons applications involving the measurement of a particular compound within a complex sample matrix by LIF make use of an affinity probe (i.e., a chromatographically purified fluorescent-labeled compound that strongly interacts with that analyte) rather than direct analyte labeling. This topic is discussed in Section 1.4.1.

A majority of the LIF detection sources for CE analysis of fluorescent-labeled compounds have employed laser excitation operating in the visible wavelength range. This is primarily due to the widespread availability of fluorescent dye chemistries that absorb radiation from such economical lasers. For example, the 442 nm line from a HeCd laser can be used to detect NDA and CBQCA derivatized primary amine of amino acid and peptides [62, 63]. Perhaps the most common LIF source used today is the 488 nm excitation wavelength produced by an air-cooled argon ion laser. This laser is being applied for detection of APTS derivatized carbohydrates [64], thiazol orange intercalated DNA [65], and various fluorescein-tagged biomolecules [66, 67].

Not all biomolecules require labeling for fluorescence detection. Some compounds endogenous to body fluids exhibit intrinsic (native) fluorescence when excited by UV radiation sources. Some of these include pyridine derivatives (NADH, pyridoxic acid) flavins (FMN, FAD, riboflavin), pteridine (biopterin, neopterin), porphyrins, and proteins

and peptides by virtue of their aromatic amino acids (phenylalanine, tryptophan, tyrosine) [68]. A disadvantage to the fluorescence properties of such compounds is their relatively poor quantum yields when compared to popular visible dyes (e.g., ϕ_F of < 0.14 and ≈ 0.5 for tryptophan and conjugated fluorescein, respectively) [69, 70]. Regardless, detection via native fluorescence does bring the advantage of being selective. Also, the alleviation of labeling procedures for these analytes can be regarded as beneficial because it ultimately results in a simpler analytical methodology. For these reasons, the development of CE application based on laser-induced native fluorescence (LINF) is of clinical interest. A major obstacle to the exploitation LINF, however, has been the lack of an economical UV laser. As discussed in Chapter 2 of this thesis, a possible solution to this problem involves the application of a modestly priced pulsed excimer laser operating at 248 nm.

1.4 Analysis using Affinity Interactions

The term affinity capillary electrophoresis (ACE) has been adopted for CE separations that make use of specific or selective affinity interactions during electrophoresis. Bioaffinity applications that have been studied include protein-protein, protein-drug, protein-DNA, lectin-sugar, and enzyme-substrate interactions [43, 71-73]. In ACE, the interactions can occur in free solution or on the surface of a solid support. Affinity interactions involving a solid-phase primarily addresses the issue of detecting dilute analytes in biological fluids. The realm of free-solution ACE encompasses two assay formats as defined by interaction kinetics. Assays involving rapid equilibrium interactions, called mobility shift assays, have been employed to characterize biomolecular-binding constants. The second free-solution mode, which is more practical in terms of clinical diagnostics, incorporates compounds that exhibit tight binding properties and dissociation rates that are relatively slow in comparison to the time of analysis - as is typically the case

with antigen-antibody interactions. The stability and specificity offered by such interactions in concert with the instrumental and separation features of CE represents a potentially powerful combination for clinical chemists. For these reasons, the development of CE-based immunoassays has received special interest.

In the following section, an overview of CE-based immunoassays is provided. For the purpose of brevity, a detailed discussion on antibody production, the concept of binding equilibria, and basic immunoassay theory is omitted. These topics are well established and may be readily found in text literature [74-76].

1.4.1 CE-Based Immunoassays

The union of CE and immunoassay technologies was first described in the early 1990's by Nielsen *et al.* [77]. This group demonstrated that the separation and detection of antigen-antibody complex from antibody and antigen (human growth hormone) in free solution using CE was possible. The description and clinical application of immuno-CE techniques that have since been developed is the subject of numerous reviews and book chapters [39, 44, 76, 78-80]. Below is a summary of the more prominent techniques used today.

CE-based immunoassays generally fall into two broad categories: solution-phase assays and solid-phase assisted assays. In the past decade, a relatively great deal of effort has gone into the development of solution-phase immunoassays. These assays have mainly employed fluorescent-labeled reagents and LIF detection since this provides the high sensitivity and detection selectivity required for many immunological tests. The interest in developing solution phase assays stems from the various advantages that this approach offers over conventional immunoassays (i.e., reductions in assay protocol steps and incubation periods).

As described in Appendix A, solution-phase assays can be subdivided into two distinct modes: noncompetitive and competitive (see Figure 1.6). With a noncompetitive (direct) assay, antigen concentration is measured by virtue of an immuno-complex peak following separation from excess unbound labeled antibody reagent that was added to the sample. A prerequisite to noncompetitive assays is that a monoclonal antibody against that antigen must be used as the affinity probe. Polyclonal antibodies cannot be used as their heterogeneity causes the formation of broad and low leveled complex peak signals that are difficult to quantitate. Competitive immunoassays rely on a binding equilibrium of free antigen and labeled antigen for a limited amount of antibody reagent. As opposed to noncompetitive assays, competitive assays may be performed with polyclonal antibodies as only the free labeled antigen peak data is required.

A notable drawback to competitive and noncompetitive assays is the difficulty in producing both homogenously labeled and biologically active protein-based affinity probes. The limitation set forth by fluorescent derivatization selectivity has thus promoted investigations into an alternative class of CE-based immunoassays; namely, solid-phase assisted assays.

Assays that employ a solid-phase capture protocol evade the need for fluorescent-labeled reagents since less demand is imposed on the selectivity and sensitivity aspects of the CE detection scheme. This is because immuno-extraction can both purify and pre-concentrate specific analytes from complex matrices prior to CE analysis. As such, many of the solid phase strategies that have been reported make use of simple absorbance detection. The primary drawbacks of solid-phase assisted assays, relative to solution phase assays, are that they tend to require more steps and longer incubation periods. However, solid-phase assisted CE assays do provide advantages over conventional immunoassays (e.g., ELISA). These benefits include low sample and reagent consumption, rapid separation procedures, and amenability to automation. With respect to assay design, the in-line and off-line types are perhaps the most common.

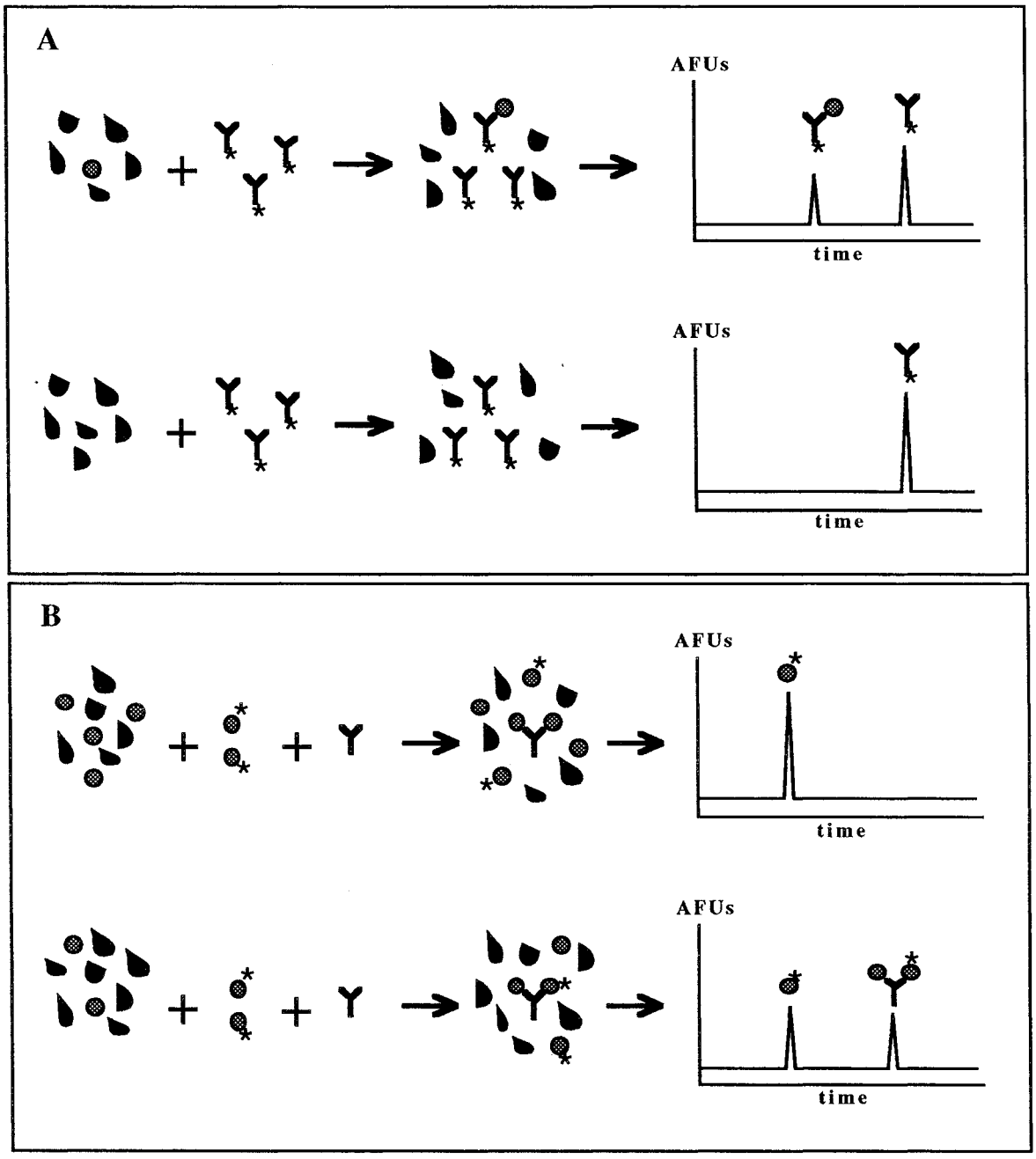

Figure 1.6 Schematic illustrations of A) noncompetitive and B) competitive free-solution CE-based immunoassays. Compounds marked with an asterisk (*) represent fluorescent-labeled reagents. Detector signal is expressed as arbitrary fluorescence units (AFUs). Refer to Appendix A for details.

As depicted in Figure 1.7 (panel A), in-line immuno-extraction assays entail the capture of an antigen by immobilized antibodies (polyclonal or monoclonal) within a capillary, followed by elution and separation of that antigen in the same capillary. Most on-line assays described thus far make use of an affinity plug, cartridge or membrane placed at the inlet end of the capillary. Although in-line CE immuno-extraction assays have been shown to be effective diagnostic tools, factors such as high costs and poor extraction-device stability remain to be addressed.

Off-line immuno-extraction assays involve a protocol whereby the interaction of an antigen with an immobilized antibody occurs in a reaction chamber other than the capillary (e.g., test tube, microtiter plate well) prior to CE separation of the supernatant components. In association with the off-line method is IS (see Figure 1.7, panel B) [81]. IS forms the basis of a procedure that is used to both identify and quantify a specific component within a complex sample by analyzing the aliquots of the sample matrix before and after analyte capture. In short, a sample is placed within a vial having sepharose beads coated with a specific immuno-fixant. Following a light vortex and bead sedimentation, a capillary is introduced into each well for sample injection. The supernatants are subjected to a CE separation and the resulting pattern is compared to the electropherogram from the original sample analysis.

1.4.2 Specific Antibody Detection

The CE-based immunoassay techniques described in the previous section are directed almost exclusively towards the quantification of compounds that do not elicit an immune response in humans. For instance, a noncompetitive immunoassay has been in serum [82]. The measurement of glucagon and insulin from the islets of Langerhans using the competitive mode was recently reported [83]. In-line immuno-analysis has been applied

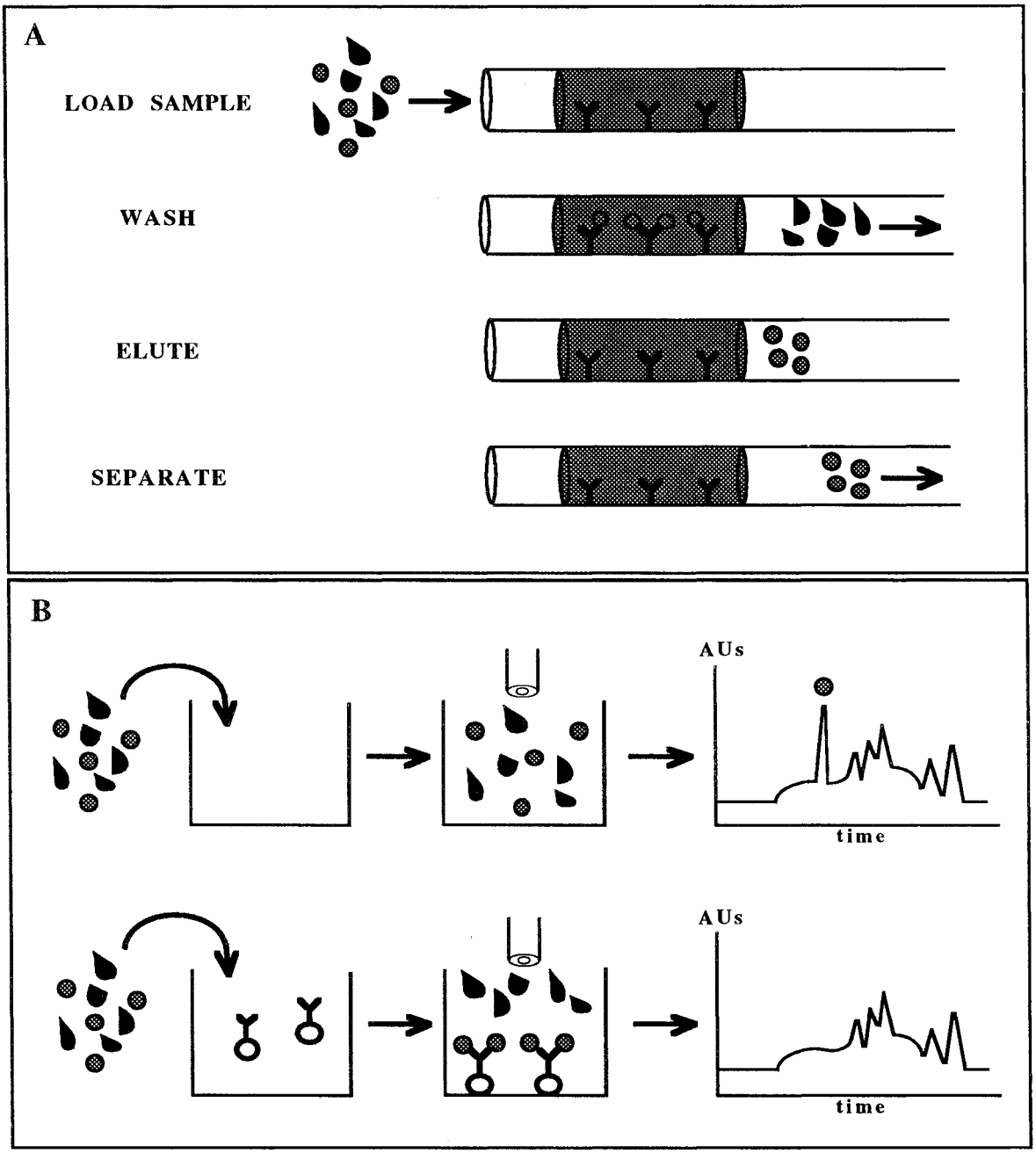

Figure 1.7 Schematic illustrations of solid-phase assisted immunoassays using CE instrumentation. Panel A depicts an example of an in-line assay configuration. An off-line method, called immunosubtraction (IS), is shown in panel B. Detector signal is in absorbance units (AUs). See text for details.

to evaluate the level of gonadotropin-releasing hormone in urine and serum [84]. Lastly, IS is routinely applied to classify the immunoglobulin subtype (IgG, IgA, IgM heavy chains, κ and λ light chains) in the SPE profile of patients exhibiting abnormal monoclonal protein spikes.

Interestingly, the detection of antigens representing viral compounds (e.g., capsid proteins) in body fluids through immuno-CE analysis has received little attention. This represents a beneficial area of research, as the application of existing CE immunoassay methodologies could prove useful in the diagnosis of viral infections. A foreseeable limitation to these assays, however, is a short temporal detection window. As is the case for HIV and hepatitis B, antigen concentrations in the serum of an infected individual are observed to decline rapidly to sporadically detectable levels following the onset of antibody seroconversion (*ca.* 3 and 24 month following initial exposure, respectively) [85]. An alternate approach to diagnosing viral diseases is through the detection of antigen specific immunoglobulins associated with the primary and secondary immune response (i.e., IgM and IgG, respectively) of a host that is challenged by an invading particle. In fact, most of the virological immunoassays used today center about the detection of specific polyclonal IgG antibodies as an indirect method to identify viral agent carriers.

A wide array of conventional immunoassay methodologies is available for specific antibody detection [86, 87]. Preliminary screening for specific antibodies is usually performed by an indirect ELISA. Briefly, an antigen coated solid-phase is used to capture any IgGs to that antigen from a serum sample. Seropositive subjects are identified, following a washing procedure (to remove all unbound components), through a color development by adding enzyme-labeled anti-human IgG and a substrate to the enzyme. General attributes to this assay include simultaneous multi-sample processing (e.g., 96 well microtiter plates), short analysis times (1 - 4 hours), low detection limits (10^{-8} - 10^{-11} M), and low operating costs [88, 89]. Although the indirect ELISA has proven to be exceptionally sensitive and specific, false-positives do occur [90, 91]. Thus, a sample that

repeatedly produces a positive result is subjected to confirmatory testing by Western blot (WB) analysis. With the WB, a standard sample of antigens (i.e., numerous viral proteins) that was separated by slab-gel electrophoresis is transferred to nitrocellulose onto which an indirect ELISA procedure is applied to determine the reactive bands. The resulting band pattern (selective markers) is subsequently used to verify a particular infection. The combined use of indirect ELISA and WB has been found to be extremely reliable: an estimated false-positive rate of 1:100,000 for a large clinical study has been reported [92]. The WB assay has therefore, and continues to be, the preferred confirmatory test for the presence of HIV-specific antibodies.

Compared to the indirect ELISA, the WB has the distinct advantage of having an antigen separation step prior to sero-analysis. In turn, this process comes at the cost of increased labor (i.e., gel preparation, nitrocellulose transfer step) and long analysis time (4 - 6 hours) as the use of slab-gel electrophoresis limits the speed of the separation. With this in mind, an improvement to the efficiency of confirmatory testing could be realized by remodeling the WB to include CE technology. In addition to providing rapid and high-resolution separations, the application of CE for specific antibody detection would be expected to bring about the same advantages displayed by existing CE-based immunoassays; that is, the means to perform a highly sensitive microvolume analysis to detect a particular compound within a complex sample matrix. It follows that the development of a confirmatory assay for the diagnosis of viral infections by CE may be of considerable value to the clinical field. As such, the concept of an indirect CE immunoassay was explored by the author. The results obtained from these efforts are revealed in this thesis.

1.5 Thesis Organization

My research activities at Concordia University have included studies into the comparison of common amine reactive probes used for the conjugation to biomolecules by CE [93], the monitoring of a conjugation between fluorescein isothiocyanate and myoglobin by CE [61], and methods for single fluorescent derivatization of peptides [60]. The material presented in this thesis focuses on work dedicated to the development of instrumental and assay techniques directly applicable to clinical CE.

Chapter 2 features the assessment of a pulsed KrF laser as an LIF excitation source for the selective detection of compounds possessing native fluorescence properties. Aspects of the laser's characteristics in relation to signal modulation are discussed along with justification for the chosen instrumental set-up. The LOD obtained for tryptophan and various proteins are reported and compared with published LODs from related investigations by other groups. Finally, results from the analysis of human serum, saliva and urine are used to demonstrate the utility of CE-LINF for profiling body fluids.

Chapters 3 to 5 deal with analysis through affinity interactions and describes the stepwise development of a novel indirect immunoassay derived from an IS-type protocol. At each stage, detection performance is demonstrated along with the assay's capability to produce a linear response for antiserum differing in specific antibody titer. Chapter 3 illustrates an overview of the assay protocol and a rationale for its design is provided. A model system involving fluorescein as a probe for rabbit anti-fluorescein is utilized to substantiate the concept of the proposed method and to characterize various assay parameters. A more clinically relevant scenario is presented in Chapter 4 with the detection of specific antibodies in serum from rabbits previously inoculated with various peptides. Furthermore, the assay's capacity to function under multiplexed conditions (i.e., the simultaneous detection of specific antibodies to different antigens) is shown. As part of a progressive evaluation for its potential use as a confirmatory test, the analysis of serum

from rabbits sensitized to HIV's p24 protein was carried out. The results of this investigation make up the material covered in Chapter 5.

Lastly, Chapter 6 incorporates some general conclusions regarding the research detailed in Chapters 2 through 5 and ends with suggestions for future work.

CHAPTER 2

Capillary Electrophoresis with Laser-Induced Native Fluorescence Detection for Profiling Body Fluids

Paquette, D.M., Sing, R., Banks, P.R., Waldron, K.C. *Journal of Chromatography B*, 714 (1998) 47-57.

2.1 Abstract

Laser-induced native fluorescence detection with a KrF excimer laser (λ = 248 nm) was used to investigate the capillary electrophoretic (CE) profiles of human urine, saliva and serum without the need for sample derivatization. All separations were carried out in sodium phosphate and/or sodium tetraborate buffers at alkaline pH in a 50 μm ID capillary. Sodium dodecyl sulfate was added to the buffer for micellar electrokinetic chromatography (MEKC) analysis of human urine. Although inherently a pulsed source, the KrF excimer laser was operated at a high pulse repetition rate of 553, 1001 or 2009 Hz to simulate a continuous wave excitation source. Detection limits were found to vary with pulse rate, as expected, in proportion to average excitation power. The following detection limits (3σ) were determined in free-solution CE: tryptophan, 4 nM; conalbumin, 10 nM; α-lactalbumin, 30 nM. Detection limits for indole-based compounds and catecholamine urinary metabolites under MEKC separation conditions were in the range of 7 to 170 nM.

2.2 Introduction

Although capillary electrophoresis (CE) and its family of related separation techniques demonstrate remarkable resolution of analytes in a relatively short time, they continue to be plagued by poor detectability below micromolar concentrations. On-column UV absorbance is the simplest and least expensive detection scheme for CE since many detectors in use today are modified HPLC detectors. However, detection capability is directly proportional to the optical pathlength, which averages only 39 μm for a 50-μm-ID capillary. This represents more than a 150-fold reduction in pathlength relative to a conventional HPLC flow cell. For biomolecules containing a UV absorbing residue, detection limits for CE separations are typically at the micromolar level (10^{-6} M) using on-

column absorbance detection. These detection limits can be improved by one to, in some cases, three orders of magnitude (10^{-7}–10^{-9} M) using modified capillaries with extended pathlengths or by on-column stacking procedures [94]. Extreme care must be used in the latter methodology, however, to maintain precision of analysis. Furthermore, in the analysis of real samples in a complex matrix, extraction is usually necessary before stacking can be accomplished.

Fluorescence detection is an alternative method for improving detection limits. Unlike absorbance, pathlength and analyte concentration are not the only parameters that influence fluorescence signal intensity. Fluorescence is also proportional to the intensity of the light source incident on the sample volume viewed by the detector. Whereas fluorescence excited by an incoherent source (i.e., lamp) improves detection limits by a factor of about 10 compared to absorbance [95], the coherent nature of lasers allows them to be focused down to the internal diameter of the capillary permitting a higher photon flux for analyte excitation. Five orders of magnitude improvement in detection limit compared to absorbance can be achieved for highly fluorescing dyes that are well matched to the laser wavelength of excitation [52]. Further reduction in the limit of detection (10^{-21} mol or 10^{-12} M) has been demonstrated with the use of a sheath flow cuvette for amino acids labeled with tetramethylrhodamine isothiocyanate (TRITC) [70].

Unfortunately, these extraordinary detection limits are for amino acids diluted after derivatization with the amine reactive fluorescent probes. The chemistry of isothiocyanate derivatization to primary amines is slow and competing hydrolysis réactions limit the concentration of analyte that can be successfully labeled. If fact, amino acids are typically derivatized at relatively high concentration (10^{-5}–10^{-4} M) before dilution and analysis. Further complications arise when derivatizing proteins that contain multiple labeling sites from lysine residue ε-amino groups. Derivatization of every available primary amine is incomplete and heterogeneous within the analyte population being derivatized resulting in a range of protein products that differ in the number and distribution of attached fluorophors.

CE can only partially separate these derivatives resulting in an analytically useless result [61].

An alternative to covalent derivatization is the use of native fluorescence, i.e., excitation of tryptophan (Trp) residues in proteins (λ_{em} ~ 320 to 380 nm). Swaile and Sepaniak were the first to demonstrate the utility of laser-induced native fluorescence (LINF) for detection of proteins separated by CE [96]. They used an argon ion laser (514.5 nm, 7 W) that was frequency doubled to produce 257 nm radiation for excitation. Although this wavelength is not optimal for Trp excitation, detection of conalbumin was possible down to 10^{-8} M - an order of magnitude lower than pathlength-extended UV absorbance detection. Lee and Yeung improved upon this detection limit by using a water-cooled argon ion laser operating at 275.4 nm, which closely matches the wavelength of maximum excitation (280 nm) for Trp. With their fluorescence detector, a limit of detection (LOD) of 10^{-10} M conalbumin was realized [97]. Unfortunately, the water-cooled argon ion laser is expensive relative to lasers typically used in CE separations [52, 61, 70]. An economical alternative to this is a low power, high repetition rate KrF excimer laser (λ = 248 nm), which represents a compromise between cost and non-ideal wavelength of excitation. A detection limit (2σ) of 10^{-9} M for conalbumin has been demonstrated using such a KrF laser with LINF detection [98].

In this study, we investigate the performance of the KrF excimer laser CE-LINF detection system operated in a quasi-CW (continuous wave) mode, rather than with gated integration as used by Chan *et al.* [98-101], for profiling body fluids. Detection limits (3σ) were evaluated for several biofluid analytes, under free-solution CE conditions and surfactant-modified CE (micellar electrokinetic chromatography, MEKC) conditions, which were used for urine analysis.

2.3 Experimental

2.3.1 Apparatus

All experiments were performed on a CE-LINF system built in-house. CE separations were carried out at ambient temperature in a 50-cm long (44 cm effective length), 50 μm ID, 185 μm OD untreated fused silica capillary, unless otherwise stated. High voltage (model CZE1000R, Spellman, Plainview, NY, USA) to drive the separation was applied at the anode (inlet) from within a Plexiglas™ safety box (fabricated in-house). Samples were introduced into the capillary electrokinetically at the anodic end by applying 1 kV for 5 seconds, timed with a stopwatch.

The LINF system, shown in Figure 2.1, was constructed on an optical breadboard (Melles Griot, Nepean, ON, Canada) to facilitate alignment of the optical components and to dampen mechanical vibrations. A model SGX-500 KrF excimer laser (Potomac Photonics, Lanham, MD, USA) was used for excitation. The 248-nm output beam was reflected from an A = 1.0 neutral density filter (Melles Griot) and two aluminum-coated mirrors, then passed through an A = 0.5 filter to reduce the average laser power to < 2 mW. The attenuated laser beam was focused onto the capillary using a UV-grade synthetic fused silica plano-convex lens (f = 10.0 mm, Ø = 5.0 mm, Melles Griot). The average laser power, which increases with pulse repetition rate, was measured at various points along the optical train and the overall transmission efficiency was found to be 5% (i.e., a 95% attenuation of power) from the laser output to the separation capillary 125 cm away. Therefore, the average excitation power reaching the capillary was *ca.* 0.6 mW when the laser was operated at 553 Hz, 0.9 mW at 1001 Hz and 1.7 mW at 2009 Hz.

Fluorescence was collected normal to the excitation beam using a 15× Reflachromat™ reflective microscope objective (N.A. = 0.58, f = 11.5 mm, Spectra-Tech, Stamford, CT, USA). Spectral filtering of Raman and incident laser scatter was achieved

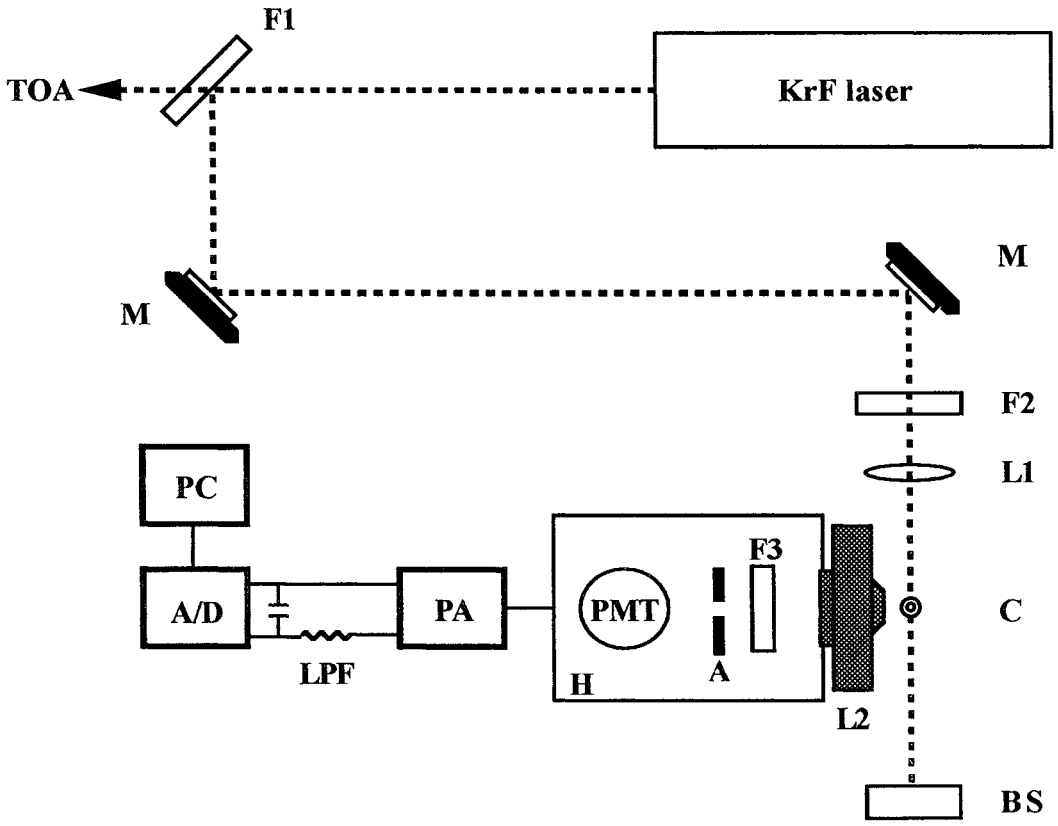

Figure 2.1 Schematic of the LINF detection system with components labeled as follows: F1, F2 = 1.0 and 0.5 absorbance filters, respectively; M = mirror; L1 = plano-convex fused silica lens; C = capillary cross-section; BS = beam stop; L2 = reflective microscope objective; F3 = UG1 glass filter; A = 1-mm aperture; PMT = photomultiplier tube; H = PMT housing; PA = picoammeter; LPF = low pass filter; A/D = data acquisition card; PC = Pentium computer; TOA = beam path to thermo-optical absorbance detector.

using a UG1 filter (60% T at λ = 350 nm, FWHM = 80 nm) from Melles Griot. Rayleigh scatter and silica fluorescence were spatially filtered by a 1-mm pinhole aperture located in the focal plane of the reflective objective. After optical filtering, the emitted fluorescence signal was detected by a model 1P28 photomultiplier tube (Hamamatsu, Bridgewater, NJ, USA) and amplified with a picoammeter (model 414 Microammeter, Keithly Instruments, Cleveland, OH, USA). The PMT was housed in a side-on, light-tight box (5 cm $\times$ 8 cm $\times$ 13 cm) and socket from Products For Research (Danvers, MA, USA). For the majority of data presented here, the picoammeter voltage output was further conditioned with a passive low pass filter (RC time constant = 0.3 second) and collected at 10 Hz on a Pentium computer via an A/D interface (National Instruments, Austin, TX, USA) with the aid of LabVIEW™ acquisition software (National Instruments). The other data, human serum electropherogram and protein LODs, were obtained by strip chart recorder (model 056-1001, Hitachi, Tokyo, Japan) connected directly to the picoammeter voltage output.

In Figure 2.1, the arrangement of optical components for fluorescence collection is not to scale. In fact, the reflective objective was threaded directly into the PMT housing and the UG1 filter and 1-mm pinhole, both 2.54-cm diameter components, fit snugly between the objective and PMT in a self-aligned fashion. As a result, no additional light-tight box was needed and the CE-LINF system was always operated with the room lights on. Care was taken, however, to block incident sunlight because the UG1 filter transmits at > 700 nm. The PMT housing and capillary were mounted on xyz translation stages (Newport, Mississauga, ON, Canada). Rapid alignment of the CE-LINF system was achieved by first centering the capillary in the excitation beam via visual inspection of the far field profile on the beam stop. Secondly, the microscope objective/PMT housing was translated across the capillary until a signal maximum was obtained for 3 μM tryptophan in buffer flowing through the capillary at 300 V/cm.

2.3.2 *Materials*

Uric acid (UA), tryptophan (Trp), homovanillic acid (HVA), 5-hydroxyindole-3-acetic acid (5HIAA), 3-indoxyl sulfate (3IXS), vanillylmandelic acid (VMA), α-amylase, conalbumin, α-lactalbumin and human serum as well as ACS grades sodium tetraborate, sodium phosphate (tribasic) and sodium dodecyl sulfate (SDS) were purchased from Sigma (St. Louis, MO, USA). ACS grade hydrochloric acid and sodium hydroxide were from BDH (Toronto, ON, Canada). In-house distilled water was purified with a multi-cartridge Millipore water filtration/deionization system before use. Fused-silica capillary was purchased from Polymicro Technologies (Phoenix, AZ, USA). Platinum wire for electrodes and microcentrifuge tubes (1.5 mL and 600 μL) for buffers were obtained from Fisher Scientific (Montréal, QC, Canada). Nylon membrane syringe filters, 0.22-μm pore size, were purchased from Chromatographic Specialties (Brockville, ON, Canada).

2.3.3 *Buffer and Sample Preparation*

Buffers were prepared using the Millipore-purified water (18 MΩ) and pH was adjusted with the appropriate volume of hydrochloric acid or sodium hydroxide solution. SDS was added to buffers before pH adjustment for separations performed in the MEKC mode. All buffers were filtered through 0.22-μm membrane filters before installation at the capillary inlet and outlet. Where indicated, MEKC buffers were irradiated for 120 min with 254 nm light in a UV Cross-linker instrument (UVC-515 UV Multi-linker, Ultra-Lum, Carson, CA, USA) to photobleach fluorescent impurities. The capillary was rinsed first with 0.1 M NaOH then with running buffer (5 column volumes during 1 minute for each solution) before each injection of body fluid or standard. Fresh urine and saliva samples collected from volunteers and human serum from Sigma were filtered through 0.22-μm

nylon filters before being diluted with running buffer. Standards were prepared in the same buffer used for the body fluid analysis.

Isolation of saliva proteins from a two-fold diluted saliva sample was performed using a Microcon-10™ microconcentrator (Amicon, Beverly, MA, USA) and repetitive washing of the retenate with running buffer after each centrifugation period (3×10 minutes at 5,585 g). The washed retenate was reconstituted in running buffer to the initial sample volume (*ca.* 600 μL) before analysis.

2.4 Results and Discussion

The advantage of LINF detection for CE is apparent: no sample derivatization is required. The LINF detection of endogenous compounds in body fluids requires that they have an appreciable molar absorptivity at the excitation wavelength and an appreciable fluorescence quantum yield in the appropriate separation buffer. For detection of proteins in body fluids by LINF, the requirement is that they contain aromatic residues, preferably Trp. The molar absorptivity is an order of magnitude higher for Trp than for either tyrosine (Tyr) or phenylalanine (Phe) at an excitation wavelength of 248 nm [102]. In addition, the intensity of Trp fluorescence is more than 100 times that of Tyr or Phe at 248 nm excitation [98]. Phe has a low fluorescence quantum yield, even when excited at its wavelength of maximum absorbance, and Tyr is easily quenched or can readily undergo energy transfer [102]. Tryptophan fluorescence also depends on the surrounding environment in that quenching can lead to a decrease in intensity, but not to the same extent as Tyr.

Sensitive detection of Trp is important in diagnostic applications because it is an intrinsic fluorophor in proteins and is excreted in urine as the free amino acid and as various metabolized forms indicative of disease [103]. Free Trp fluorescence, when excited at 280 nm, is highly pH dependent, in that a 2-fold increase in fluorescence intensity is

seen when increasing pH from 8 to 10.2 [69]. Therefore, a working range of pH 9-10 was used in all our work to take advantage of this pH dependence of free Trp fluorescence and also to minimize protein adsorption to the capillary wall.

2.4.1 LINF Detection

Nanomolar detection limits for tryptophan-containing polypeptides [98] and other fluorescing analytes [99, 101, 104] have been demonstrated for LINF using the same pulsed KrF laser used in this work along with a gated integration (boxcar) detection scheme. Gated integration is a phase sensitive detection technique for low duty-cycle modulated signals such as those obtained with pulsed laser excitation (high duty-cycle signals are best handled by phase-locked amplifiers). Phase sensitive detection is typically used to enhance the S/N ratio of modulated signals, provided the dominant noise is additive. Gated integration provides an additional advantage in that it permits temporal discrimination of the laser scatter from the fluorescence, as long as the temporal profiles of the two differ to some extent. Most pulsed lasers, including the KrF laser used in this work, suffer from relatively large pulse-to-pulse fluctuations that contribute in a multiplicative fashion to the overall noise in fluorescence intensity. As well, in the absence of analyte, the measured intensity for on-column laser fluorescence is generally dominated by laser scatter and/or background fluorescence. Even under ideal conditions, the fundamental (shot) noise would still be multiplicative rather than additive. Consequently, the S/N ratio improvements expected for phase sensitive detection would be limited.

Given the high repetition rate of the KrF laser (up to 2009 Hz), and in view of the above considerations, we have investigated LINF detection performed in a quasi-CW mode rather than with gated integration of the fluorescence pulses. This approach was recently used to monitor the refolding pathway of a large protein [105], although the authors did not

provide the necessary data to compare the performance of gated to non-gated integration detection schemes. Figure 2.2 shows the relationship between the excitation pulse rate and fluorescence emission before (panels A-C) and after (panels D-F) conditioning with a low pass filter (RC = 0.3 second) for a continuous flow of 30 nM Trp in buffer (500 V/cm, 6 μA). Figure 2.2 panels A-C clearly show the pulse-to-pulse variations in emitted light. These large fluctuations mimic the incident laser profile and appear in the scattered light, background fluorescence and analyte fluorescence. Such variations are not discriminated against by phase sensitive detection, as mentioned above. The emitted pulses in panels A-C are broad (*ca.* 0.8 mseconds at half height) compared to the excitation pulses (*ca.* 60 nseconds), due to the response of the picoammeter. The traces in panels D-F show the effect of low-pass filtering of the fluorescence pulses, using a 0.3-second time constant. Data were collected at 10 Hz and plotted on the same scale for each pulse repetition rate. While the average fluorescence intensity increased as a function of the pulse repetition rate (i.e., the average excitation power), the noise on the Trp signal stayed essentially constant. The same trend was seen for the background signal from tetraborate buffer in the absence of Trp (data not shown) with the net result of a higher S/N ratio for higher pulse repetition rates.

Figure 2.3 shows the electropherogram of a 2-nL (apparent volume [106]) injection of 30 nM Trp. The Trp LOD (3σ) for our CE-LINF detection system (KrF laser operated at 1001 Hz) was determined to be 4 nM. This compares favorably with Chan's LOD (2σ) of 3 nM Trp for a 15-nL injection and KrF excimer LINF detection with gated integration in a 75 μm ID capillary [98], and Lee and Yeung's LOD, also at 2σ, of 2 nM Trp obtained by argon-ion LINF at 275.4 nm without gated integration [97]. We found that our LOD improved to 1 nM when the KrF laser was operated at 2009 Hz, taking advantage of the higher incident power versus noise characteristics described above. However, LOD measurements at 2009 Hz were obtained using a shorter capillary (33 cm total length, 758

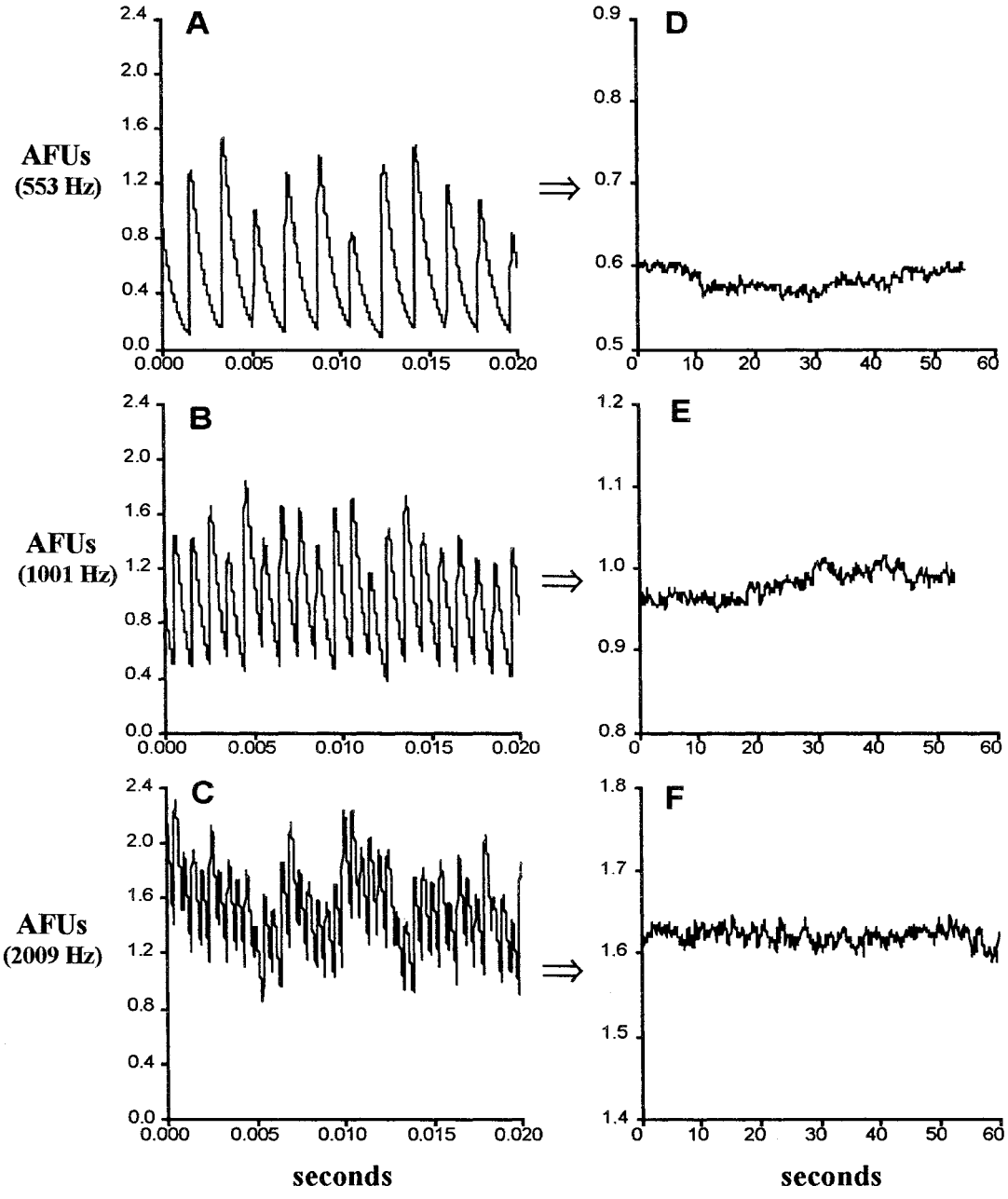

Figure 2.2 Representative traces of the picoammeter signal output (A-C) and low-pass filtered output (D-F) from the CE-LINF detector operated at 553 Hz (A and D), 1001 Hz (B and E), 2009 Hz (C and F) for 30 nM Trp in 5 mM sodium tetraborate buffer (pH 10.0), 500 V/cm, 6 μA.

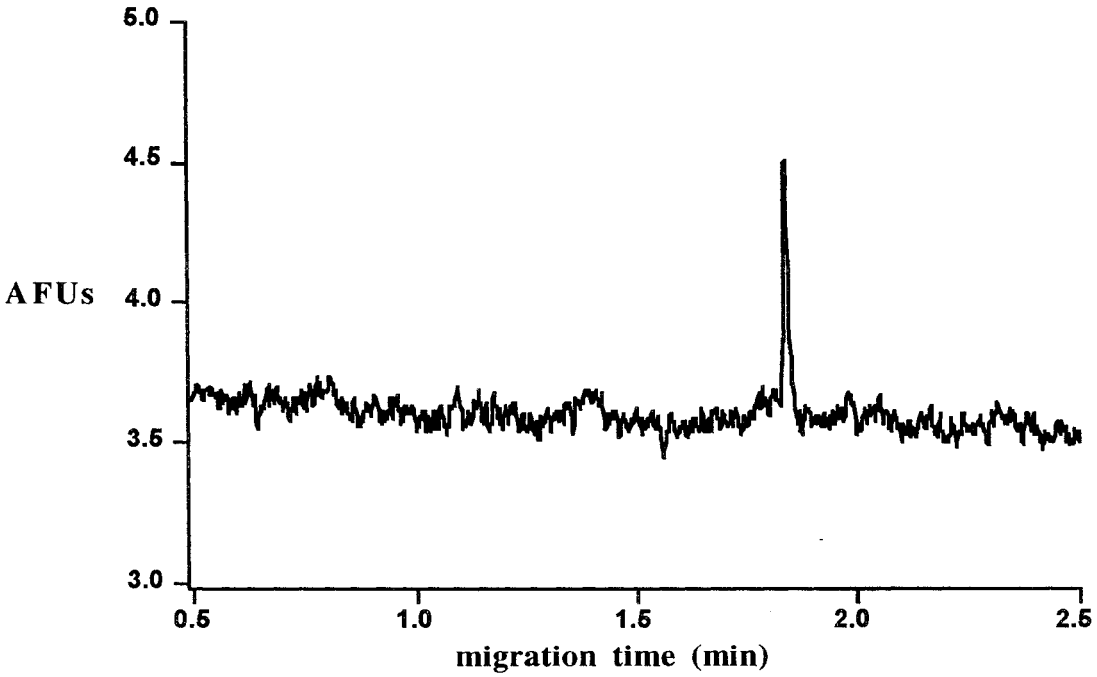

Figure 2.3 Capillary electropherogram of 30 nM Trp at 500 V/cm in a 5 mM sodium tetraborate (pH 10.0) buffer. Laser excitation was at a pulse rate of 1001 Hz.

V/cm, 10 μA) to ensure that Trp eluted (data not shown) before the capillary cracked under the stress of the high incident laser power and separation voltage. In our laboratory, the KrF laser is used concurrently for the LINF work presented here and for thermo-optical absorbance (TOA) detection of peptides [107]. Because it is a shared laser (Figure 2.1), excitation was performed at a pulse rate of 553 Hz (the optimum for TOA detection) instead of 1001 or 2009 Hz for profiling body fluids.

Detection limits for proteins depend on the number of Trp residues present and, to some extent, the local environment of those residues. For example, the LODs (3σ) of egg white conalbumin and bovine α-lactalbumin were determined to be 10 nM and 30 nM, respectively, for our CE-LINF system. Fan *et al.* reported a similar LOD for P22 tailspike endorhamnosidase protein detected with their KrF excimer LINF system [105]. Conalbulmin has 10 Trp residues whereas α-lactalbumin has 4 (obtained from NCBI Entrez Protein Query database). Therefore, we were surprised to find that both of our protein detection limits were worse than that of Trp. It can be postulated that the proteins interacted with the capillary wall and that adsorptive broadening of peaks increased the LOD, which we had calculated based on peak height. Also, Trp residues would be in a different microenvironment than free Trp amino acid, thus the fluorescence characteristics would vary.

We observed blue and white luminescence of the capillary at all pulse repetition rates, which was also reported by Lee and Yeung for excitation at 275.4 nm [97], and non-specific fluorescence from the borate buffer. Both sources of background signal were not completely eliminated by the UG1 filter after being efficiently transmitted by the Reflachromat™ reflective objective, which is a mirror-based microscope objective. After prolonged exposure of the capillary to 248-nm excitation, we could distinctly see red fluorescence, which may be due to damage of the fused silica [108]. This red luminescence was not caused by heating of the capillary.

2.4.2 Body Fluid Profiles

Reports on the use of CE with UV absorbance detection for body fluid analysis are numerous [109, 110]. For some applications, the analyte of interest is at sufficient concentration that direct injection and quantification by CE-UV is reliable. However, sensitivity issues arise in the determination of less abundant proteins and protein precursors, which may be indicators of a diseased state [111]. Accurate quantification by CE-UV becomes problematic at the micromolar level. Therefore, 100-fold better detectability achieved with CE-LINF is advantageous and increases the working range for analyte determination. Of equal importance is the selectivity that LINF detection affords. Caslavska *et al.* [112] demonstrated the utility of fluorescence detection in CE as a means of simplifying urine profiles compared to CE-UV, where electropherograms become crowded by the vast number of endogenous urinary compounds.

2.4.3 Human Serum

The detection selectivity offered by CE-LINF with the KrF excimer laser was investigated using various body fluids obtained commercially or from volunteers working in our laboratory. Figure 2.4 shows the CE-LINF profile at pH 10.0 of a 60-fold dilution of standard human serum. Such profiles obtained using UV absorbance detection, and their use for identifying gammopathies and other protein disorders, have been reported numerous times [35, 113-115]. To the best of our knowledge, this is the only example of a serum profile by CE-LINF. Although the use of LINF detection for profiling serum may not provide many distinct advantages over UV absorption, its use in quantifying pre-albumin is one possible application. Pre-albumin, which can be an important indicator of nutritional status, inflammation, malignancy, cirrhosis of the liver and Hodgkin disease

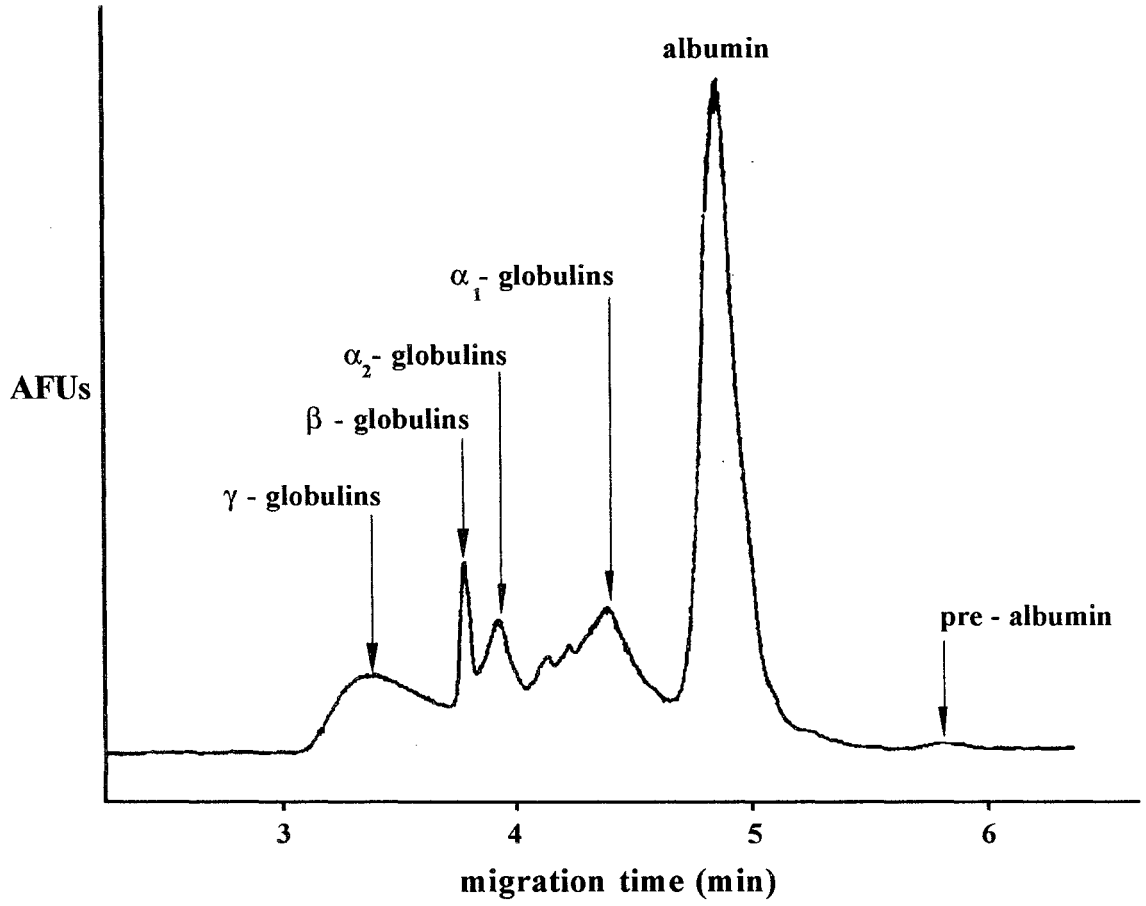

Figure 2.4 Capillary electropherogram of human serum, filtered (0.22 μm) then diluted 60-fold in buffer before injection. Separation was carried out at 300 V/cm in a buffer consisting of 5 mM sodium tetraborate (pH 10.0).

[114, 116], is generally found in serum at micromolar concentrations, i.e., close to the LOD for CE with UV absorbance detection [114, 117]. Because pre-albumin is rich in Trp, facile analysis by CE-LINF with the KrF excimer laser is expected. We are investigating this application further.

2.4.4 Human Saliva

There are few reports on the CE analysis of endogenous components in saliva [118]. Figure 2.5 shows the CE-LINF profile of human saliva before (panel A) and after (panel B) filtration to remove compounds of nominal M_r <10,000 Da. One of the main components secreted in saliva is α-amylase, the enzyme responsible for hydrolysis of amylose and amylopectin [119]. The saliva sample was spiked with an α-amylase standard to confirm its migration time at 2.35 min. Trace B in Figure 2.5, which is the retenate from filtration and should contain only high M_r compounds, showed that smaller species, perhaps Trp-containing peptides, co eluted with α-amylase and may contribute in part to the shoulders seen on the main peak in trace A. The source of some of these peptides may be due to proteolytic degradation of saliva proteins because no protease inhibitory pretreatment was implemented, such as addition of PMSF (phenylmethylsulphonyl fluoride) [120]. Identification of other salivary components such as lysozyme, kallikrein [119] and trace endogenous species by CE-LINF may be undertaken in the future.

2.4.5 Human Urine

Urine is a complex biological matrix. Rapid screening to give a chromatographic fingerprint of urine or any other mammalian biological fluid can provide useful qualitative

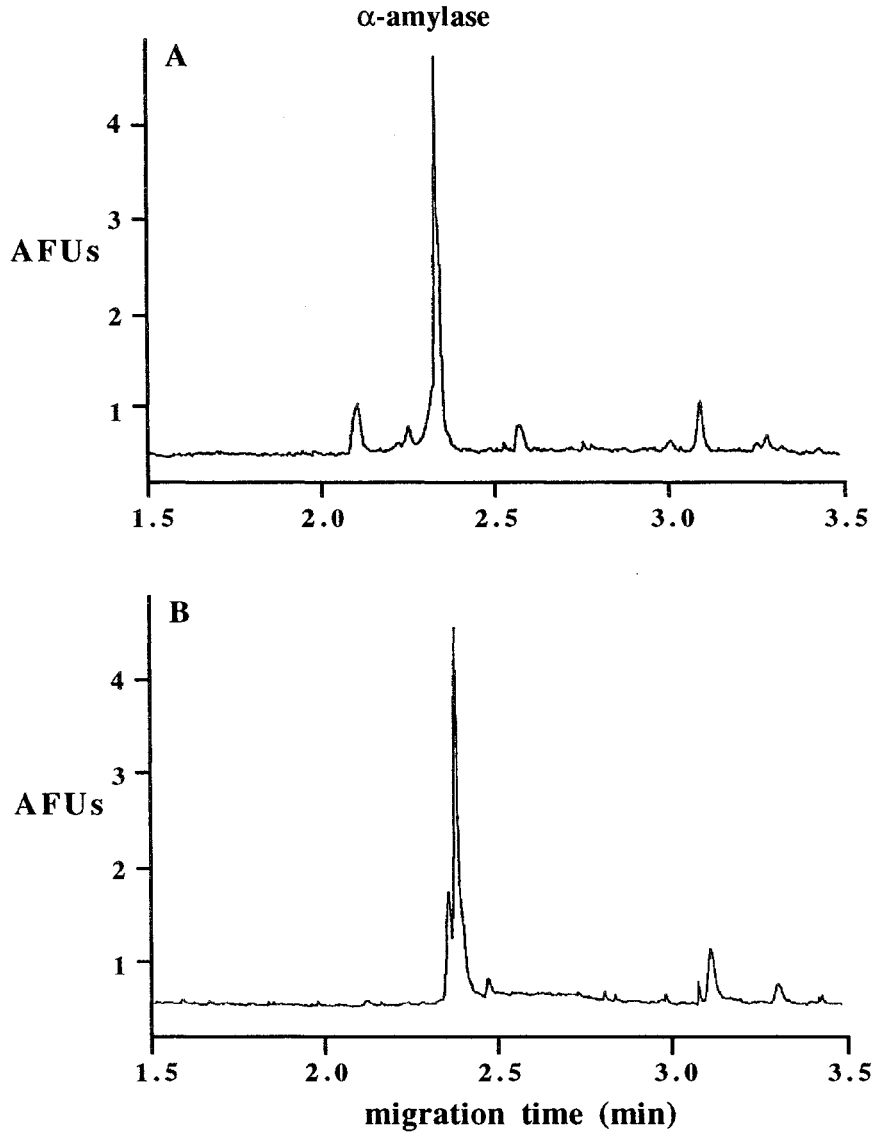

Figure 2.5 Capillary electropherogram of human saliva. Separation was carried out at 400 V/cm in a buffer consisting of 5 mM sodium phosphate (pH 10.2). (A) Saliva sample was filtered (0.22 μm) then diluted tenfold in buffer before injection. (B) Saliva sample was filtered (0.22 μm), diluted twofold, then passed through a 10 000 M_r cut-off filter and the retenate was reconstituted in buffer before injection.

and semi-quantitative information, but usually only for one or a few classes of compounds at a time. GC and HPLC are perhaps the most widespread analytical techniques for screening urine [121-123]. MEKC-LINF represents a complementary technique for the selective detection of endogenous and exogenous compounds in urine. MEKC can potentially provide better separation selectivity than free-solution CE because many urinary components, such as Trp and 5-hydroxytryptophan, are not well resolved by the latter technique (data not shown).

The application of CE and MEKC to monitoring underivatized fluorescent compounds, either endogenous or exogenous, in body fluids has been reported by several research groups [100, 101, 104, 112, 124, 125]. Figure 2.6 shows the MEKC-LINF electropherogram of diluted urine. Native fluorescence detection simplifies the urine profile, compared to multi-wavelength absorbance detection [126], because only that class of analytes that fluoresce are specifically detected. The peaks for six endogenous species known to be in urine were identified by spiking urine samples with the appropriate standards and monitoring their peak co-migration. This method is not necessarily conclusive, nor does it exclude the possibility that another unidentified fluorescent component of urine may also co-elute with the spiked standard. However, conditions for the MEKC separation were chosen based on the work from Thormann's group [112] who confirmed the assignment of these peaks by matching the multi-wavelength absorption spectra collected during electrophoresis. We did not attempt further optimization of this separation.

Detection limits (3σ) were initially determined for 5HIAA, HVA and VMA in the same MEKC separation buffer as that used in Figure 2.6. Only a 10-fold improvement in detectability was seen for all three species with respect to non laser-induced methods of fluorescence detection (λ_{ex} = 220 nm) [112]. In fact, our detection limits were on the same order of magnitude (*ca.* 1 μM) as those reported by Simon and Nicot [127] for phenylglyoxylic and mandelic acids, analytes similar to HVA and VMA, determined by CE

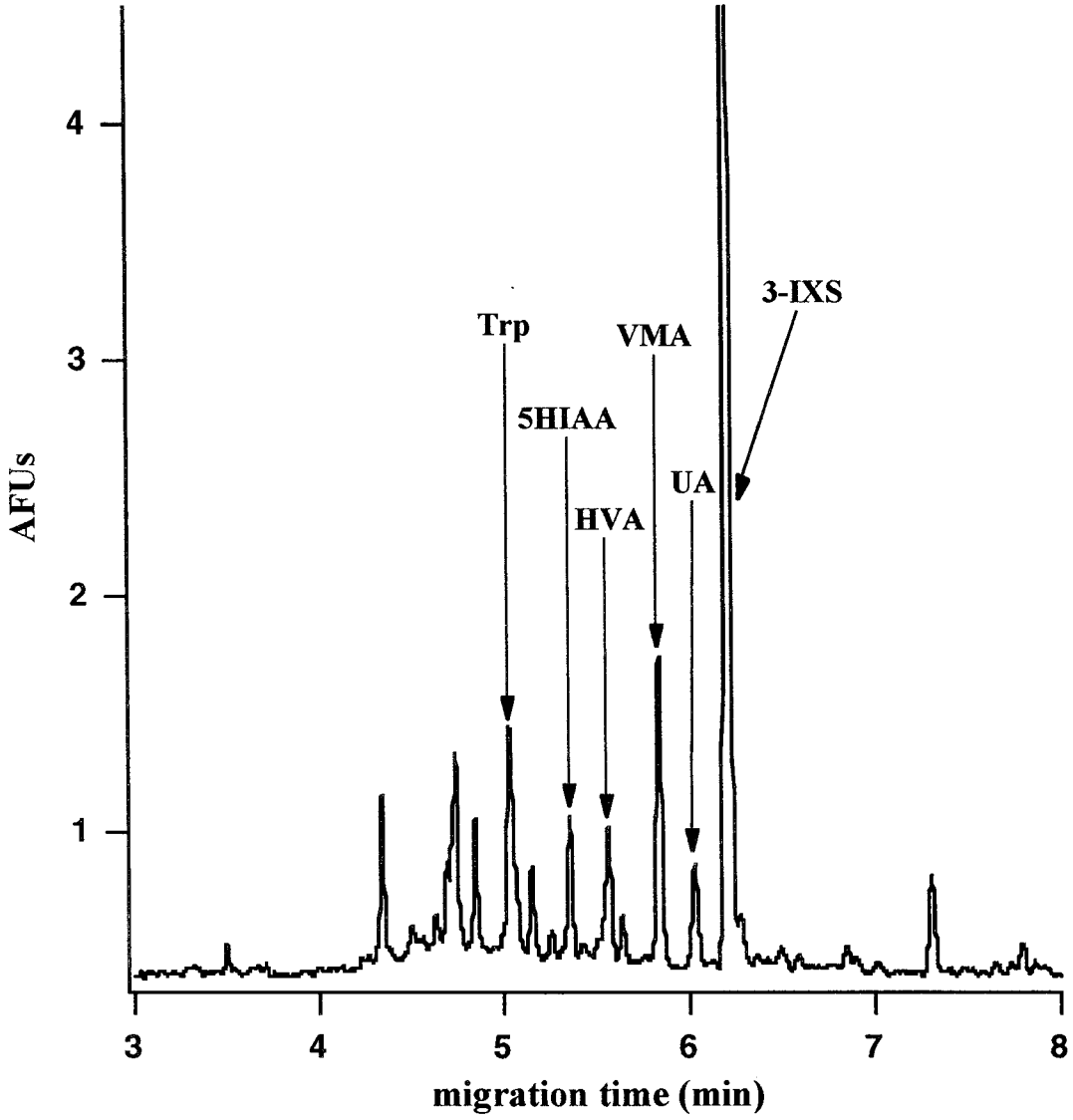

Figure 2.6 MEKC electropherogram of human urine. Separation was carried out at 300 V/cm in a buffer consisting of 6 mM sodium tetraborate, 10 mM sodium phosphate, 75 mM SDS (pH 9.3). The following endogenous compounds were identified by spiking with standards: UA, uric acid; Trp, tryptophan; 5HIAA, 5-hydroxyindole-3-acetic acid; 3IXS, 3-indoxyl sulfate; HVA, homovanillic acid; VMA, vanillylmandelic acid.

with UV absorbance detection (λ = 255 and 210 nm). Impurities in SDS have been shown to contribute to background fluorescence [99] and the presence of micelles has been observed to increase the LOD by a factor of two [98, 99, 101]. Indeed, we found a 6-fold improvement in the LODs of 5HIAA, HVA and VMA when the MEKC buffer was irradiated with UV light for 120 min to photobleach fluorescent impurities. Further improvements in LOD were seen when the concentration of SDS was lowered from 75 to 50 mM and when sodium phosphate was eliminated. These results are summarized in Table 2.1 along with the normally excreted amounts of these compounds. Our LOD of 7 nM for 5HIAA (Table 2.1) compares well with that obtained by Chan *et al.* [101] (5.8 nM at S/N = 2) for the same buffer, excluding 5% acetonitrile. The improvements in LOD for 5HIAA in buffers B and C (Table 2.1) were observed to be inversely proportional to the current generated in the capillary (data not shown). Apparently, Joule heating in the capillary affects the analyte emitted fluorescence. We are investigating this relationship further. Although the three metabolites in Table 2.1 have high normal excretion levels that could be quantitated by CE-UV, the inaccuracy associated with closely eluting peaks from other UV-absorbing components makes fluorescence detection, a selective technique, an attractive alternative for urine analysis.

2.5 Conclusions

Native fluorescence is an attractive approach for detecting biological compounds because the need for derivatization chemistry associated with visible-LIF detection is eliminated. LINF detection with a KrF excimer laser, operated at high repetition rates without gated integration, was used. Applications of this detection system were demonstrated by obtaining free-solution CE profiles of human saliva and serum and an MEKC profile of human urine. It was shown that the selective detection offered by this

Table 2.1 Normal level, medical importance and buffer-dependent LOD of three fluorescent components found in urine: 5-hydroxyindolacetic acid (5-HIAA), homovanillic acid (HVA), and vanillylmandelic acid (VMA).

Compound	Normal level[a]	Relevance	LOD at 3σ (nM) in buffer:		
			A[e]	B[f]	C[g]
5HIAA	2 mg/day (<50 mM)[b]	Carcinoid tumor screening[c]	33	15	7
HVA	6 mg/day (<40 mM)	Neuroblastoma and hepatic encephalopathy screening[d]	800[h]	-	170
VMA	3.5 mg/day (<45 mM)	Neuroblastoma and hepatic encephalopathy screening[d]	500[h]	-	150

a) [128]

b) Based on 1 L/day typical volume of urine excreted [128]

c) [112, 129]

d) [121, 122, 130, 131]

e) 6 mM borate, 10 mM phosphate, 75 mM SDS (pH 9.1), UV irradiated for 120 min

f) 6 mM borate, 10 mM phosphate, 50 mM SDS (pH 9.1), UV irradiated for 120 min

g) 5 mM borate, 50 mM SDS (pH 9.1), UV irradiated for 120 min

h) Extrapolated from background S/N obtained for 5HIAA measurement

technique could provide qualitative and, in future, quantitative information on various endogenous species in these body fluids.

Although comparatively low detection limits for Trp and urinary compounds were obtained, we are continuing to investigate further improvements in LOD. For example, ratioing the KrF excitation pulses to the fluorescence emission pulses will eliminate pulse-to-pulse fluctuations and should substantially improve S/N. Secondly, better spatial discrimination of silica fluorescence from analyte fluorescence might be achieved with a smaller pinhole aperture (ideally 0.75 mm) and/or the use of sheath flow detection techniques.

2.6 Acknowledgments

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CHAPTER 3

Detection of Specific Antibodies using Immunosubtraction and Capillary Electrophoresis Instrumentation

Paquette, D.M., Banks, P.R. *Electrophoresis* 2001, 22, 2391-2397.

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3.1 Abstract

Solution-phase immunoassays based on capillary electrophoresis (CE) separations have been shown to be rapid and simple to perform. The potential for sample matrix interference and incompatibility with multiplexing conditions for antibody detection when dealing with real samples, however, has prompted the development of an assay that utilizes an immunosubtraction methodology. A model assay for the detection of specific antibodies that relies on solid-phase extraction, CE and laser-induced fluorescence (LIF) detection is described. The method, called immunocapture/immunosubtraction (ICIS), incorporates an antibody capture/purification protocol using magnetic particles. The detection of specific antibodies is achieved by CE-LIF analysis of a probe solution following incubation with the captured antibodies. As an example of the ICIS-CE assay's capabilities, the relative quantification of anti-fluorescein in serum is presented.

3.2 Introduction

The ability to detect antigen-antibody interactions, via precipitation reactions or through the use of various antibody or antigen labeling strategies, has provided a means for quantifying analytes of interest within complex sample matrices for over four decades [132]. Immunoassays continue to play a vital role in analytical chemistry, particularly in the realm of the biological sciences, as attested by their incorporation in many of the diagnostic protocols performed by modern clinical analyzers [133, 134]. Such immunoassay analyzers provide several important features in light of the efficiency demands set upon today's clinical laboratory. Some of these features include full automation, high-throughput capabilities and microscale sample and reagent volume manipulation. It thus follows that the development of new immunoassays should cater to these specifications. Immunoassays

based on capillary electrophoresis (CE) separations and laser-induced fluorescence (LIF) detection of fluorescent immune complexes represents an approach having these characteristics.

CE is a technique that is recognized for its rapid, high-resolution separations and microliter sample volume requirements. When coupled to an LIF detector, limits of detection surpassing most antibody-antigen dissociation constants (K_d = 10^{-8} to 10^{-11} M) are readily attained for highly fluorescent species [52, 135, 136]. This provides the necessary assay dynamic range to be achieved for many biologically relevant compounds. Furthermore, the ongoing development of automated instrumentation able to simultaneously sample 96 well microtiter plates has made CE amenable to high-throughput analysis [137].

Numerous examples of CE-LIF-based immunoassays describing the detection and quantification of specific analytes within complex matrices may be found in the literature [78, 138]. In general, such assays are useful provided that the antigen of interest is present at nanomolar concentrations or higher [139]. Thus, the utility of CE-LIF-based immunoassays may be compromised in the case of viral infection diagnosis (e.g., human immunodeficiency virus, hepatitis B) where viral-specific antigen detection requires highly sensitive assays such as the direct enzyme-linked immunosorbent assay (ELISA) [85, 140]. An alternate approach in adapting CE for usage in immunodiagnostics involves the development of an immunoassay based on the detection of host antibodies specific for viral antigens. This is plausible since viral specific antibody concentrations in serum tend to remain high during infection relative to antigen concentrations – this approach forms the basis of many diagnostic tests used today. The design, and preliminary characterization, of a technique for the detection of specific antibodies in serum that depends on the separation power of CE is described. Attributes of the assay method relative to conventional CE-based immunoassays are also discussed.

3.3 Materials and Methods

3.3.1 Reagents and Buffers

Unless stated otherwise, reagents were purchased from Sigma (St. Louis, MO). All buffers were prepared using water obtained from a Barnstead NanoPure System (18 MΩ-cm). Immunocapture/immunosubtraction (ICIS) assays were performed using rabbit anti-fluorescein (supplied as 1.1 mg/mL of partially purified polyclonal product) obtained from Molecular Probes (Eugene, OR). Flat bottom, medium binding, 96 x 360 μL well RIA polystyrene microtiter plates (Corning Costar, Corning, NY) were used as the sample reservoirs. The microtiter plate wells were blocked overnight at 4°C with a 0.05% bovine γ-globulin solution prepared in a 50 mM phosphate/50 mM NaCl pH 7.4 (PBS) buffer prior to use. Assays involving complex sample matrix conditions were performed in serum from non-immune challenged rabbits (Biogenesis, Poole, England). Rabbit immunoglobulin G (IgG) was captured with non-uniform 1 μm diameter superparamagnetic particles coated with goat anti-rabbit IgG (PerSeptive Biosystems, Framingham, MA). Magnetic separations were carried out by applying 1.75 cm diameter, pot magnets (Visual Planning, Montreal, PQ) to the bottom of the microtiter plate wells. Washing procedures were accomplished with the PBS buffer. Stock solutions of fluorescein and BODIPY-FL (Molecular Probes) were prepared by dissolution with *N,N*-dimethylformamide to individual concentrations of 100 mM. Probe solutions were prepared by combining aliquots of the stock solutions followed by dilution with a PBS buffer to the indicated nanomolar concentration. Capillary zone electrophoresis (CZE) separations of the probe solutions were performed using borate buffer solutions that were adjusted to the indicated pH using concentrated sodium hydroxide or hydrochloric acid.

3.3.2 ICIS-CE Assay Overview

A schematic illustration of the solid-phase assisted immunoassay protocol is displayed in Figure 3.1. Serum from a particular host is placed in a microtiter plate well containing magnetic particles. The magnetic particles, coated with antibodies specific for a class of host immunoglobulins, capture a population of the antibodies thereby immobilizing them to the solid-phase. A magnetic separation allows the magnetic particles to be retained and a majority of the sample's concomitant species to be removed. The particles are repeatedly washed (1 to 5 times) using wash buffer re-suspension/magnetic separation/wash buffer extraction cycles. An aliquot of a standard solution (probe solution) comprised of a fluorescent-labeled antigen(s) and an internal standard is then added to the well. A recognition event between the captured antibodies and the antigen(s) results in a decrease of free solution antigen(s). This decrease in antigen concentration is detected by separating the probe solution supernatant by CE and comparing the antigen/internal standard peak signal ratios.

3.3.3 Particle Washing Characterization

Characterization of the assay washing procedure was performed with a 100 μL aliquot of magnetic particle slurry that had been previously washed four times in PBS buffer. The washed particles were placed in a microtiter plate well containing 200 μL of undiluted rabbit serum. Following a magnetic separation, the supernatant was removed and analyzed using a UV-absorbance spectrophotometer. The particles were re-suspended with 290 μL of wash buffer. Cycles of magnetic extraction and supernatant removal were repeated until minimal absorbance at 280 nm was observed.

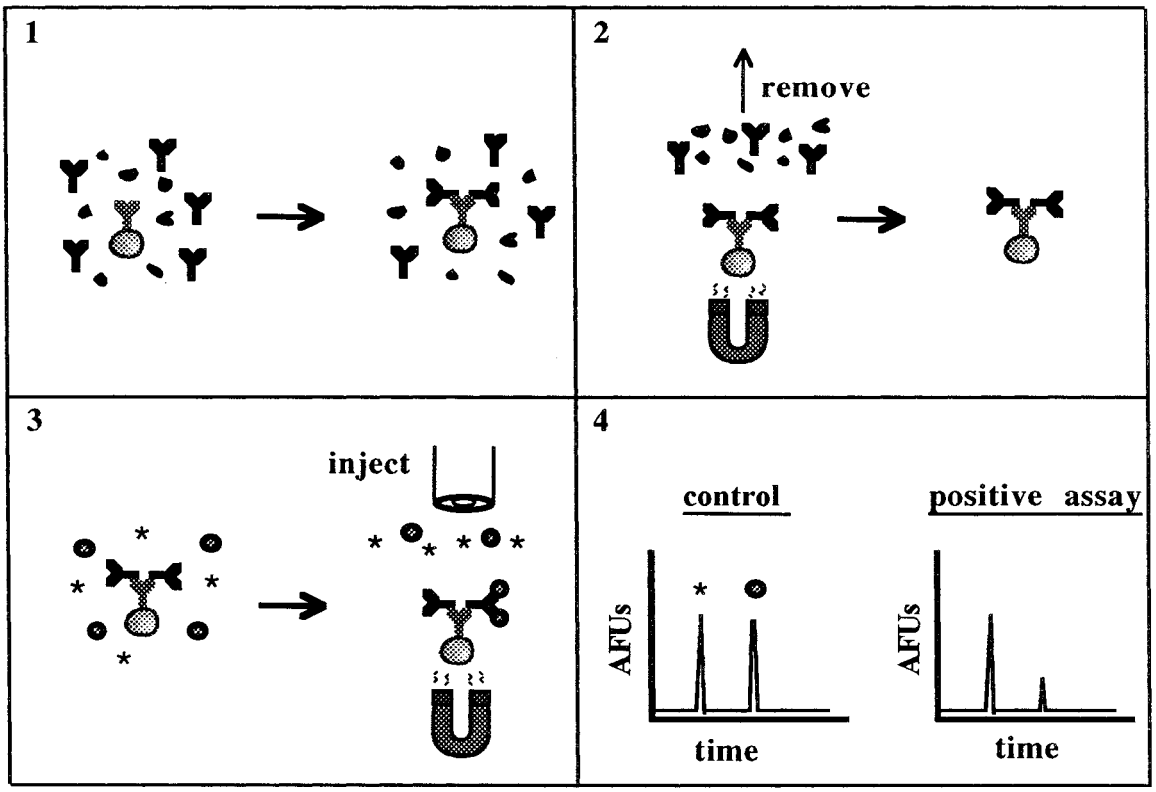

Figure 3.1 Protocol of the ICIS assay for the detection of specific antibodies. 1) Antibodies from the biofluid are captured by magnetic particles coated with secondary antibodies. 2) Magnetic separation allows non-immobilized compounds to be removed via micropipette. The particles are repeatedly washed with wash buffer/magnetic separation cycles. 3) A solution containing a fluorescent internal standard (*) and a selected fluorescent-labeled antigen (●), called a probe, is added to the washed particles. Antigen recognition by any immobilized host antibodies causes a decrease in free solution antigen concentration. The probe solution supernatant is subsequently analyzed with CE-LIF. 4) An electropherogram displaying a change in internal standard/probe signal intensity ratio relative to a control experiment infers the presence of antibodies specific for that probe in the biofluid.

3.3.4 ICIS-CE Assay and Calibration Curve

Demonstration of ICIS-CE assay feasibility was investigated by spiking 350 μL of rabbit serum with 100 μL of undiluted anti-fluorescein solution. A 100 μL aliquot of the resulting solution was added to a microwell containing 100 μL of goat anti-rabbit IgG magnetic particle slurry. The well contents were subjected to two cycles of a 2 minute low speed vortex with an intermittent 2 minute static incubation. The particles were then washed three times with 270 μL of PBS buffer. Ten minute magnetic separations were used to isolate the particles at the bottom of the well in each step. Supernatant from the well following each wash was removed via micropipette in the following manner. A Plexiglas™ plate with an aperture was placed over the well and the micropipette tip was inserted until the diameter of the hole prevented further insertion. With the aperture that we used, 20 μL of supernatant and the magnetic particles remained in the bottom of the well after the extraction process. A 100 μL volume of 5 nM BODIPY-FL/3 nM fluorescein solution was added to the washed particles to probe for captured anti-fluorescein. Incubation of the particles with the probe solution was performed during four 2 minute low speed vortex/2 minute intermittent static cycles. After the completion of a final magnetic separation, 50 μL of the supernatant was removed and placed into a 250 μL glass CE vial. The solution in this vial was subsequently analyzed with a CE-LIF system. Concurrently, a control assay was performed using 100 μL of unspiked rabbit serum as the sample solution.

A calibration curve was prepared by spiking 10 μL of stock anti-fluorescein solution in 300, 435, 600, 800 and 1000 μL of rabbit serum. A 100 μL aliquot of each solution was placed into individual wells containing 100 μL of magnetic particle slurry. The particles were then washed and probed for the presence of anti-fluorescein using the same assay protocol described above.

3.3.5 Separation and Detection

Separations were performed at ambient temperature with an ATI-Unicam Crystal 310 CE system using a 60 cm (45 cm effective length) × 50 μm ID × 186 μm OD untreated fused-silica capillary (Polymicro, Phoenix, AZ). Since the CE instrument is not designed to perform injections from microtiter plate wells, the probe solution from each assay was analyzed by transferring a 50 μL aliquot of well supernatant into a 250 μL glass CE vials prior to analysis. In all cases, injection of sample into the anodic end of the capillary was carried out with a 25 mbar pressure for 0.1 minutes. Electroseparations were driven by applying 25 kV. Following each separation, a capillary washing protocol consisting of a 0.1 M NaOH flush (1 bar for 0.3 minutes) and a run buffer rinse (1 bar for 0.7 minutes then 1 bar plus 25 kV for 1 minute) was implemented. Detection of fluorescent compounds was made with an external laser-induced fluorescence detection system, constructed in-house, that has been previously described [67]. Briefly, the output of an air-cooled argon laser (488 nm) was focused onto the capillary window (1 cm) using a 6.3 × microscope objective. Fluorescence, and light scatter, was collected at a right angle to the excitation source with a 16 × microscope objective. Rayleigh scatter from the collected radiation was spatially filtered by a 800 μm pinhole located in the focal plane of the collection objective. Spectral filtering of Raman and Rayleigh scatter was achieved using a bandpass filter and cut-off filter arrangement selective for passage of fluorescein fluorescence wavelengths (λ_{max} = 520 nm). Fluorescence was detected by a photomultiplier tube (PMT) set at -900 V. The current produced by the PMT was filtered and converted to a voltage by a low pass filter prior to being collected by a data acquisition board.

3.4 Results and Discussion

3.4.1 Rationale for Immunosubtraction Assay

The utility of CE-based immunoassays as analytical tools has been, and continues to be, demonstrated by various research groups [141-144]. The interest in CE-based immunoassay stems from the ability to efficiently separate antigen and antibody-antigen complexes and to selectively detect one or both of these compounds following the separation procedure. In most cases, fluorescent-labeled antigens or antibodies (or their fragments) are used as probes (tracers) in either competitive or direct solution-phase assay formats. The design of these assays has focused on the detection and concentration measurement of particular antigens such as drugs [136, 143, 145-147], hormones [148-150], peptides [139, 141], and proteins [142, 151, 152]. Alternatively, relatively few reports can be found which present antibodies as the analytes of interest [145, 147, 153-155]. The purpose of this work was to further demonstrate the usefulness of CE for specific antibody detection. The detection of host antibodies which recognize particular antigens has been widely exploited for the diagnosis of viral infections [85]. Assays which function on this premise, called indirect immunoassays, rely on the innate ability of a host's immune system to develop specific and high affinity antibodies to foreign substances which enter the body.

Although CE-based solution-phase immunoassays have been successfully employed for detecting specific antibodies in complex matrices [145, 156], a few drawbacks exist. Firstly, some samples (e.g., serum, ascites fluid) may require dilution in order to obtain respectable analyte peak signals and to avoid sample matrix signal interference and/or nonspecific probe interactions [157] (see Appendix B). The need for such dilution could have a negative impact on the detection limits of an assay - particularly in cases where the antiserum contains low concentration or low affinity antibodies to the

probe(s). A second and more critical restriction relates to the multiplexing capabilities of the assay. For these assays, separation conditions that favor antigen-antibody complex stability are normally required; that is, the complex should remain intact throughout the separation. The development of an indirect immunoassay for broad spectrum screening or fingerprinting analysis, however, would require a probe solution comprised of numerous antigens. Separation buffers having extremely high or low pH, surfactants, organic solvents or other buffer additives could therefore be necessary in order to attain sufficient resolution of the antigen zones. The use of such separation buffers, however, could contribute to the dissociation of immunocomplexes early during electrophoresis thereby contributing to assay interference [143, 146]. An added requirement is the need to separate the complex zone, which can be broad with polyclonal antibodies, from all probe zones [38, 150]. Given that complex zones are often observed in CE-based [139, 141, 142, 146, 147], an increasing demand is set upon the separation condition as the probability of fluorescent complexes/free probe co-migration increases with increasing probe solution complexity.

Since our aim was to develop a robust indirect immunoassay that would be minimally affected by sample matrix effects and that could readily function under multiplexing conditions, a hybrid technique based on immunosubtraction [13] was designed. The method relies on the solid-phase capture of antibodies followed by CE-LIF analysis of the supernatant from a standard solution of fluorescent antigen(s) that was incubated with the immobilized antibodies.

3.4.2 ICIS-CE Assay: Design and Characterization

In designing and characterizing the ICIS-CE approach, several factors contributing to assay performance were addressed. These, in general, related to probe solution

characteristics and choice of solid-phase extraction material. Conditions for an assay compatible probe solution require that the solution i) be near neutral pH and moderate in ionic strength to promote antibody-antigen binding and ii) be lower or similar in ionic strength to the separation buffer to avoid electrodispersion. Electrodispersion is a phenomenon encountered in CE separations when the sample plug injected into the capillary contains higher conductivity than the electrophoresis buffer [158] (see Appendix B). The resulting separation produces poor analyte peak symmetry and low separation efficiency. As such, a balance in probe solution buffer ionic strength composition must be found which promotes antibody-antigen interactions in the assay well and that minimally affects the efficiency of the electrophoretic separation step that follows. A rudimentary investigation involving 5 nM BODIPY-FL/3 nM fluorescein solutions prepared in various phosphate/NaCl buffers and 100 mM borate pH 9.2 as the separation buffer was performed. In short, a compromise between high sample ionic strength and minimal analyte electrodispersion was obtained with the described PBS buffer (data not shown). Furthermore, BODIPY-FL/fluorescein solutions prepared in this buffer, and separated in the borate buffer, consistently produced peak (height and area) signal ratio values having CVs of < 3% (n = 3, data not shown). This level of probe solution analysis precision is of relative importance given the subtractive nature of the assay.

Superparamagnetic particles coated with anti-rabbit IgG antibodies were chosen as the solid-phase capture agent for a number of reasons. Magnetic extraction permits efficient purification of immobilized analytes. Since the particles do not exhibit a magnetic memory following magnetic separations, no magnetically-induced aggregation occurs and re-suspension of the particles is easily achieved. The ability to suspend and mix the particles allows antibody-antigen reactions to occur more rapidly relative to static surface capture methods [159]. The non-uniform surface of the particles presents a large surface area, relative to spherical particles, which provides for high binding capacity [160]. Furthermore, the use of immobilized secondary antibodies ensures capture of specific

classes of host antibodies (e.g., IgG) and that their biological activity is retained. Lastly, the magnetic manipulation of particles is compatible with microtiter plate robotics rendering the ICIS-CE assay adaptable to full automation.

Preliminary characterization of the immunocapture protocol included investigations into incubation conditions that promoted efficient host antibody capture, the time required to magnetically extract the particles from solution, the number of wash steps needed to significantly reduce the presence of concomitant species and the incubation procedure that provides steady state antigen (probe) binding by the captured host antibodies. Incubation studies were performed by using a standard sample and assay protocol which altered only in a particular incubation parameter (data not shown). In short, these studies indicated that host antibody capture and probe binding saturation occurred using two and four cycles of 2 minute low speed vortex with a 2 minute intermittent static incubation, respectively. It was also observed that magnetic isolation of the particles from undiluted serum is readily achieved in under five minutes with the magnets at hand. Finally, the results displayed in Figure 3.2 indicate that significant removal of extraneous serum components is obtained following three wash cycles. Since the sample matrix concentration is reduced by over 10 fold following the first wash, only a single wash step may be necessary for reducing matrix interference to a respectable level. We chose, however, to wash our particles three times to ensure reliable assay results. The characterized protocol was employed in demonstrating the qualitative and quantitative aspect of the ICIS-CE approach.

3.4.3 ICIS-CE Assay

Figure 3.3 displays electropherograms from the analysis of probe solution aliquots incubated with magnetic particles which were pre-exposed to rabbit serum and rabbit serum spiked with anti-fluorescein. Subtraction of fluorescein was readily attained with no

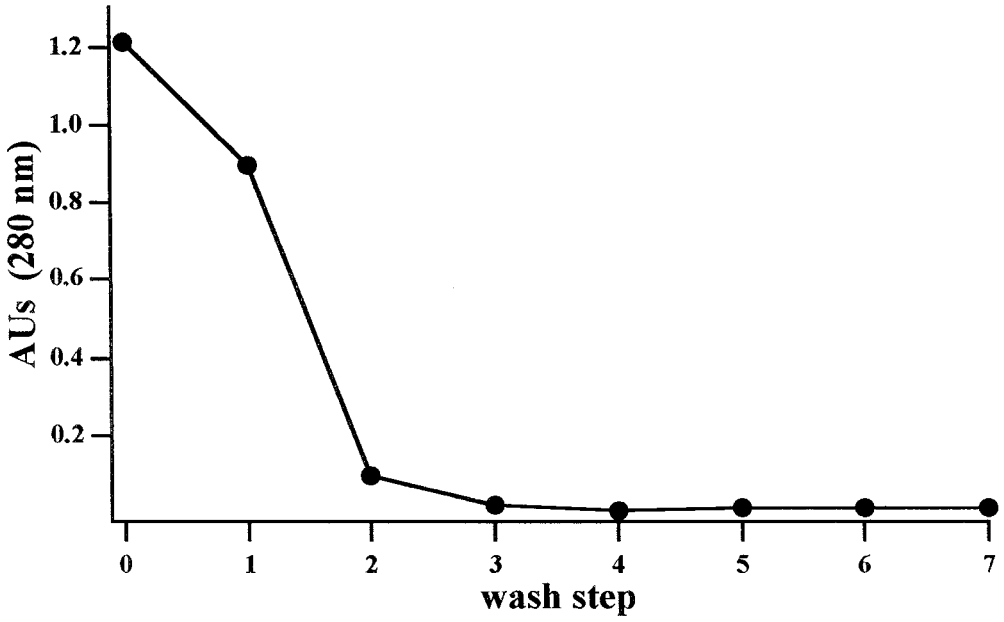

Figure 3.2 Determination of residual serum protein content following the washing of magnetic particles within a microwell.

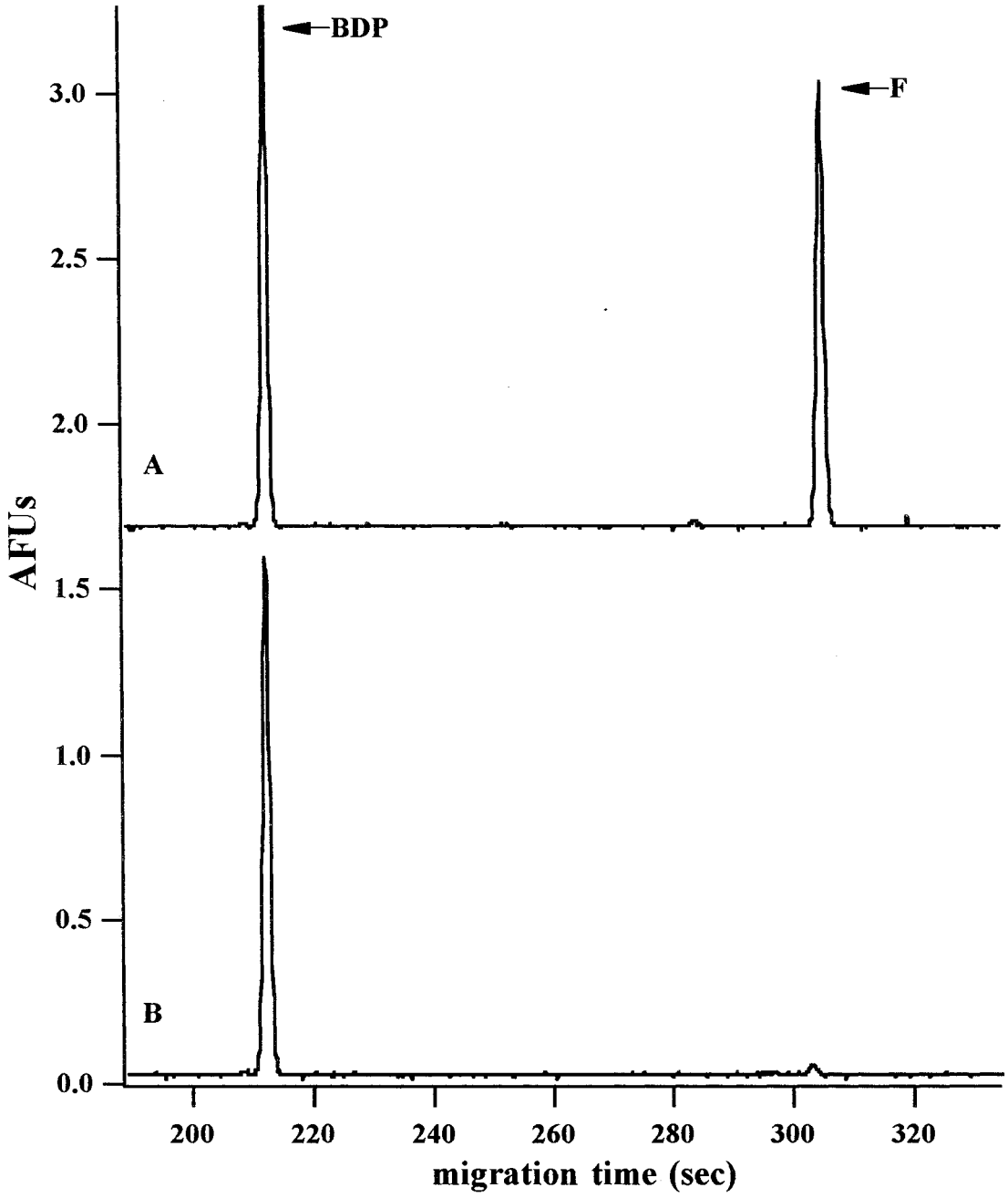

Figure 3.3 Electropherograms from the analyses of probe solution supernatants following incubation with magnetic particles which were pre-exposed to serum (A) and rabbit serum spiked with anti-fluorescein (B). Separations were performed in a 100 mM borate pH 9.2 buffer. Peak identities: BODIPY-FL (BDP); fluorescein (F).

evidence of matrix interference. Furthermore, these results demonstrate that the presence of specific antibodies, amongst a population of captured IgG, could be detected. Calculations based on the concentration range of IgG in serum from non-immune challenged rabbits (*ca.* 10-15 mg/mL [161-163]) indicate that the sample was comprised of approximately 0.5 % anti-fluorescein (relative to total sample IgG). Since concentrations of up to 1 mg/mL of antigen-specific IgG in serum are possible in hyperimmune cases [74], these results imply that the ICIS-CE technique could be regarded as an alternative to conventional indirect immunoassay techniques (e.g., indirect ELISA, Western blot). Additional calculations suggest that the sample initially contained >100-fold excess IgG relative to the binding capacity of the particles (*ca.* 150 picomole per 100 μL of particle slurry [160]). Of the subpopulation of antibodies captured by the particles, it is estimated that a low picomole quantity of immobilized anti-fluorescein was responsible for the observed immunosubtraction of fluorescein from the probe solution supernatant.

In addition to the assay's qualitative capabilities, relative quantification of specific antibodies is also made possible when excess sample antibodies are initially present in the capture step. In this case, a competition occurs between spurious and specific antibodies for a limited number of binding sites on the solid-phase. Hence, serum samples containing larger relative amounts of specific antibodies will ultimately produce greater probe subtraction in comparison to samples containing lower quantities. As such, the assay should be useful in not only detecting specific antibodies but also in monitoring the relative level (i.e., titer) of these compounds over the course of a host's immune response. An example of this aspect of the assay is exhibited in Figure 3.4 which displays representative electropherograms from the analyses of serum samples containing various quantities of anti-fluorescein. The peak signal data from these experiments were used to generate the calibration curve ($r = 0.996$) shown in Figure 3.5. An extension of the anti-fluorescein dilution in serum beyond *ca.* 0.03% anti-fluorescein, relative to all other IgG in serum, produced a plateau in BODIPY-FL/fluorescein peak signal ratio (data not shown).

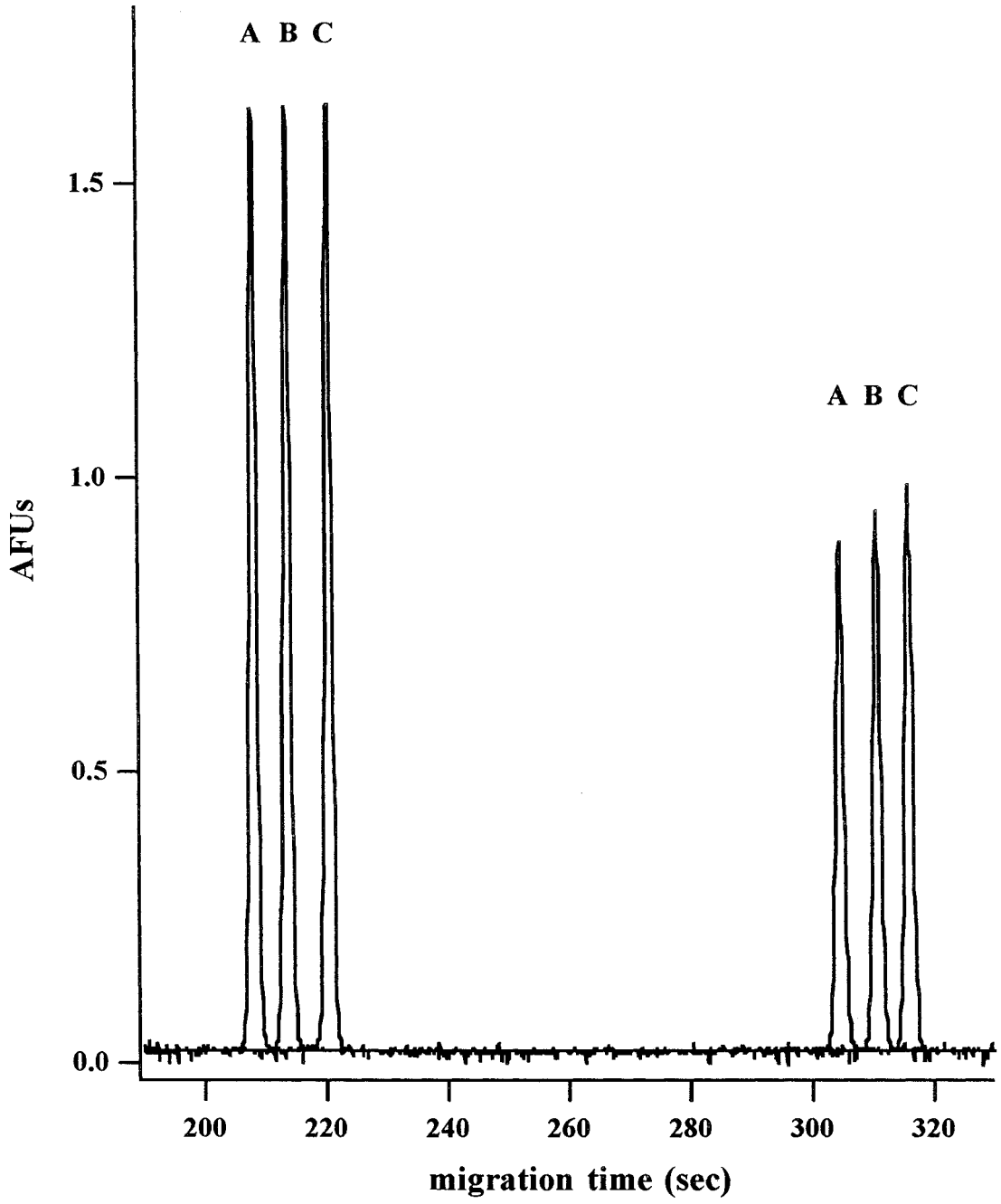

Figure 3.4 Probe solution supernatant electropherograms obtained from ICIS assays involving 300 (A), 600 (B) and 1000 μL (C) of serum spiked with 10 μL of anti-fluorescein stock solution. Separations were performed in a 100 mM borate pH 9.2 buffer. The B and C electropherogram migration times were shifted for clarity purposes.

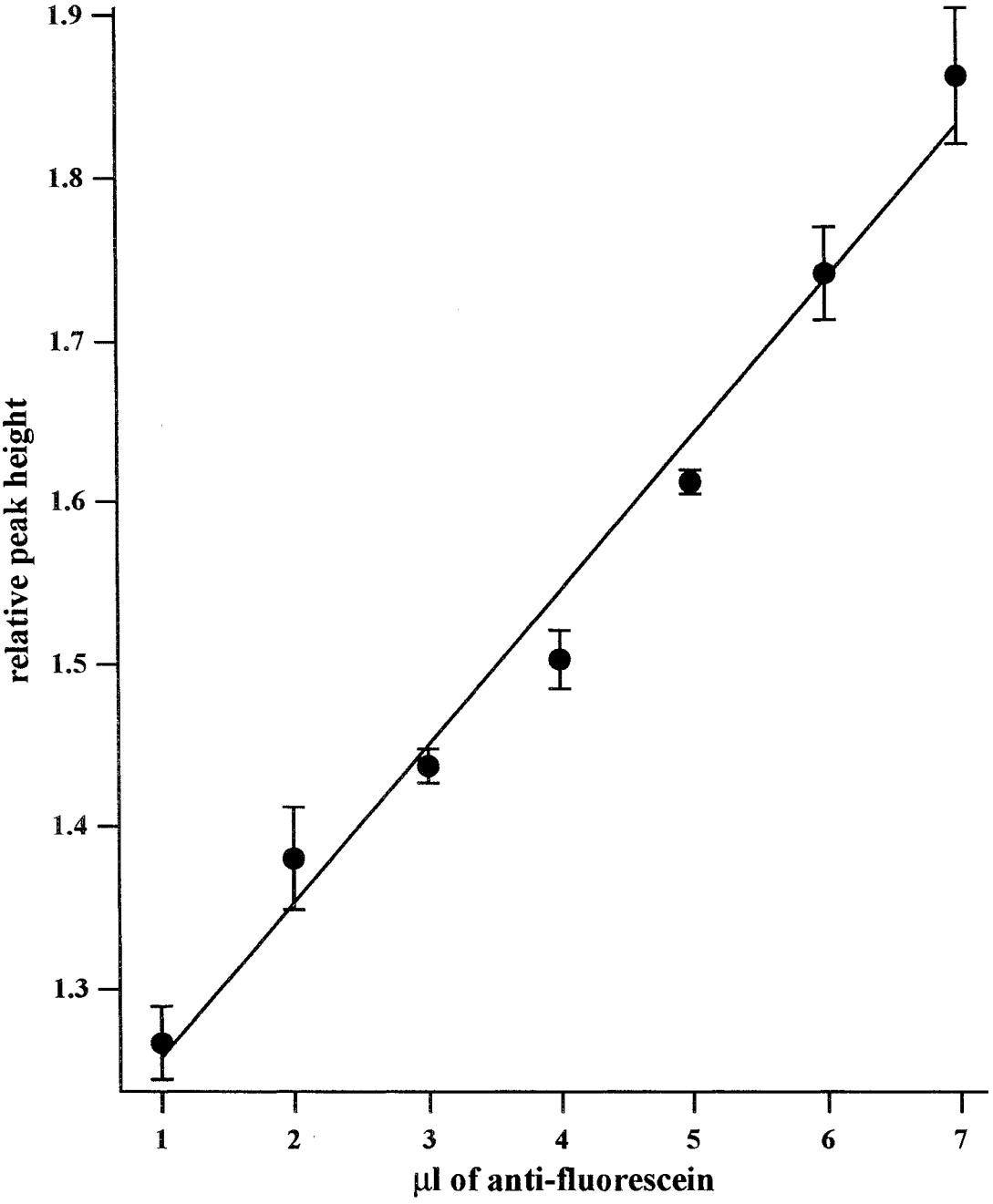

Figure 3.5 Immunocapture/immunosubtraction assay standard curve for the anti-fluorescein/fluorescein system. The data is present as BODIPY-FL/fluorescein peak height ratio values.

Additional experiments have shown that the detection of lower relative proportions of specific antibodies can be achieved by increasing the amount of solid-phase captured antibodies (i.e., by using larger amounts of particles in the capture step) and/or utilizing lower concentrations of antigen in the probe solution (to be published). Finally, intra-assay precision experiments involving a 435 μL serum sample spiked with 15 μL of anti-fluorescein solution produced a CV of < 1% (n = 4, data not shown).

3.5 Concluding Remarks

The development of indirect CE-based immunoassays has, as of yet, been relatively unexplored in the realm of diagnostic immunology. The model assay presented within represents an alternative to indirect assays now in use by clinical laboratories for the diagnosis of viral infections. The attractiveness of the demonstrated technique stems from the potential for high-throughput analysis through the use of existing microtiter plate robotics and fully automated multi-capillary CE-LIF systems capable of sampling from standard microtiter plates. The technique is also amenable to assay multiplexing. In theory, a probe solution consisting of a multitude of fluorescent-labeled antigens, representing various epitopes, could be used to probe for captured specific antibodies provided that appropriate separation conditions are found. This aspect of the immunosubtraction assay is presently being investigated by our group.

3.6 Acknowledgments

This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) and Fonds pour la formation de chercheurs et l'aide à la

recherche (FCAR).

CHAPTER 4

Peptide-Based Assay for Indirect Detection of Specific Antibodies using Capillary Electrophoresis and Laser-Induced Fluorescence Detection

Part 1 of 2: Analysis of Serum from Rabbits Immunized with Various Neuropeptides

Paquette, D.M., Skinner, C.D., Banks, P.R. Submitted to: *Analytical Biochemistry* (November 2002)

4.1 Abstract

The design and characterization of an indirect immunoassay based on immunocapture/immunosubtraction (ICIS) and capillary electrophoresis (CE) with laser-induced fluorescence detection is described. The ICIS-CE method was employed to detect specific antibodies in antiserum from rabbits immunized against the neuropeptides: bradykinin, neurotensin and substance P. Linear responses for antiserum differing in levels of anti-bradykinin and anti-neurotensin were obtained demonstrating the assay's usefulness for monitoring antibody titer. Furthermore, ICIS-CE was shown to readily function under multiplexed conditions by analyzing mixtures of antisera. Increased assay sensitivity was shown to correlate with increasing amounts of solid-phase-capture material and decreasing concentrations of fluorescent probe. Although an off-line ICIS-CE interface was utilized, assay design features such as solid-phase extraction with magnetic particles and microwell volume compatibility allow for facile integration with existing microplate robotics and multi-capillary instrumentation technologies. This proposed at-line configuration would promote the protocol to a fully automated assay with high-throughput capabilities.

4.2 Introduction

The complexity of biological matrices often necessitates the application of an analyte extraction step prior to chromatographic or electrophoretic analysis. In the past decade, a growing interest towards biofluid microanalysis has led to the development of methods that combine capillary electrophoresis (CE), a rapid and high resolution separation technique, with solid-phase extraction [43, 44, 80, 164]. In addition to removing interfering compounds, solid-phase extraction is well suited for CE since this process allows for analyte preconcentration, which in many instances is required due to the poor concentration

detection performance exhibited by the ubiquitous CE on-column UV/Vis detector. The process also reduces the concentration of salt, which can be significant in crude biosamples, associated with the analyte of interest. This is beneficial because high salt concentrations can be detrimental to CE separation efficiency [157]. Numerous types of non-selective and selective solid-phase extraction methods have been utilized in collaboration with CE, for example: C18, divinylbenzene, immobilized metal ions and lectins [43, 165-167]. In dealing with biofluids, however, it is often attractive to employ solid-support material having immobilized antibodies (i.e., immunoaffinity coating) as antibody-antigen interactions provide for efficient and specific analyte purification. Indeed, solid-phase immunoaffinity CE has been applied to measure various compounds within serum and/or urine including methamphetamine [168], immunoglobulin E [169], immunoreactive gonadotropin-releasing hormone [84], paraproteins [30, 81, 170], and cytokines [171].

In categorizing, from an instrumental point-of-view, the various solid-phase extraction-CE schemes that have been described, four levels of integration exist as defined and reviewed by Veraart *et al.*: in-line, on-line, at-line, and off-line [80]. In-line generally entails analyte extraction in the separation capillary path through the use of a device at or near the capillary inlet. Examples of such devices involving immunoaffinity interactions include a microsphere packed cartridge [168], a microcapillary bundle cartridge [172], a plug of magnetic beads [173], and a coated section of the capillary inner wall [171, 174]. Although promising for certain applications, this approach presently suffers from insufficient sample loadability, the need to frequently replenish or replace the device, and poor robustness when dealing with real samples. On-line and at-line solid-phase extraction CE set-ups involve coupling via a transfer line or a robotic interface, respectively. A limited number of on-line protocols have been reported due to the mechanical difficulties or poor CE separation efficiency associated with such instrumentation (references within [80]). The at-line system represents a compromise between ideal sample pretreatment handling and

robust CE analysis. Here, solid-phase extraction is performed externally and a plug of the resulting extract is introduced into the capillary in an automated fashion. The off-line mode is similar to the at-line mode but differs in that the reservoir containing the extracted analyte(s) is not robotically loaded into the CE instrument. The inherent simplicity and robustness of the off-line mode approach has made it popular amongst solid-phase extraction CE practitioners. However, since the off-line mode has the disadvantage of being laborious, the incorporation of at-line functionality for applications that require higher throughput is beneficial in many instances.

In relation to off-line immunoaffinity CE is a technique called immunosubtraction (IS). IS is routinely employed in the clinical laboratory for characterizing serum paraproteins associated with multiple myeloma [30, 81, 170]. Curiously, IS involves CE analysis of serum following a solid-phase extraction procedure, but the extracted material itself is not analyzed. With this method, a series of serum aliquots are placed within test tubes each containing Sepharose beads coated with a different specific immunosorbent reagent. Following incubation and bead sedimentation, a plug of the resulting supernatant is subjected to a CE separation. By comparing the CE protein profile of native and immunosubtracted serum sample aliquots, the type of protein that is being overproduced by the body is identified.

It came to our attention that the utility of IS could be extended, through the incorporation of a preceding immunocapture step, to include the detection of antigen-specific antibodies [175]. This technique, called immunocapture/immunosubtraction (ICIS) CE, is similar to conventional IS in that a particular type of serum protein is immobilized from the sample off-line onto a solid-phase. The technique differs from the conventional approach, however, since the IS analysis is performed on a standard solution comprised of selected fluorescent-labeled antigens and not on the serum itself (see: ICIS Procedure in Materials and Methods Section for details). Furthermore, a microtiter-plate-based protocol involving solid-phase extraction with magnetic particles was developed. These features

were included so as to make ICIS-CE compatible with existing microtiter plate robotics and multi-capillary instrumentation hence making the assay amenable to at-line integration and thus high-throughput analysis.

In order to demonstrate the feasibility of ICIS-CE for specific-antibody detection, an assay scheme based on rabbit antiserum analysis was designed [175]. In the first step (immunocapture), magnetic particles coated with goat anti-rabbit IgG are incubated with an antiserum sample in a microtiter plate well. The magnetic properties of the particles and their affinity for rabbit immunoglobulin G (IgG) allows for efficient extraction and purification of a population of rabbit antibodies from the sample. In the assay's second step, an IS procedure is applied to detect the presence of specific antibodies, amongst all of the particle-captured antibodies, through the use of an appropriate probe solution. In this paper, the qualitative, quantitative and sensitivity aspects of a peptide-based assay are presented. The utility of the ICIS-CE approach for multiplexed analysis is also demonstrated.

4.3 Materials and Methods

4.3.1 Chemicals

Monobasic sodium phosphate, dibasic sodium phosphate, borax, sodium hydroxide, casein and normal human serum were purchased from Sigma Chemical Inc. (St. Louis, MO). Partially purified rabbit polyclonal anti-fluorescein and BODIPY-FL were from Molecular Probes (Eugene, OR). Fluorescein-labeled neuropeptides (fluo-substance P, fluo-neurotensin and fluo-bradykinin) were kindly donated by Advanced Bioconcept (Montreal, QC). Rabbit polyclonal antiserum to neurotensin and bradykinin were obtained from Oncogene Research Products (Cambridge, MA) and Peninsula Laboratories Inc. (San

Carlos, CA), respectively. Purified rabbit polyclonal anti-substance P was acquired from SeroTec (Raleigh, NC) and normal rabbit serum from Biogenesis (Kingston, NH). Superparamagnetic particles coated with goat anti-rabbit IgG, supplied as a suspension of non-uniform 1 μm sized particles, were from PerSeptive Biosystems (Framingham, MA).

4.3.2 Buffers and Solutions

All buffers were prepared using purified water (18 MΩ specific resistance) from a Barnstead NanoPure System. Borate buffer pHs were obtained by the addition of concentrated sodium hydroxide. Phosphate buffer pH was adjusted to 7.4 by mixing appropriate volumes of 10 mM monobasic and dibasic sodium phosphate solutions. The wash and probe solution buffers, 10 mM phosphate/0.015% casein pH 7.4 (PC buffer), were prepared daily by dissolving 3 mg of casein in 20 mL of the phosphate buffer. A stock solution (100 mM) of BODIPY-FL was prepared in *N,N*-dimethylformamide (DMF) while individual stock solutions of the fluo-neuropeptides were made by dissolving the product vial content (2 nmole) in 5 μL of DMF with subsequent dilution to 100 μL with purified water. Aliquots of the BODIPY-FL and fluo-neuropeptides stock solutions were mixed and diluted to the indicated probe solution component concentration in PC buffer.

4.3.3 ICIS Procedure

A detailed description of the ICIS protocol has previously been described [175]. Briefly, a microliter volume of sample (e.g., serum) from a host suspected of containing antibodies to a particular antigen is placed into a microtiter plate well containing an aliquot of superparamagnetic particle slurry. Since the particles are coated with antibodies specific

for a certain class of antibodies produced by that host, a population of these antibodies from the sample is captured by the particles. Purification of the immunocaptured sample antibodies is subsequently performed through a series of wash cycles. A wash cycle includes: a magnetic separation, removal of the supernatant, and re-suspension of the particles in a wash buffer. Magnetic separation is accomplished by application of a strong magnet to the underside of the microtiter plate at the well location. Immobilization of the particles to the bottom of the well allows the supernatant to be extracted via micropipette. Re-suspension of the particles in a wash buffer is carried out with the aid of a brief vortex following displacement of the magnet from the bottom of the well. Detection of particle-captured antibodies specific for the antigen of interest is achieved by re-suspension of the particles in a solution comprised of fluorescent-labeled antigen (probe) and a fluorescent internal standard. Thus, IS of the solution-phase probe occurs with the presence of captured antibodies which recognize the probe. The decrease in probe concentration, relative to the internal standard, is detected using peak signals obtained by analyzing the probe supernatant with a CE system equipped with a laser-induced fluorescence (LIF) detector.

Past characterization of the ICIS protocol using a model assay indicated that immunocapture of host antibodies by the particles is complete with 2 cycles of 2 minute low speed vortex and 2 minute static incubation. Magnetic isolation of the particles from each of the samples, wash and probe solutions is accomplished within 5 minutes. It was also found that removal of most of the extraneous sample compounds occurs using three wash cycles (300 μL of wash buffer per cycle). Finally, IS by the particle-captured antibodies, which recognize the probe, is accomplished using 4 cycles of 2 minute low speed vortex and 2 minute static incubation, and one final magnetic separation for supernatant recovery.

4.3.4 Separations and Detection

The separation of probe solution constituents were performed by applying 25 kV at the injection end of a capillary using a ATI-Unicam (Mississauga, ON) model Crystal 310 system. Since this instrument is unable to perform injections from microtiter plate wells, an aliquot (170 μL) of probe solution supernatant from each assay well was transferred to 4 mL glass CE vials containing 170 μL of PC buffer prior to analysis. The increased volume was necessary in order to provide sufficient sample for injection. All injections were achieved by applying 30 mbar of pressure for 0.1 minute to the sample vials. Untreated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ) of indicated lengths having 50 μm ID and 186 μm OD were used for the separations. Detection was performed at a window approximately 10 cm from the grounded end of the capillary with a previously described LIF detector system [67]. The detection window was prepared by removal of the capillary polyimide coating (*ca.* 1 cm section) using a flame.

4.3.5 Antiserum Analysis

For anti-bradykinin detection in real sample conditions, seven microtiter plate wells containing 10 μL of normal human serum and 6 μL of bradykinin antiserum were individually spiked with 2, 3, 4, 5, 6, 7 and 8 μL of normal rabbit serum. The volume in each well was adjusted to 50 μL using PC buffer. The contents of the wells were thoroughly mixed and 50 μL of goat anti-rabbit IgG superparamagnetic particle slurry was added to each well. Particle-captured rabbit IgGs were probed for the presence of anti-bradykinin using 200 μL of a 5 nM fluo-bradykinin/5 nM BODIPY-FL solution. Anti-neurotensin containing samples were prepared by placing 1, 3, 7, 10, and 13 μL of normal rabbit serum in individual wells containing 3 μL of neurotensin antiserum and 15 μL of

normal human serum. Each sample volume was adjusted to 50 μL by adding the appropriate volume of PC buffer. The resulting solutions were vigorously agitated prior to the addition of 50 μL of superparamagnetic particle slurry. A 200 μL volume of probe solution comprised of 5 nM fluo-neurotensin/5 nM BODIPY-FL was subsequently used to probe for immunocaptured anti-neurotensin in each well.

4.3.6 Characterization of Assay Sensitivity

The effect of superparamagnetic particle slurry volume on assay sensitivity was investigated by incubating 25, 50, 75, and 100 μL of particle slurry with excess neurotensin antiserum in individual microtiter plate wells. Corresponding control experiments were performed by placing the same volume of particle slurry in normal rabbit serum. Relative quantification of the amount of particle-captured anti-neurotensin in each well was subsequently achieved using 200 μL of 5 nM fluo-neurotensin/5 nM BODIPY-FL probe solution. The effects of probe concentration on assay sensitivity was examined using 50 μL aliquots of particle slurry (per well) which were exposed to samples comprised of 15 μL of human serum spiked with 1.75 μL of neurotensin antiserum. The surface of particles in each well was probed with 5, 1, 0.75, 0.5, 0.1, and 0.05 nM fluo-neurotensin/5 nM BODIPY-FL solutions. The resulting IS was determined by comparing the fluo-neurotensin/BODIPY-FL peak signal ratio from the assays with those from the corresponding control experiments; i.e., particle slurry exposed to 16.75 μL of human serum.

4.3.7 Assay Multiplexing

Samples were prepared in separate microtiter plate wells containing 50 μL of PC buffer by adding one or more of the following: 1.5 μL of anti-substance P, 6 μL of anti-bradykinin, 3 μL of anti-neurotensin. For the control experiment, 10 μL of normal rabbit serum was placed within a well containing 50 μL of the PC buffer. Following brief agitation of the microtiter plate, 100 μL of magnetic particle slurry was added to each sample containing well. The detection of specific antibodies captured by the particles in each assay was performed using a probe solution comprised of fluo-substance P, fluo-bradykinin, fluo-neurotensin, and BODIPY-FL, each at a 1 nM concentration.

4.4 Results and Discussion

4.4.1 Anti-Peptide Detection

It was previously shown by our group that polyclonal anti-fluorescein within whole serum could be effectively detected with ICIS-CE using fluorescein as the probing compound [175]. The focus of this work was to demonstrate the detection of antibodies against compounds which more closely represent relevant biological antigens (e.g., viral protein epitopes). Short peptides were selected as probes for assay development since this class of compounds can be readily synthesized with sequences that correspond to major linear epitopes on immunogenic proteins and, unlike most proteins, can be obtained homogeneously labeled with a fluorescent tag at high purity. Preliminary characterization of probe solutions consisting of fluorescein-labeled peptides, neurotensin and bradykinin, and an internal standard (BODIPY-FL) revealed poor CE run to run reproducibility in terms of probe/internal standard signal ratios. This was due to slow adsorption of the peptides onto

the wall of glass CE vials between analyses. We found that adsorption was significantly reduced by incorporating 0.015% casein (wt/vol.) as part of the probe solution make-up (see Appendix C). Under these conditions, peak signal ratio reproducibility values of < 3% (CV, n = 6) were typically obtained. For our purpose, this level of probe solution precision was deemed sufficient for specific antibody detection.

Figure 4.1 displays ICIS-CE data from the analysis of bradykinin and neurotensin antiserum samples which were spiked with increasing amounts of normal rabbit serum. These results indicate that the detection of solid-phase-captured anti-bradykinin and anti-neurotensin was successfully achieved, through IS, using the corresponding probe solution. Furthermore, the electropherograms illustrate that the serum's antibodies undergo a competitive process during the immunocapture step when limited amounts of solid-phase binding sites are available. As the fraction of anti-peptide decreases there is less IS resulting in a larger probe peak. Indeed, the assay's ability to quantitatively measure, in a relative fashion, the degree of specific antibodies within different serum samples is exhibited by the calibration curves in Figure 4.2.

4.4.2 Sensitivity Considerations

The detection of reactive antibodies by IS inherently requires that a decrease in probe signal, relative to an internal standard signal, is registered. Since it is generally undesirable to search for a small difference between two large signals, two strategies that can contribute to increases in assay response were examined. The first involved the effects of varying probe concentration. In theory, the range for maximum assay sensitivity should be attained with an amount of probe similar to the amount of corresponding binding sites on the solid-phase. Indeed, a series of assays using a fixed amount of immobilized antibodies from neurotensin antiserum produced a sharp increase in subtracted fluo-

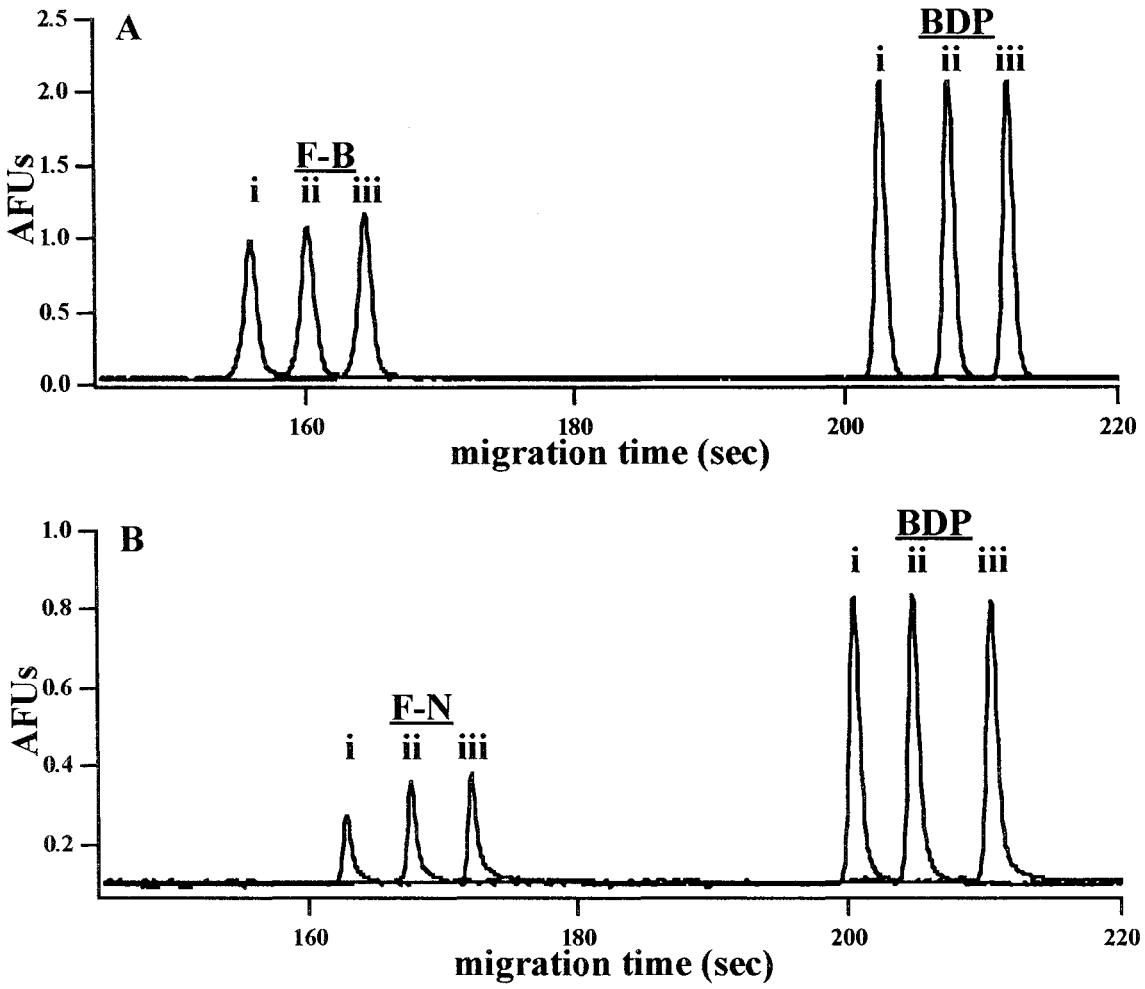

Figure 4.1 Detection of bradykinin and neurotensin specific antibodies at various serum titers using ICIS-CE. A) Samples composed of 6 μL of bradykinin antiserum and 10 μL of human serum spiked with i) 2 μL ii) 5 μL and iii) 7 μL of normal rabbit serum. Probe solution: 5 nM fluo-bradykinin (F-B)/5 nM BODIPY-FL (BDP). B) Samples containing 3 μL of neurotensin antiserum in 10 μL of human serum and i) 1 μL ii) 10 μL and iii) 13 μL of normal rabbit serum. Probe solution: 5 nM fluo-neurotensin (F-N)/5 nM BODIPY-FL. The (ii) and (iii) electropherogram migration times were shifted for clarity purposes. Separation parameters: 60 cm capillary, 50 mM borate pH 9.5 buffer.

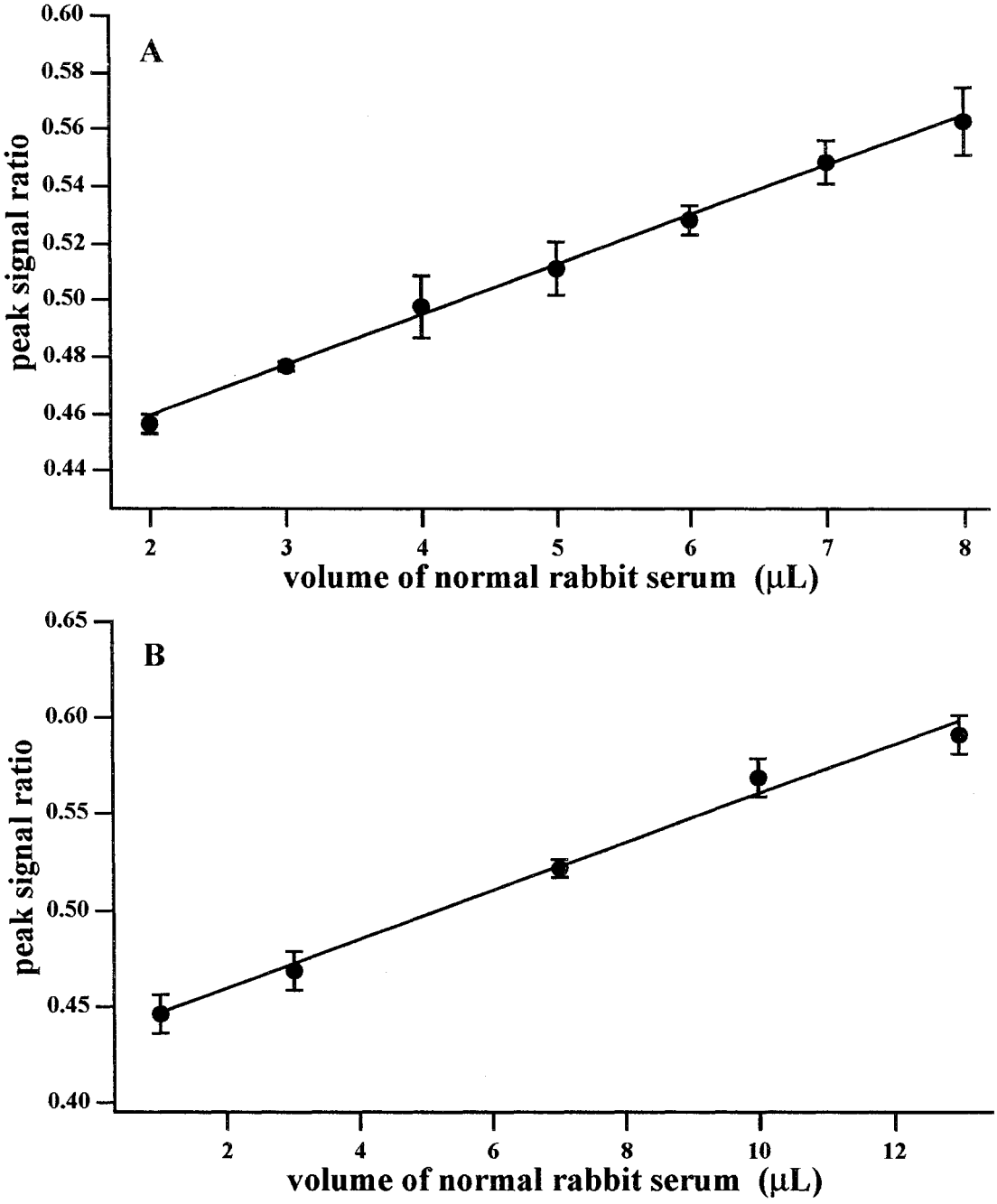

Figure 4.2 ICIS-CE calibration curves for A) anti-bradykinin (r = 0.999) and B) anti-neurotensin (r = 0.996) at various titers in serum. The ordinate scale values represent fluo-peptide/internal standard peak height ratios. Each data point is an average of three replicate analyses. The error bars are one standard deviation.

neurotensin when the concentration of this probe was lowered (Figure 4.3). However probe concentration cannot, in practice, be decreased indefinitely since sensitivity is also dependent upon the average dissociation constant (K_d) of the polyclonal antibodies for that probe. Basic equilibrium constant theory for protein-ligand interaction states that little complexation occurs when the concentration of protein or ligand is significantly below the system's K_d value [176, 177]. Thus, keeping in mind that antibodies produced early in an immune response usually have much lower affinity ($K_d < 10^{-4}$ M) than those produced later (K_d up to 10^{-12} M), an accommodation between probe solution concentration and average antibody affinity must be found in order to obtain the desired assay sensitivity. With the neurotensin and bradykinin antisera utilized in the assays described above, we estimate that average K_d values in the nM range were (at least) in effect. It should be noted that, generally speaking, the quantities of total antibodies captured with 50 μL of particle slurry from mature antiserum should provide concentrations of immobilized specific antibodies in the tens of nM when probed with 200 μL of labeled antigen solution. These calculations are based on the reported binding capacity of the particle slurry (0.2 mg/mL) [160], and that antigen-specific antibody levels of up to 10% of the total IgG population (*ca.* 13 mg/mL IgG in rabbits) can exist in serum [74].

The second parameter affecting assay sensitivity examined was the influence of particle slurry volume. By increasing the number of capturing particles within a well, a greater fraction of the total population of serum antibodies can be immobilized provided that there is excess an amount of antibodies in the original sample volume. This, in the end, increases the effective antibody concentration available to interact with the corresponding probe hence driving the formation of more complex (i.e., greater IS). As expected, the curve in Figure 4.4 illustrates that lower quantities of solid-phase capture material produces inferior IS results when a fixed concentration of probe is used. Although the total volume of standard microtiter plate wells (360 μL) limits the amount of particle slurry that can be placed in a well, we found that the manufacturer's slurry could be easily concentrated. That

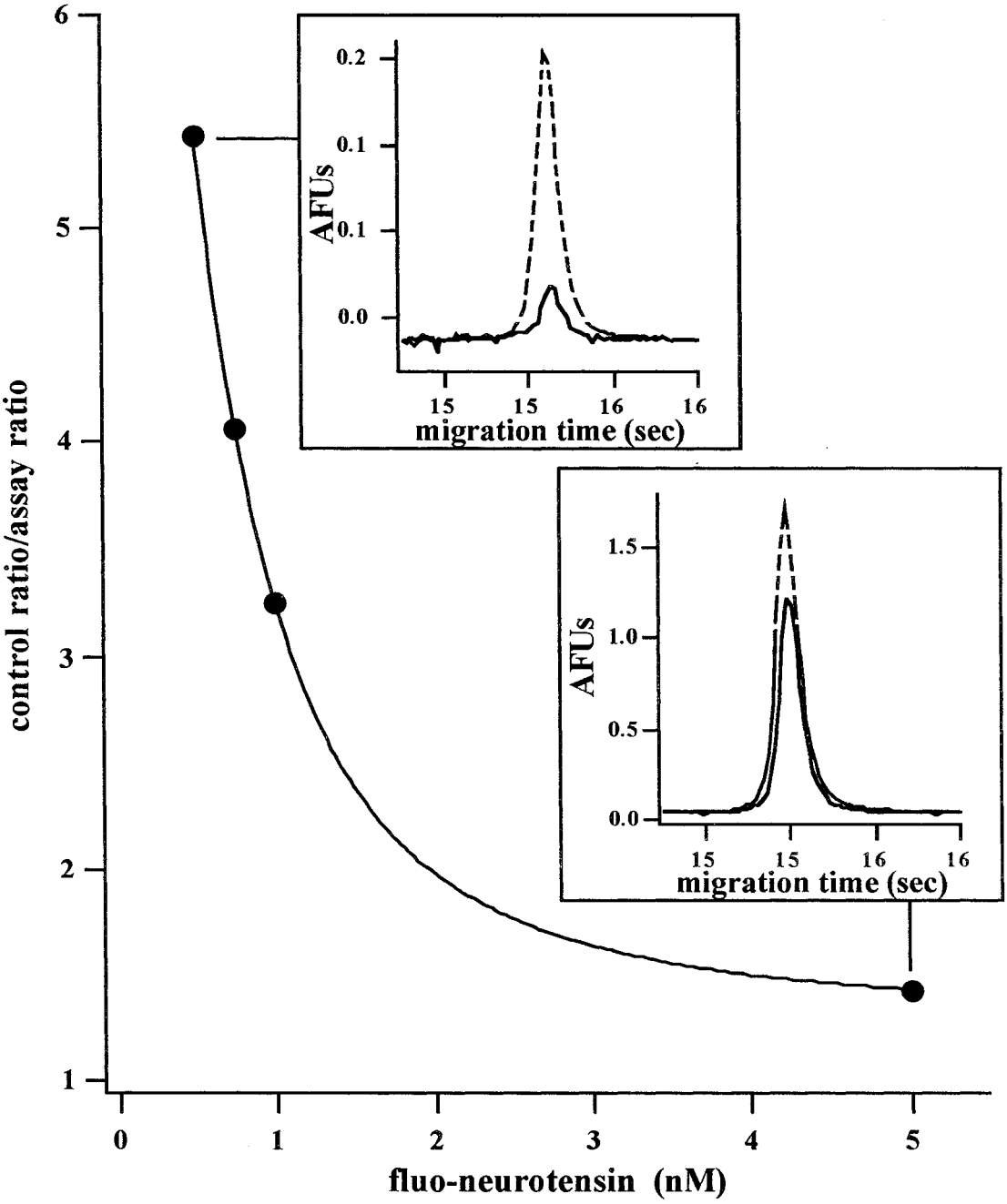

Figure 4.3 ICIS-CE assay sensitivity with respect to probe concentration. The insets are overlays of fluo-neurotensin peaks of an assay (solid trace) over the corresponding control experiment (dashed trace).

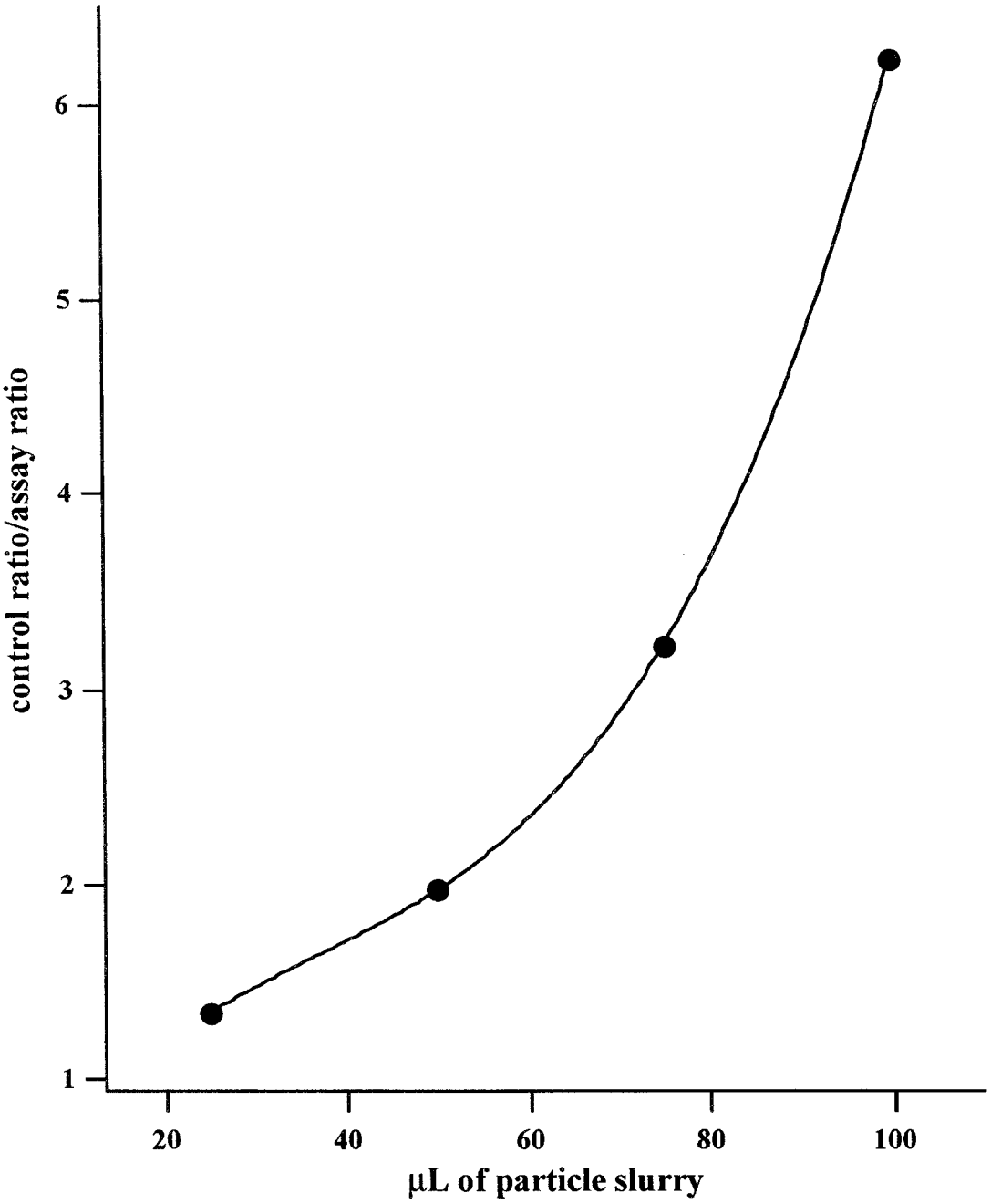

Figure 4.4 ICIS-CE assay sensitivity with respect to volume of superparamagnetic particle slurry. The presented data correspond to fluo-neurotensin/BODIPY-FL peak height ratios from assay and corresponding control experiments.

is, by isolating the particles to the bottom of the product vial with a magnet and subsequently drawing out a majority of the supernatant buffer, an almost ten fold increase in slurry concentration can be achieved. In fact, concentrated slurry has allowed us to enhance the detection of low concentrations of antibodies in antiserum against HIV p24 capsid protein (see Chapter 5).

4.4.3 Multiplexed Assays

An important attribute of the ICIS technique is that a number of different antibodies reactive toward different antigens can be simultaneously detected by a single assay. This is accomplished, via the separation power of CE, by employing a probe solution containing a multitude of labeled antigens. As an example, Figure 4.5 shows assay results from serum samples differing in anti-peptide content. Assay response to the sample make-up from this set of experiments, in terms of percent probe signal recovery, is presented in Table 4.1. Again, the degree of IS for that probe can be attributed to fraction of corresponding antibodies to the probe present in the sample when a limited amount of solid-phase binding site is used. In a separate experiment, IS of all fluo-peptides was observed when anti-fluorescein containing serum was assayed (data not shown). Consequently, these results suggest that ICIS-CE could be a useful approach for epitope mapping, cross-reactive studies, monitoring immunological maturation to a particular epitope, or for determining the specificity of antibody products from various vendors. The technique could also be applied as a test for diagnosing viral and bacterial infections. A probe solution consisting of peptides representing antigenic sites on proteins from different viruses (or viral strain), for example, would function as a screening assay. Moreover, a probe solution having an assortment of probes representing proteins from a particular virus might provide an attractive alternative to Western blot analysis - a laborious assay routinely used in clinical

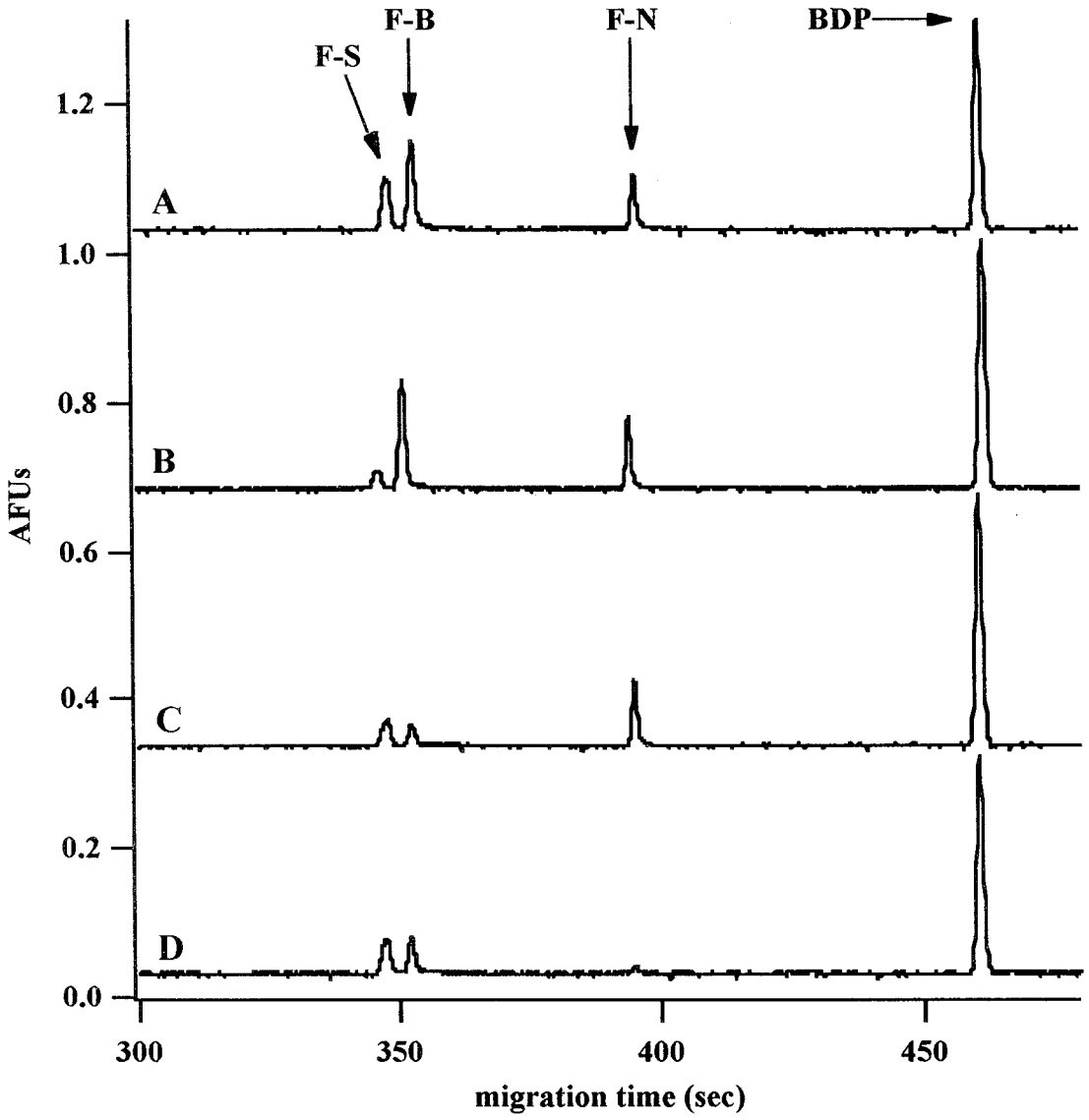

Figure 4.5 Multiplexing capability of the ICIS-CE approach for the detection of specific antibodies. A solution comprised of fluo-substance P (F-S), fluo-bradykinin (F-B), fluo-neurotensin (F-N) and BODIPY-FL (BDP), each at a 1 nM concentration, was used to probe the surface of the magnetic particles for specific antibodies. Superparamagnetic particles incubated with A) normal rabbit serum and serum spiked with B) anti-substance P; C) anti-substance P and anti-bradykinin; D) anti-substance P, anti-bradykinin and anti-neurotensin. Separation parameters: 90 cm capillary, 50 mM borate pH 10.0 buffer.

Table 4.1 Percent recovery of peptide probe/BODIPY-FL peak areas ratios, as normalized to the ratio of the control assay, with samples varying in antiserum content. The exhibited data were calculated from the experimental results illustrated in Figure 4.5.

Sample content	Signal recovery (%)		
	Fluo-substance P	Fluo-bradykinin	Fluo-neurotensin
10 μL serum (control)	100	100	100
1.5 μL of anti-substance P	27	101	101
1.5 μL of anti-substance P 6 μL of anti-bradykinin	43	25	99
1.5 μL of anti-substance P 6 μL of anti-bradykinin 3 μL of anti-neurotensin	64	47	-*

* Fluo-neurotensin peak could not be integrated due to the weak signal.

laboratories as a confirmatory test for the diagnosis of viral infections such as HIV. The feasibility of the latter application is under study by our group.

4.5 Conclusions

Solid-phase extraction of antibodies from serum with subsequent IS analysis of an antigen-containing probe solution was found to be an effective means for detecting specific antibodies. The method, termed ICIS-CE, can be completed within 1.5 hours, is devoid of sample matrix interference and has the capacity to perform quantitative measurements with respect to antibody titer. Given its multiplexing capabilities, microscale volume dimensions and amenability to automation, it can be envisioned that the ICIS-CE assay could become a valuable tool in the fields of both immunodiagnostics and bioresearch.

4.6 Acknowledgments

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CHAPTER 5

Peptide-Based Assay for Indirect Detection of Specific Antibodies using Capillary Electrophoresis and Laser-Induced Fluorescence Detection
Part 2 of 2: Analysis of Serum from Rabbits Immunized with the HIV Protein p24

Paquette, D.M., Skinner, C.D., Banks, P.R. Submitted to: *Analytical Biochemistry* (November 2002)

5.1 Abstract

Selected peptides representing human immunodeficiency virus (HIV) p24 protein epitopes, as recognized by the immune system of rabbits, were utilized as probes for detecting p24 antibodies by immunocapture/immunosubtraction capillary electrophoresis (ICIS-CE). Immunosubtraction of the peptide probes was observed when analyzing the serum of rabbits inoculated with p24. Neuropeptide and HIV gp120 epitope probes failed to display any immunosubtraction using the same serum demonstrating the specificity of the p24 probes for that protein. The obtained results suggest that ICIS-CE may, with further development, function as a third generation Western blot-like test for viral infection confirmatory diagnosis.

5.2 Introduction

From a historical perspective, the onset of immunoassays as a clinical tool for the screening of viral infections began by commercially licensed enzyme-linked immunosorbent assays (ELISA) that used antigens derived from whole virus lysate (i.e., first generation ELISA) [91]. Subsequently, ELISAs involving recombinant proteins as immobilized antigens (second generation) gained acceptance during the late 1980's as such tests were not subjected to antigen contamination by cell culture material. Further assay development ensued in the early 1990's with the application of synthetic peptides that emulate antigenic sites on viral proteins as the immunosorbent [178]. Immunoassays that employ synthetic peptides are in principle superior to protein-based assays because such tests are less costly and, in theory, more specific than assays that use whole proteins [179]. In fact, the application of synthetic peptides representing antigenic protein sequences has made the third generation indirect ELISA one of the most specific and sensitive

immunological screening methods for the human immunodeficiency virus (HIV) [180].

As stated in Chapter 1, the possible occurrence of false-positive HIV results by the indirect ELISA screening method necessitates positive diagnosis verification by Western blot (WB) analysis [181]. With the WB, an increase in serological detail is obtained through the interpretation of a developed band pattern produced by an ELISA-type analysis on a previously separated viral protein sample. Since the separation is done according to protein size, a defined immunological composition can be assessed based on the identity of the proteins.

Band pattern criteria (selective markers) for positive interpretation, in general, require that certain members of HIV's *gag* and/or *env* gene products be reactive to host antiserum [90, 182]. These proteins normally include p24 and p31 from the *gag* genomic region and gp120, gp41 and gp160 from the *env* genomic region. Interestingly, the WB has gone through a similar first to second generation evolution. However, little work devoted towards the development of a third generation WB has been reported [183]. As such, the focus of this chapter is to present preliminary results from the analysis of antiserum against HIV's p24 protein by ICIS-CE, a WB-like assay that uses synthetic peptides as indirect selective markers.

5.3 Methods and Materials

5.3.1 Reagents

Two > 95% purified α-amino fluorescein-labeled peptides representing HIV-1 IIIB p24 epitopes, that we called 15aa and 13aa (sequences: Ala-Phe-Ser-Pro-Glu-Val-Ile-Pro-Met-Phe-Ser-Ala-Leu-Ser-Glu and Gly-Pro-Ile-Val-Gln-Asn-Leu-Gln-Gly-Gln-Met-Val-His, respectively) were synthesized by NRC-Biotechnology Research Institute (Montreal,

QC). Two other highly purified peptides, also labeled with fluorescein at their α-amino terminal, representing HIV-1 IIIB gp120 epitopes were purchased from Sheldon BioTechnology Center (Montreal, QC): Val-Glu-Gln-Met-His-Glu-Asp-Ile-Ile-Ser-Leu-Trp-Asp (pep13) and Ile-Arg-Ile-Gln-Arg-Gly-Pro-Gly-Arg-Ala-Phe-Val-Thr-Ile (pep14). Fluo-neurotensin and fluo-bradykinin were generously donated by Advanced Bioconcept (Montreal, QC). BODIPY-FL and fluorescein, used as an internal standard, were obtained from Molecular Probes (Eugene, OR). Normal rabbit serum and goat anti-rabbit immunoglobulin G (IgG) coated superparamagnetic particles were from Biogenesis (Kingston, NH) and PerSeptive Biosystems (Framingham, MA), respectfully. Water utilized to make all solutions was obtained from a Barnstead NanoPure System (18 MΩ-cm). All other reagents (borax, monobasic and dibasic sodium phosphate, casein, *N,N*-dimethylformamide and normal human serum) were purchased from Sigma (St. Louis, MO).

5.3.2 Probe Solution Preparation and Analysis

Stock solutions of 10 mM 13aa, 15aa, pep13 and pep14 and 100 mM fluorescein were prepared separately by dissolution of the solid products in *N,N*-dimethylformamide (DMF). Fluo-neurotensin and fluo-bradykinin stock solutions were made by dissolving 2 nmoles of each component in 5 μL of DMF with subsequent dilution to 20 μM with purified water. All stock solutions were stored at -20°C until needed. Probe solutions were prepared daily by combining and diluting the appropriate stock solution aliquots in 10 mM phosphate/0.015% casein pH 7.4 buffer to the indicated component concentrations.

All probe solution analyses were performed on the CE laser-induced fluorescence (LIF) detector instrument described in Chapter 3. Unless indicated otherwise, probe solution components were separated by applying 25 kV to a 50 μm ID (180 μm OD)

untreated-fused silica capillary having a total length of 60 cm. Detection was carried out at 15 cm from the capillary outlet (cathodic end) through a 1 cm section where the capillary's protective polyimide coating was removed with a flame. Sample introduction was accomplished at the anodic end of the capillary by applying 30 mbars of pressure for 6 seconds to the sample vial. Unless indicated otherwise, 50 mM borate pH 9.2 was employed as the separation buffer. Between analyses, the following capillary washing and re-equilibration procedure was practiced: 1 bar for 30 seconds of 0.1 M NaOH, 1 bar for 45 seconds of separation buffer, and 1 bar plus 25 kV for 60 seconds of separation buffer.

5.3.3 Assay Protocol

All immunocapture/immunosubtraction capillary electrophoresis (ICIS-CE) assays were performed using the previously described protocol (see Chapters 3 and 4). In order to increase the degree of probe immunosubtraction, concentrated magnetic particle slurry was utilized for some assays. Preconcentration to the indicated level was typically achieved by removing the appropriate amount of supernatant from a 1 mL volume of slurry with a micropipette following magnetic immobilization of the particles to the bottom of a 1.5 mL centrifuge tube.

5.3.4 Calibration Curve

Samples were prepared in individual microtiter plate wells by combining 1.0, 2.5, 4.0, 5.5 and 7.0 µL of normal rabbit serum with 10 µL of p24 antiserum and 20 µL of human serum. For each sample, antibody capture was accomplished using 200 µL of 3 fold concentrated superparamagnetic particle slurry. A 200 µL solution consisting of 0.5

nM 15aa and 0.5 nM fluorescein was used to probe the surface of the particles in each assay well.

5.4 Results and Discussion

5.4.1 Probe Selection

The progressive investigation of ICIS-CE's effectiveness as an indirect immunoassay technique required that antiserum against a protein, as opposed to a hapten (Chapter 3) or a peptide (Chapter 4), be analyzed. As a model system, rabbit antiserum against p24, an HIV capsid protein, was chosen. This protein has traditionally been utilized as an indirect serological marker for HIV since anti-p24 antibodies appear early in human serum and remain at high concentrations until the commencement of acquired immune deficiency syndrome, or AIDS [85]. In addition to its diagnostic and prognostic implications, p24 has proven to be useful in animal model assay development since it has a highly conserved amino acid sequence, is immunogenic, and has well characterized epitopes in several species, including rabbits [179, 184-186]. As such, short linear antigenic sequences detected through Pepscan analyses were used [179], in collaboration with the known amino acid sequence of p24 (obtained from NCBI Entrez Protein Query database), to identify possible ICIS-CE probe candidates (see Appendix D). Sequence criteria included the absence of a cysteine residue(s) and the minimal presence of arginine and methionine residues. In general, short peptides incorporating cysteine(s) have been observed to produce variable and unstable CE separation results. This is likely attributed to cysteine oxidation [176]; i.e., the production of intra- or inter-peptide disulphide bonds during the course of repeated peptide solution analyses. A prerequisite for probe solution separation for the investigated assay was the use of high pH separation conditions since

fluorescein, which was used to tag the peptide probes, appreciably fluoresces only when in a dianionic form (> pH 8) [187]. However, the side chains of arginine residues are positively charged over a broad pH range and interact with the negatively charged wall of an untreated fused-silica capillary when in alkaline environment. This interaction leads to considerable peak tailing and thus increasingly poorer separation efficiencies for those peptides having one or more arginines as part of their sequence. For this reason, peptides containing arginine(s) were discriminated against. It should be noted that the buffer pH limitation brought upon by the use of a fluorescein tag could be readily overcome by labeling peptide probes with a pH insensitive dye such as BODIPY and tetramethylrhodamine based dyes [188]. Probes labeled by this fluorophor would bring the advantage of low pH separation where interactions between arginine (and lysine) residues and the capillary wall would be minimized due to the protonation of the capillary's inner surface silanol groups [19]. In addition to cysteine and arginine, peptide sequences with methionines were avoided because of this residue's susceptibility to be oxidized by air to sulfoxide and sulphone forms [176]. Such an amino acid modification was anticipated to negatively impact the recognition process between antibodies and methionine containing probes. To this end, peptides corresponding to the amino acid sequences 1-12 and 31-45 of p24 were chosen. These peptides, synthesized with a fluorescein tag at their α-amino terminal were found to produce single peaks when analyzed by CE-LIF at high pH (see Figure 5.1). With the incorporation of an internal standard and 0.015% casein (to reduce peptide/vial wall adsorption), variations in probe solution peak signal ratios of < 5% where typically obtained over the course of several hours of analyses (see Appendix E).

5.4.2 ICIS-CE Assays

A series of ICIS-CE experimental results illustrating the effectiveness of the selected peptides as probes for anti-p24 detection were conducted. In a preliminary study, a reduction in both 13aa and 15aa peak signal intensities was obtained with a p24 antiserum assayed sample when compared to a normal serum assayed sample (Figure 5.1). As previously mentioned, an advantage of the ICIS technique over the solution-phase CE approach is that nonspecific interactions are limited due to the incorporation of a selective extraction step in the former assay's protocol. Thus, probe subtraction was strongly attributed to specific interaction with p24 antibodies since anti-rabbit IgG coated magnetic particles (pre-blocked with casein) were used in the immunocapture step. Although the 13aa peptide was shown to serve as a useful probe for detecting antibodies directed towards p24, the relatively poor peak symmetry and signal exhibited by this compound with the utilized separation conditions encouraged further assay characterization with only the 15aa peptide.

It has been previously demonstrated that changes in antibody titer can be monitored with the ICIS-CE assay (Chapters 3 and 4). The immunosubtractive response of 15aa to p24 antiserum samples spiked with varying amounts of normal rabbit serum is illustrated in Figure 5.2. As expected, a linear increase in 15aa probe signal was obtained with samples containing decreasing percentages of anti-p24. It should be noted that this calibration curve, when compared to curves from previous model assays, was obtained at the expense of *ca.* 2 fold concentrated magnetic particle slurry requirement. That is, a greater amount of particles was needed per assay to obtain sufficient probe subtraction than what was utilized in the past. This is due to lower relative amounts of specific antibodies in antiserum for that epitope when a protein, which will typically have numerous antigenic sites, rather than the epitope itself (e.g., a neuropeptide, Chapter 4) is used as the immunogen. It is thus anticipated that even more concentrated magnetic particle slurry would be required in order

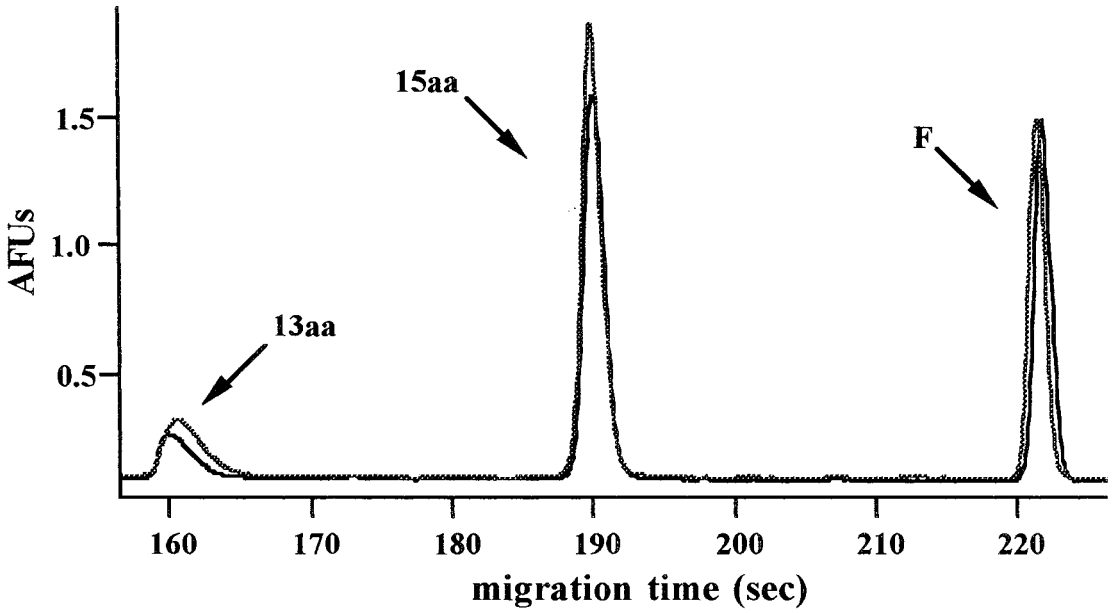

Figure 5.1 Detection of p24 antibodies in rabbit antiserum by ICIS-CE using a 10 nM 13aa, 4 nM 15aa and 3 nM fluorescein (F) probe solution. The overlaid black and gray electropherograms are the assay and control experiments, respectively.

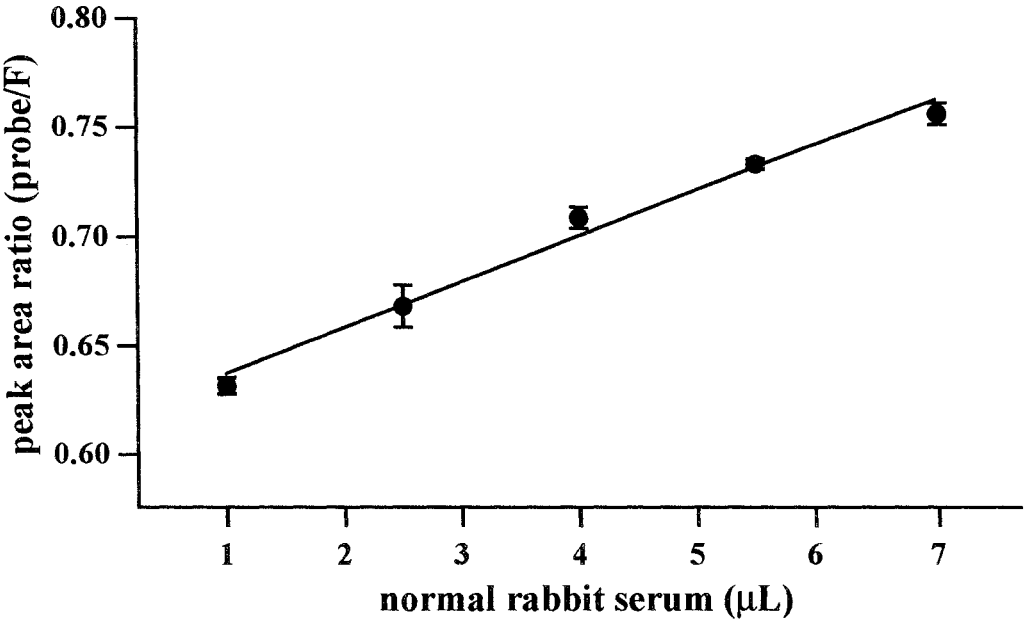

Figure 5.2 Calibration curve representing ICIS-CE assay response to 15aa probe immunosubtraction by antiserum varying in p24 antibody titer. Error bars represent one standard deviation.

to detect antibodies to a particular antigen site if whole virus was used as the immunogen.

An investigation into the 15aa probe's specificity for anti-p24 detection was carried out in two separate studies. In the first study, a solution consisting of the 15aa peptide, two fluo-neuropeptides and two fluorophors were used to probe the surface of antibody-capturing magnetic particles that had been pre-exposed to p24 antiserum. Figure 5.3 displays the electropherograms obtained from analysis of the probe solution supernatant for this assay and the corresponding control. Expanded views of the fluo-neuropeptide, fluorescein and BODIPY-FL overlaid peaks reveal little difference in peak signal intensities between the control and assay experiments for these compounds. The 15aa peptide peak overlay expanded view, however, indicates that specific immunosubtraction of this substance took place. In this case, the relatively weak subtraction of 15aa probe that occurred is due to the small volume of magnetic particle slurry that was used in the assay's immunocapture step (i.e., 100 μL, unconcentrated). The specificity of the 15aa peptide for anti-p24 detection was demonstrated in a second study with a solution that included fluorescein and two fluorescein-labeled peptides derived from antigenic amino acid sequences on HIV-1's gp120 protein [189]. As seen in Figure 5.4, subtraction of 15aa was obtained while no significant signal changes were recorded for all other probe solution compounds. The pep14 probe did, however, display relatively poor precision (>7%, see Appendix E) for both the assay and control experiments. This is presumed to stem from unfavorable separation conditions (i.e., alkaline buffer and an untreated-fused silica capillary) for this arginine residue-containing probe.

Together, the results discussed above indicate that an immunological response towards a particular viral protein can be readily detected by ICIS-CE. Since specific antibodies were detected using probes in sub-nanomolar range, these investigations also demonstrate that the technique may provide comparable detection capabilities to those of the standard WB (*ca.* 10^{-10} -10^{-11} M [74, 190]). In fact, detection limits in the low picomolar levels for fluorescein and fluorescein-labeled peptides are routinely obtained by the CE-LIF

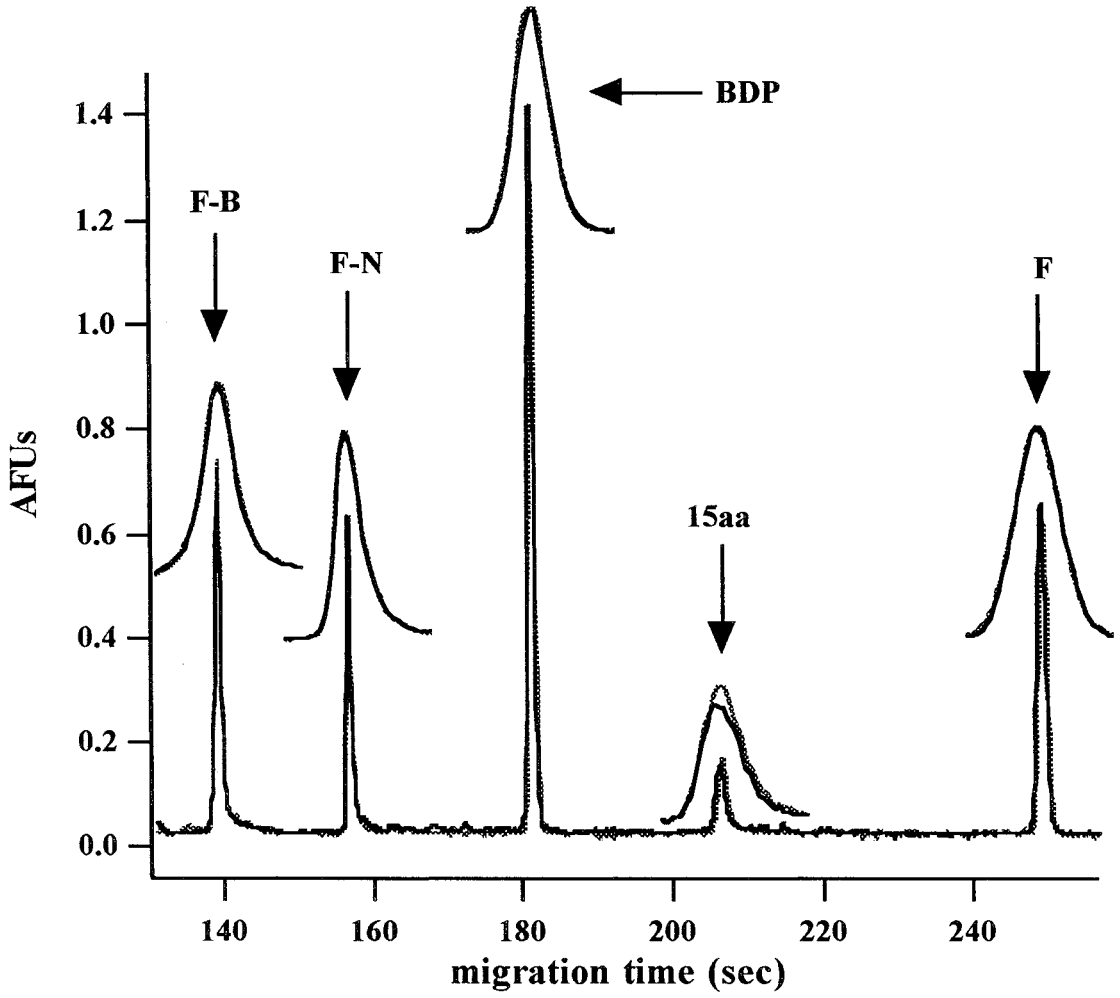

Figure 5.3 Specific immunosubtraction of the 15aa peptide, amongst various peptide and non-peptides fluorescent probes, by p24 captured antibodies. The control and assay wells were probed with a solution representing 0.5 nM of each component. The control experiment is represented by the gray trace. Separation was performed in a 90 cm capillary with a pH 10.0 run buffer.

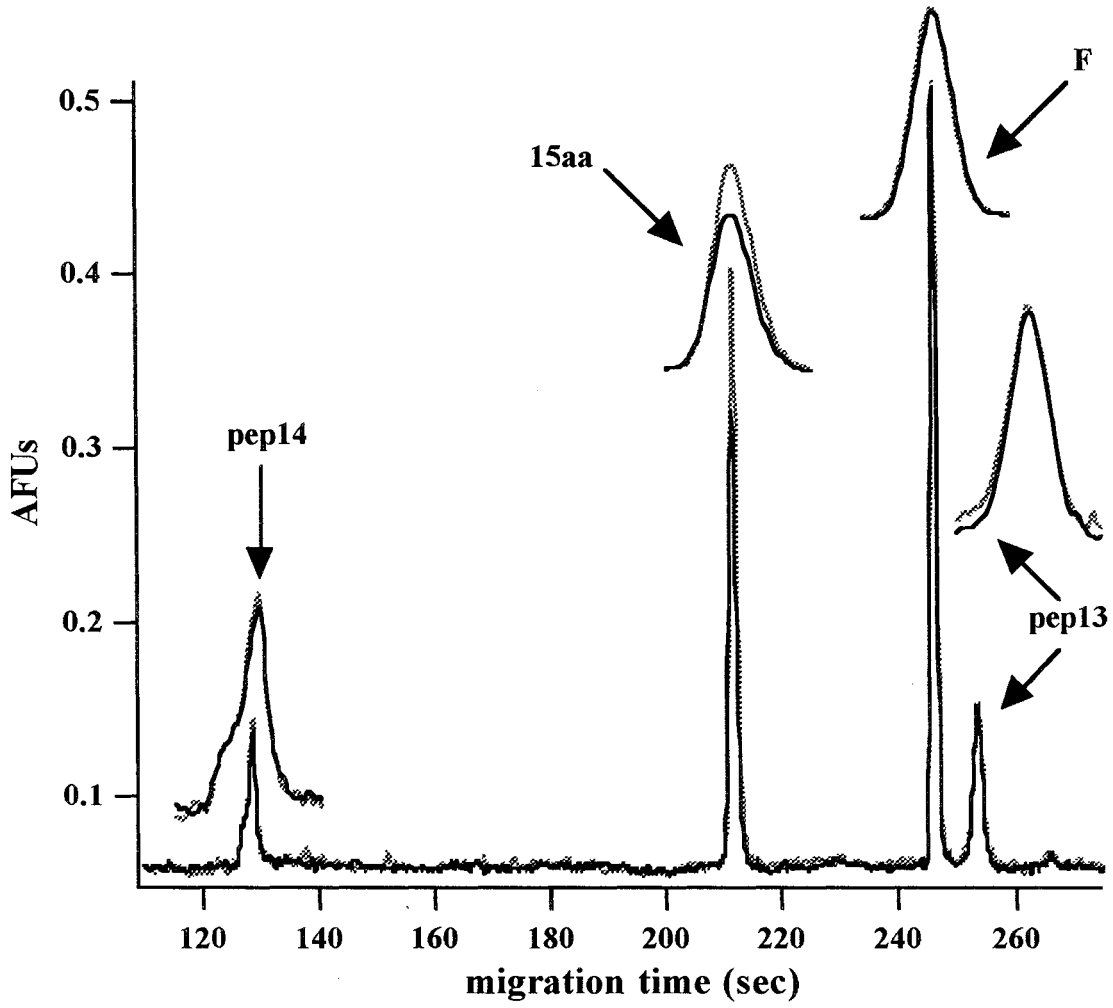

Figure 5.4 Demonstration of ICIS-CE assay specificity for p24 antibody detection using a probe solution consisting of peptide sequences representing p24 (15aa) and gp120 (pep13 and pep14) HIV protein epitopes and fluorescein (F). Probe solution component concentrations were 5, 1, 5 and 1 nM, respectively. The gray trace represents the control experiment. A 54 cm capillary was used for the separations.

instrument used in our laboratory. As such, the ability to detect specific antibodies by ICIS-CE may be limited by the level of antiserum maturation for that epitope (i.e., relative amount and average K_d of the antibodies), rather than the probe's limit of detection by LIF. Given this, ICIS-CE is expected to perform with, at least, similar diagnostic sensitivity and specificity to that displayed by the conventional WB. Thus, it is envisioned that the next step in characterizing and assessing ICIS-CE for use as a confirmatory assay would involve the analysis of serum of rabbits inoculated with whole HIV virus with subsequent assay performance as compared to the WB.

5.5 Concluding Remarks

The work presented in this chapter strongly suggests that the ICIS-CE assay, when combined with its multiplexing capabilities (Chapter 4), could provide serological details analogous to those obtained by the conventional WB. In addition to its potential function as a third generation confirmatory assay, several other features of ICIS-CE help to warrant its further development. In general, the WB is ill-suited for high-volume situations [182]. This is mainly due to the laboriousness and complexity of the WB protocol, and its lengthy separation, membrane transfer, incubation, and color development times. Thus, notable advantages of ICIS-CE over the WB are that the assay protocol is relatively simple, that antigen solution separation is achieved at a greatly reduced time scale, and that separation and detection are performed in one step. This decrease in protocol procedure not only saves time, which also manifests itself in cost effectiveness, but is expected to increase assay reliability. Finally, a notable attribute of the WB is that several samples may be processed at one time. This feature, as discussed in Chapter 4, could be equally realized by ICIS-CE through the use of existing automated laboratory instrumentation. Moreover, ICIS-CE can presumably be adapted to simultaneously determine the identity of viral sub-type infections.

Although the detection capacity is highly dependent on separation capabilities of CE for the chosen set of labeled antigens (e.g., HIV-1 peptide epitopes), a strategy based on antigens tagged with a different fluorophor (HIV-2 peptide epitopes) together with wavelength resolved detection would permit the use of highly complex probe solutions. This would undoubtedly increase the immunological fingerprinting capacity that could be achieved by a single assay and, in turn, enhance the overall efficiency of the diagnostic assessment with respect to viral sub-type infection.

5.6 Acknowledgments

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CHAPTER 6

Conclusions and Future Work

6.1 General Conclusions

The high resolving power and microscale dimension of CE make this separation technique an attractive bioanalytical tool. Despite these characteristics, CE often cannot be applied toward many clinical applications because it is not capable of separating the vast assortment of compounds that make up whole biofluids and/or provide the required LOD for a large number of clinically relevant analytes found within these samples. As such, the materials presented in this thesis described successful strategies that address the fundamental complexities associated with real sample analysis by CE. The two explored approaches share the common application of LIF for sensitivity and selective compound detection. By incorporating off-line solid-phase immuno-extraction as an additional assay protocol dimension, a highly effective method for determining the presence of compounds with affinity characteristics is readily achieved. Below is brief appraisal of these studies and their importance to the field of clinical diagnostics.

6.1.1 CE Analysis of Body Fluids by Selective Detection

The research in Chapter 2 demonstrates both the practicality and potential utility of CE-LINF in a clinical setting. As previously stated, the most beneficial aspect of LINF is the dispensing of labeling procedures normally associated with LIF detection. In addition to this favorable attribute, native fluorescence induction by laser excitation in an on-column arrangement is rugged and simple in instrumental design. It also offers a compromise between absorbance and derivatization/LIF detection schemes in terms of detectability performance for compounds that exhibit substantial native fluorescence when stimulated by UV radiation. It should be mentioned that biofluid analysis by CE-LINF brings about additional advantages that are not necessarily represented by the research encompassed

within Chapter 2. The technique has been shown to be useful for clinical samples that demand careful handling, that are limited in quantity, and/or that contain low analyte concentration (e.g., single cell content analysis [191-193]). For these situations, negation of a derivatization procedure for sensitive and selective detection greatly simplifies sample manipulation and makes the technique cost-effective, less susceptible to variations in sample pre-treatment, and does not lead to substantial sample dilution. Also, the LINF allows for sample preservation (i.e., only a few nanoliters of native sample per analysis are required) which is beneficial in cases where the clinical chemist desires to perform subsequent complementary tests on that sample. These characteristics, together with the disclosed investigations in Chapter 2, strongly suggest that CE-LINF could become an important tool for diagnosing and/or monitoring various diseases and metabolic disorders

A noted disadvantage of LIF detection for CE is that the price of continuous-wave (CW) lasers that emit at wavelengths shorter than 600 nm increases exponentially. The problem is further exacerbated for an LINF scheme because CW lasers that operate in the deep UV wavelength range (200-300 nm) are generally more complex those that function in the visible spectrum. Chapter 2 showed that a relatively simple and economical KrF laser discharging at 248 nm in a pseudo-CW fashion could be directly applied as a UV source for LINF. Compared to high power argon ion laser sources, which are typically used in LINF, added features are that this laser i) does not require frequency doubling of its emitted light ii) is air-cooled rather than water-cooled and therefore much less expensive, and iii) has a considerably smaller foot-print. Perhaps most impressive is that the LOD for tryptophan obtained by the investigated detection system falls within the same range as that attained by argon ion based LINF detectors. Overall, these characteristics appear to make the pulsed KrF excimer laser an attractive UV radiation source for biological sample analysis by CE-LINF.

6.1.2 Body Fluid Analysis using Solid-Phase Immobilization and CE-LIF

The amalgamation of off-column solid-phase immuno-extraction and CE-LIF analysis represents a particularly powerful methodology for complex sample analysis. The designed approach, termed ICIS-CE, brings together the robustness of classical immunoassays and the practicality of modern analytical instrumentation. The ICIS procedure not only provides for assay specificity, but also reduces the complexity of the sample introduced into the capillary (Chapter 3). The minimization of matrix interference, in turn, allows for multi-probe solution to be analyzed by CE-LIF with a high level of precision and sensitivity (Chapter 4). Most importantly, the projected capacity to interface multi-sample ICIS processing with multi-capillary CE-LIF analysis in an at-line fashion can be envisioned to greatly contribute to the efficiency of a clinical laboratory. Indeed, the concept of microtiter plate processing by a liquid handler coupled to subsequent multi-capillary analysis has been recently realized in the realm of DNA sequencing [194], which attests to the validity of the idea.

Through a series of experiments that gradually increased in clinical relevance, preliminary results showed that the ICIS-CE could be a promising alternative to the WB for specific antibody detection (Chapter 5). Although the assay was characterized using serum as antibody source, a similar assay protocol could be applied for detecting viral antibodies in saliva and urine. This approach is attractive as the collection of these fluids is non-invasive, rapid, economical and can be easily collected by minimally trained personnel [88, 195-197]. Also, the infectious capacity of oral and urine fluids is negligible when compared to blood [197]. In general, the amount of antibody in urine and saliva is less than in serum. The concentrations of saliva and urine IgG in healthy individuals, for example, have been measured to be in the range of 4.7-1200 nM (233 nM average) and 0.3-273 nM (25 nM average), respectively, as compared to 25-240 mM (100 mM average) in serum [198]. In HIV-infected subjects, the concentration of IgG in urine and saliva samples tend

to be slightly enhanced, ranging from 80-3000 nM in saliva and 3-700 nM in [198, 199]. ICIS-CE analysis is well suited for saliva and urine analysis since automated solid-phase immunocapture allows for host antibody pre-concentration through repeated sample application to the assay well, thereby compensating for the naturally low levels of antibodies in this fluid. The presented work also lays the foundation for further studies aimed at detecting antibodies for diagnosis of various disease states associated with immunological reactions. For example the assay is readily adaptable for specific IgE detection, which implicates the characterization of allergies in a patient [200].

6.2 Future Outlooks

Although the designed CE-LINF and ICIS-CE techniques were shown to be well suited for their intended niche applications, it is equally important to put the described work into perspective. Outlined in the following two sections are immediate concerns relating to the techniques and possible directions that may be undertaken to further enhance their performance for their intended purposes. A third and final section is included to give a brief outlook on the prospective adaptation of CE for the clinical laboratory.

6.2.1 LINF

It is clear that the instrumental simplicity and detection selectivity offered by LINF is an advantageous approach for analyzing biological samples. Indeed, CE-LINF related clinical applications continue to appear in the literature [191, 201-204]. To the best of our knowledge, however, no assays employing the described detector have been reported since our publication (i.e., Chapter 2). This indicates that the KrF laser is not recognized as an

adequate source for biocompound native fluorescence excitation in CE. Albeit less expensive than most common UV light emitting lasers, it is believed that the detection performance of a KrF-based LIF detector is not perceived as being sufficiently beneficial when considering the associated cost of the laser. This conclusion is supported by persisting publications on applications involving LINF detection using the large frame frequency doubled argon ion laser [191, 192, 203, 204], and the existence of an ongoing search for an alternate UV laser source. With respect to the latter, Chan *et al.* have described the utility of a solid-state laser (λ = 266 nm) for LINF detection after separation by CE [205]. Similar to the KrF laser, this laser provides a few mW of quasi-CW power allowing for nanomolar LODs to be attained for various indole-containing compounds. Moreover, the laser is thermoelectrically-cooled, highly compact, and reasonably priced making it a more attractive LINF excitation source. Another example is the recent use of an economical metal vapor NeCu laser (*ca.* $10,000 U.S.), operating at 248.6 nm, for LINF analysis of serotonin-related analytes within a single cell [203]. In this study, an LOD for serotonin was found to be comparable to that achieved with a frequency-doubled argon ion laser (27 nM versus 21 nM, respectively).

In consideration of the overall method of analysis, the LINF LODs obtained by the described on-column KrF-based detector may not be fundamentally low enough for many diagnostic markers. In order to achieve better detection sensitivity, UV-LINF systems involving the application of different lasers for particular classes of molecules are needed. For example, optimized excitation wavelength for indole-containing compounds and catecholamines using newly developed HeAu and HeAg air cooled lasers sources (291.8 and 224.3 nm, respectively) are expected to provide enhanced LODs for these species [203]. In addition to optimizing analyte excitation, measures to maximize fluorescence signal collection can be implemented. Although less straightforward than on-column detection, application of a sheath-flow cuvette detection cell (post-column) greatly reduces the amount of scattered light and background fluorescence gathered by the collection optic,

which ultimately leads to a substantial decrease in LOD (see Table 1.2). Another aspect to detector design that has been shown to be beneficial in terms of data collection, is the use of a charged-coupled device (CCD), rather than a PMT [192, 203, 206]. A CCD-based detector brings the notable advantage of wavelength-resolved fluorescence detection; that is, analytes having different emission profiles can be identified on the basis of both their electrophoretic migration and fluorescence spectra. In the end, it is the opinion of this author that continued demonstration of native fluorescence detection for real sample analysis using these instrument components will positively impact the acceptance CE-LINF as a bonafide clinical tool.

6.2.2 ICIS-CE

A logical continuation to the work presented in Chapter 5 would be to undertake an animal model study involving whole virus inoculation. Due to the vast number of antigenic sites typically associated with viral particles, a foreseeable problem with the ICIS-CE approach will be the presence of lower antibody fractions for the selected probes. Thus additional measures to augment ICIS-CE assay sensitivity will probably be needed. To increase the capacity of immobilized antibodies per assay, thereby increasing probe immunosubtraction (Chapter 4), larger volumes of concentrated particle slurry (i.e., > 500 μL) could be used in collaboration with a deep well plate. Also, probes comprised of two or more epitopes could be employed. This would result in pronounced subtraction since there would a greater proportion of immobilized antibodies to that probe. Such an approach would also capitalize on immunosubtraction through avidity interactions; that is, enhanced probe extraction by lower affinity antibodies via numerous simultaneous binding interactions. To an extreme, and in relation to the detection scheme described in Chapter 2, this concept could be applied towards a second-generation type immunoassay by using a

combination of whole recombinant viral proteins as a probe solution in concert with CE-LINF analysis. On the other hand, probable drawbacks would be some loss in assay specificity and sensitivity compared to the third-generation test developed in Chapters 3 to 5.

Given that the anticipated sensitivity issue can be overcome by the above suggestions, the suitability of the ICIS-CE assay as a confirmatory assay for viral infection diagnosis ultimately depends on its potential capacity to outperform the WB. A direct comparison between the conventional WB and automated ICIS-CE with respect to assay sensitivity, specificity and throughput using real clinical samples will be needed. As next steps, assuming that the ICIS-CE approach is found sufficiently advantageous over the WB, a method validation procedure would need to be performed (see Appendix F) and an investigation into the reliability of the indirect ELISA/ICIS-CE combination for diagnosing viral diseases would be required. Since the ICIS-CE was designed as a peptide based assay, making it more specific than the second generation-based WB assay, it can be anticipated that less overall indeterminate diagnostic test results (e.g., a 10-20% indeterminate rate with the indirect ELISA/WB has been reported [91]) would be obtained.

6.2.3 Projected Future of Clinical CE

Aside from the development methods that address the inherent difficulties associated with real-world sample analysis by CE, such as those presented in this thesis, a foreseeable advancement in the realm of microscale electrophoresis that will impact clinical CE is the evolving concept of microfluidic-based separations (as reviewed in [207]). Microchip technology allows for the integration of a dense array of extremely short separation channels within a single chip. In general, microfluidic chips offer the advantages of multi-capillary instrumentation with the added benefit of ultra-rapid analysis times.

Thus, the adaptation of LINF and ICIS to microfluidic-based separations, for example, can be expected to enhance the efficiency of such techniques as diagnostic tools – these providing potential avenues to more effective testing procedures and earlier disease detection.

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APPENDIX A

Clinical Applications of CE

Harvey, M.D., Paquette, D.M., Banks, P.R. *Journal of Liquid Chromatography & Related Technologies*, 24 (2001), 1871-1879.

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Introduction

Capillary electrophoresis (CE) is a relatively new analytical separation technique in the clinical laboratory. The clinically relevant attributes of CE include: high resolution, rapid analysis time, nanoliter sample volume requirements, reduced costs, and full automation. In turn, the CE user is faced with the task of obtaining physiologically relevant detection limits and avoiding sample matrix interference. For this separation technique to be utilized routinely in the clinical laboratory, selectivity, reproducibility, and throughput must match if not exceed that of traditional slab gel and HPLC-based techniques. In discussing the clinical applications of CE it is also necessary to draw the distinction between various sample types.

Biomolecules of clinical interest have been analyzed by CE in various sample matrices. The majority of work has focused on the detection of bioanalyte standards added to different buffers and complex solutions. These numerous studies provide an important framework for the development of CE-based clinical assays and demonstrate the immense potential for this technique in the clinical laboratory. Of greater interest to the clinical chemist, and the focus of this review, is the qualitative/quantitative determination of biomolecules endogenous to real human sample matrix. CE can be used effectively in the analysis of biomolecules found in real sample matrices such as serum, urine, cerebrospinal fluid, seminal plasma, saliva, cell plasma, and tissues. Presented herein are a variety of clinical applications of CE divided into three main categories: biofluid profile analysis, immunoassays, and clinical CE analysis using polymer sieving media. Future trends of CE in a clinical setting will be discussed.

Biofluid Profile Analysis

Serum

Electrophoresis in the clinical laboratory is routinely used for the diagnosis of myeloma, a malignant disorder distinguished by the presence of monoclonal protein in serum. Serum protein analysis has traditionally been performed by cellulose acetate electrophoresis (CAE) or agarose gel electrophoresis (AGE) under non-denaturing conditions. However, assets such as on-line UV detection, full automation, rapid analysis time and capillary array instrumentation has made capillary zone electrophoresis (CZE) an attractive alternative to CAE and AGE .

Due to the complexity of the serum protein population, components are normally separated into 6 characteristic regions, defined as γ, β_2, β_1, α_2, α_1 and albumin. Abnormalities in a serum profile are thus characterized by distinct peak shapes with diagnostic correlations obtained using the relative protein concentration in each region via peak areas. Since an augmentation of the diagnostic interpretation is often necessary, classification of the monoclonal protein is performed using immunosubtraction. Immunosubtraction is performed by incubating a serum sample with a solid phase having immobilized antibodies specific for a protein or a particular epitope on that protein. The recognized antigen is retained by the solid phase allowing for the determination of its identity through a comparison of the subtracted CZE profile with that of a control profile (Figure A-1).

Urine

Electrophoretic analysis of urine is performed primarily for the detection and quantification of Bence Jones protein, a protein associated with multiple myeloma, and to

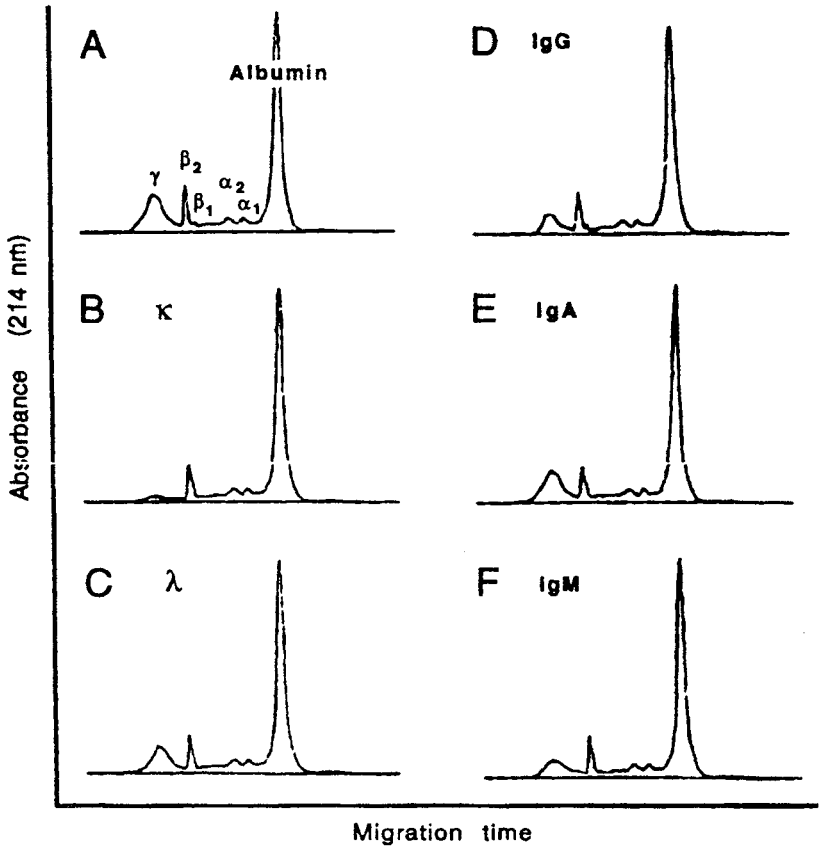

Figure A-1 Capillary zone electrophoresis analysis of human serum showing 6 characteristic protein zones (panel A). Identification of IgMκ monoclonal protein in the serum profile using sequential immunosubtraction procedures (panels B through F). Modified figure from Oda, R.P., Clark, R., Katzmann, J.A., Landers, J.P. in *Electrophoresis* (1997), 18, 1715, with permission.

detect and distinguish between glomerular and tubular proteinurias. Diagnosis of such conditions has, in the past, involved electrophoresis of concentrated urine using CAE, AGE or high-resolution AGE. UV detection of Bence Jones protein and proteins indicative of renal dysfunction (β_2-microglobulin, retinol binding protein and α_1-microglobulin) from unconcentrated urine vindicates the practicality of CE in the clinical laboratory. Analysis of various endogenous compounds in urine including amino acids, peptides, small ions and organic anions without sample pretreatment can also be successfully performed using the various modes of CE and a host of detection schemes.

Seminal Plasma

Seminal plasma profiles are useful in the assessment of accessory sex gland function. The seminal plasma levels of various proteins such as transferrin, prostate specific antigen (PSA), and albumin are of clinical importance. CE has been performed for the determination of small molecular weight compounds and the identification of transferrin, α_1-antitrypsin, albumin and PSA zones in seminal plasma. Though few examples of CE seminal plasma profiles exist, improved resolution and the use of immunosubtraction is expected to make these profiles clinically useful.

Cerebrospinal Fluid (CSF)

CE is ideal for CSF analysis due to its many advantages over the traditional separation of CSF components using AGE. Staining and destaining procedures are unnecessary, profiles are obtained on the order of minutes versus hours, and little CSF (microliter volume) is required. CSF protein electrophoresis is clinically important in the detection of oligoclonal bands associated with multiple sclerosis. CE is effectively used in the diagnosis of multiple sclerosis by providing high resolution of these oligoclonal bands

in the γ-globulin region of a CSF profile. This separation technique has also been demonstrated useful in rapid screening of multiple sclerosis without the need for sample preconcentration.

Cell Plasma

Analysis of the contents of single human cells is yet another example of the clinical utility of CE. Since the inner diameter of a capillary is typically less than 100 μm, one can position a single cell in the end of a capillary where it is lysed, its contents derivatized and separated electrophoretically. The low concentrations of intracellular species necessitate the use of detection schemes such as laser-induced fluorescence (LIF). Quantification of proteins, enzymatic activity, ions and small biomolecules within single human erythrocytes can be achieved using CE. Perhaps the most common use of CE in a clinical setting for the analysis of cellular contents is in screening for hemoglobin variants (Figure A-2). The hemoglobin profiles of patients can be obtained in under 20 minutes, providing important information on hemoglobinopathies, diabetes, and thalassemia syndromes. Furthermore, the use of CE for obtaining cell profiles is attracting considerable attention in the realm of functional genomics and the early diagnosis of disease.

Immunoassays

Immunoassays provide a means for clinical laboratories to specifically and quantitatively measure minute amounts of analyte (antigen) in complex sample mixtures. Immunoassays center about the use of antibodies raised against a clinically relevant molecule. Such immunoglobulins inherently exhibit high specific binding affinity for their antigen. The high sensitivity of immunoassays stem from conjugation of antibodies or

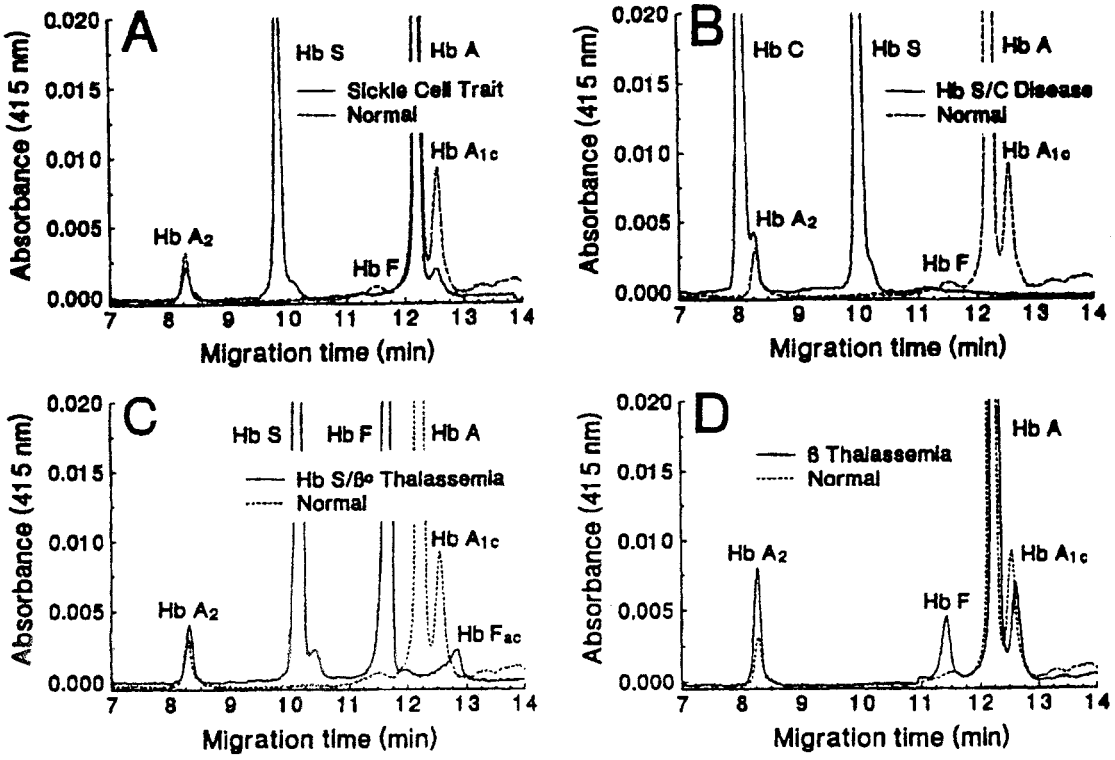

Figure A-2 Comparison of hemoglobin variants in normal blood and those from blood from subjects with sickle cell traits (panel A), Hb S/C disease (panel B), Hb S/β^{o}-thalassemia (panel C), and β-thalassemia (panel D). Figure and caption from Hempe, J.M., Craver, R.D. *Clinical Chemistry*, 40 (1994), 2288, with permission.

antigens with labels based on fluorescence, chemiluminescence, radioactivity, or enzymatic amplification.

Two modes of immunoassays exist for CE: competitive and direct. Competitive immunoassays typically involve the use of an antigen conjugated to a highly fluorescent dye and an antibody, or a fragment of an antibody, which recognizes that antigen. Briefly, a set amount of antigen-dye conjugate is added to the sample of interest followed by the addition of a known quantity of antibody. Incubation of the resulting solution allows for endogenous antigens to compete with labeled antigens for a limited number of antibody binding sites. CE-LIF analysis of a nanoliter-size sample aliquot provides separation and detection of the free labeled antigen and the fluorescent antigen-antibody complex. A calibration curve using the signal intensities of the free labeled antigen, the fluorescent complex or their ratio can then be constructed. Quantification of the steroid hormone cortisol in human serum, which can indicate adrenal malfunction, is an example of a real sample competitive CE immunoassay.

Direct CE immunoassays rely on the use of fluorescently labeled antibodies or antibody fragments (called affinity probes). In this approach, an excess quantity of affinity probe relative to the antigen is incubated in the sample solution to ensure complete antigen capture. Following the injection of a small sample aliquot into the capillary, an electrophoretic separation of the excess affinity probe and fluorescent complex is performed. LIF detection of the fluorescent complex provides a means to determine antigen concentration via peak intensity and a pre-constructed calibration curve. The measurement of human serum immunoglobulin A concentration has been described using this method.

The utility of CE-based immunoassays in a clinical setting is, to date, limited. The use of labeled antigens for competitive immunoassays has been confined to assays involving small antigenic molecules which contain few derivatizable sites. Furthermore, the complex chemistry associated with the homogenous labeling of antibodies and antibody fragments and the difficulty in separating bound from unbound affinity probes has made

direct assays nontrivial. Despite these drawbacks, CE in the realm of clinical immunoassays remains attractive. This is due, in part, to advantages over conventional immunoassays that include the dispensing of sample clean-up and washing protocols, low reagent consumption, reduced incubation period, rapid analysis and the potential for simultaneous assay of multiple analytes.

Clinical CE Analysis Using Polymer Sieving Media

Polyacrylamide and/or agarose slab gel electrophoresis is commonly used in the clinical laboratory for the separation of proteins and nucleic acids. Both these techniques bear significant time and labor costs and are, at best, semiautomated. CE is proving to be a viable high-throughput alternative, particularly in the realm of genetic analysis. The use of polymer solutions (non-gel) in CE allows for the separation of bioanalytes according to differences in mass. The major advantage of polymer sieving media over chemical gels is that they can be easily replaced after every analysis. In addition, several sieving media have been developed and the separation conditions optimized for the rapid, high resolution (single-base substitution) separation of DNA restriction fragments and polymerase chain reaction (PCR) products. Detection schemes using LIF and fluorescent labeling reagents provide an alternative to time consuming staining/destaining procedures and isotopic labeling. Typical methods for the detection of polymorphisms in the human such as restriction fragment length polymorphism (RFLP), variable tandem repeats (VNTR), microsatellite analysis, and single strand conformation polymorphism (ssCP) can be readily performed using CE. In this section, emphasis is placed on the analysis of clinically relevant diagnostic nucleic acids obtained from cells of human patients.

CE has been used effectively in the analysis of RFLP products for the DNA diagnosis of diseases such as cancer, cystic fibrosis, Alzheimer's, and Duchenne muscular

dystrophy. CE-based ssCP analysis of the p53 tumor suppresser gene from white blood cells of normal and multiple myeloma patients can be used in the detection of p53 gene mutations and in genetic screening for cancer. Differences in the number of base-pair repeats can be determined by VTNR analysis using CE. This has been applied to the quantification of repeats in human apolipoprotein B alleles for assessing the risk of coronary heart disease. Genomic instability of specific short tandem repeats (microsatellites) at specific loci in the human genome can be determined by CE. For example, the CE profile of PCR products from tumor and normal DNA of a colorectal cancer patient can be obtained in less than 15 minutes. Other diseases to which microsatellites CE analysis has been successfully applied include Kennedy disease, cystic fibrosis, and pre-natal diagnosis of Down's syndrome.

As a final note, CE is also applicable to the analysis of antisense DNA in biofluids such as serum and urine, and reverse-transcriptase PCR products for the detection/quantification of various viruses such as Hepatitis C and HIV-1. Of particular interest is the adaptation of such genetic analyses to high-throughput CE instrumentation as has been done successfully in the case of DNA sequencing.

The Future of CE in the Clinical Laboratory

Although CE has proven to be competitive with conventional instrumentation methodologies, it remains to be seen whether CE-based assays will displace existing clinical assays. An increase in acceptance for CE in the clinical laboratory will require the development of assays having high accuracy, precision, robustness and appropriate dynamic ranges. From an instrumental point of view, foreseeable developments in CE technology that will have a dramatic impact on its promotion to the clinical laboratory include improved limits of detection, ultra-rapid analysis time, and high sample throughput.

The issue of detecting dilute analytes in biological fluids has been addressed by several research groups. On-line solid-phase preconcentration (spPC) through the use of bead containing microcartridges or membranes having non-specific or specific affinity coatings have been shown to enhance sensitivity by up to 1000 fold. A unique feature of spPC is that sample clean-up/desalting allows for additional information on the analyte when coupled to mass spectrometric, nuclear magnetic resonance or circular dichroism detection schemes.

The advent of microchip CE holds great promise for ultra-rapid analysis in the clinical laboratory. The fabrication of narrow channels within glass or fused silica substrates is made possible through the application of photolithography and chemical etching techniques. Due to the open geometry of the microchip, very short separation distances can be used resulting in exceptionally fast sample component separations. For example, analysis of human serum samples subjected to a competitive immunoassay for cortisol was shown to be complete in under 30 seconds. Microchip technology also allows for the integration of dense channel arrays within a single chip, thereby providing a means for high-throughput sample analysis.

The concept of arrays in the capillary format, catering to high throughput in a clinical setting, has been realized by the recent introduction of a commercial 7 capillary CE instrument specifically designed for serum protein electrophoresis analysis. The routine use of CE in the clinical laboratory will largely depend on the development of such high-throughput technologies.

Suggested Reading

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APPENDIX B

Sample Matrix Interference

Observations and Discussion

Effects of sample matrix interference and electrodispersion in CE are illustrated in Figure B-1 when dealing with complex biological fluids such as serum. Undiluted, 10-fold and 100-fold diluted rabbit serum solutions (492.5 μL final volume) were spiked with 5 μL of a 0.1 μM fluorescein solution and 2.5 μL of a 1 μM BODIPY-FL solution. The resulting solutions were briefly vortexed and subsequently analyzed by CZE. Although electrodispersion of the fluorescent probes zones can be minimized by increasing the run buffer ionic strength relative to the ionic strength of the sample (Figure B-1, trace B to A), elimination of background signal interference and/or nonspecific probe interactions with sample components requires serum dilution by at least 10 fold (trace C to B). Rabbit serum not spiked with BODIPY-FL and fluorescein produced similar results to trace C (data not show).

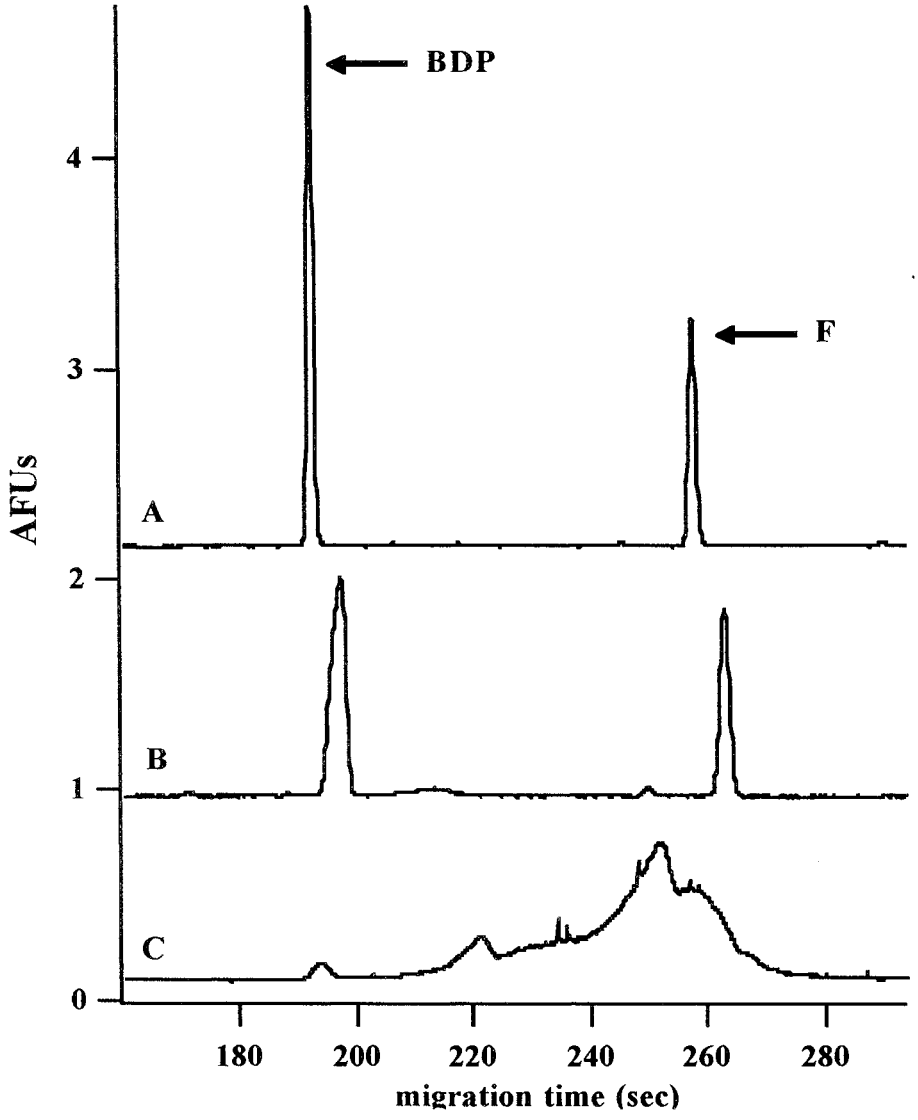

Figure B-1 Sample matrix effects on BODIPY-FL (BPD) and fluorescein (F) in 100 (A), 10 (B) and 0 (C) fold diluted rabbit serum. The BODIPY-FL and fluorescein in each case were at final concentrations of 5 and 1 nM, respectively. The separation buffer was 50 mM borate pH 9.5.

APPENDIX C

Neuropeptide Adsorption

Observations and Discussion

The utility of ICIS-CE as a viable assay requires that any reductions in peptide-based probes be the product of interaction with specific antibodies captured by magnetic particles, and not due to non-specific adsorption to the surface of sample vials, microtiter plate wells, or the magnetic particles. Preliminary characterization indicated that all three fluo-peptides were adsorbed by the walls of glass sample vials. Fluo-neurotensin was found to be particularly susceptible to this process (see Figure C-1). The adsorption effect was found to be even more pronounced with sample containers made of plastic (i.e., 1.5 mL centrifuge tubes and microtiter plate wells). As such, an extensive investigation aimed towards finding an effective blocking protocol to obtain stable and reproducible peak signals for nM fluorescein-labeled neuropeptide containing probe solutions was undertaken. Blocking solutions comprised of 10 mM phosphate pH 7.4 with bovine serum albumin (BSA), bovine γ-globulin (BGG), casein and casein hydrosylate ranging from 0.005% to 0.1% (wt/vol.) were assessed.

Investigated were the effectiveness of glass and plastic sample vial pre-blocking (overnight incubation, followed by ambient temperature drying) with the inclusion of a blocking agent as part of the peptide probe solution makeup. This combination was found to significantly reduce peptide adsorption to both plastic and glass containers (see Figure C-2 for an example). Additional characterization showed that the pre-blocking procedure could be bypassed by merely including a blocking agent in the probe solution. Furthermore, concentrations of larger than 0.015% blocking agent were found to produce a sustained plateau in peptide/internal standard ratio. Having determined a means to peptide minimized adsorption, focus was placed on reproducibility. Although probe solutions that incorporated 0.015% BSA, BGG or (to a lesser extent) casein hydrosylate were found to produce respectable precision for peptide probe/internal standard peak height and area ratios (i.e., < 10%), probe solutions containing casein repeatedly produced slightly superior

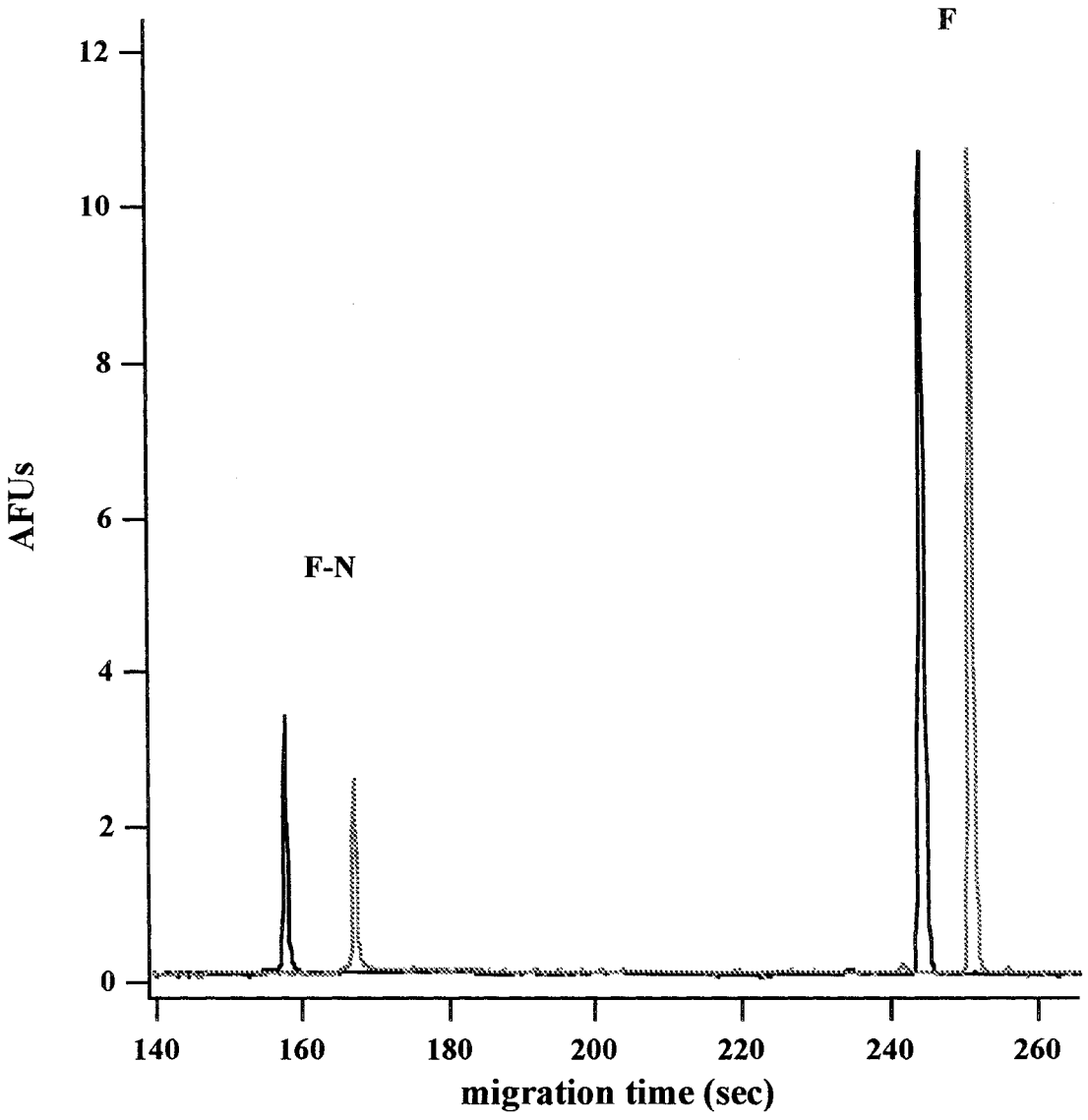

Figure C-1 Example of temporal adsorption phenomenon observed for fluo-neurotensin in an unblocked glass sample vial. A 10 nM fluo-neurotensin (F-N)/5 nM fluorescein (F) solution in 10 mM phosphate pH 7.4 buffer was analyzed immediately after preparation. The black and gray traces represent the first and third analyses from sequential runs, respectively. The gray electropherogram was offset for clarity purposes. Separation were performed in a 25 mM borate pH 9.25 buffer at 25 kV using a 45 cm (effective length, 60 cm total length) capillary having a 50 μm ID and 186 μm OD.

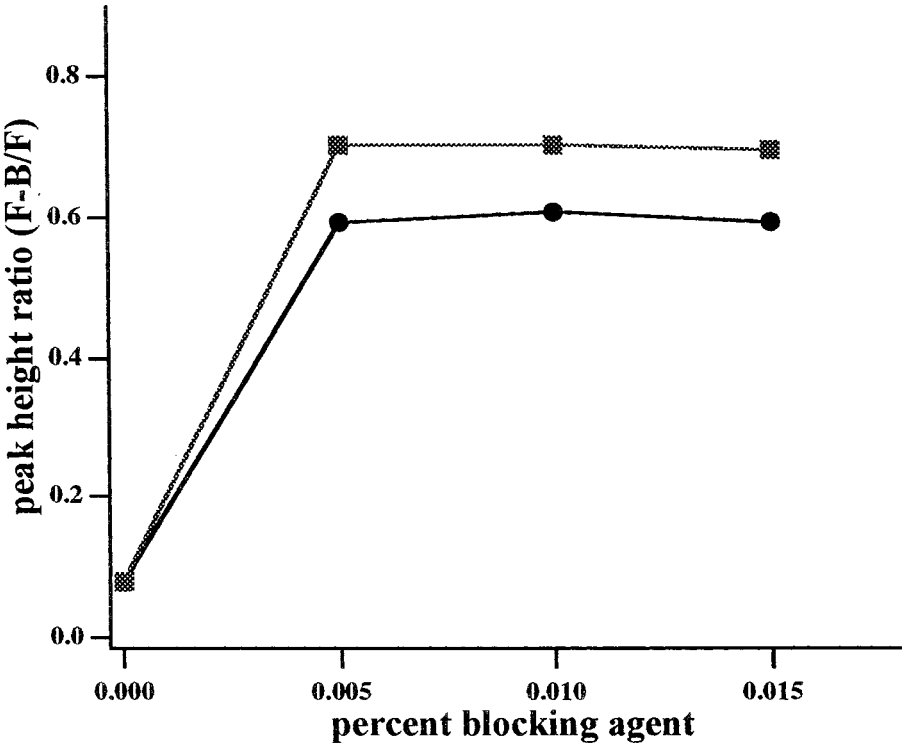

Figure C-2 Effects of BSA (black trace, square marker) and BGG (gray trace, round marker) as blocking agents against fluo-bradykinin adsorption to plastic and glass sample vial containers. Probe solutions containing 10 nM fluo-bradykinin/5 nM fluorescein in 0, 0.005, 0.010 and 0.015% blocking agent were incubated in pre-blocked plastic 1.5 mL centrifuge tubes for 30 minutes and transferred to unblocked glass sample vial before being analyzed by CZE-LIF. Pre-blocking of the centrifuge tubes was achieved by overnight incubation in the same percent blocking solution used to prepare the probe solution. The contents were removed, and the tubes were allowed to dry prior to initiating the experiment.

results. Table C-1 presents typical reproducibility data obtained for a fluo-peptide solution prepared in the phosphate/casein buffer that was incubated in either glass or plastic sample vials (over 30 minutes).

In a final series of studies, the effect of probe solution exposure to empty microtiter plate wells, wells having magnetic particles, and wells having magnetic particles that were pre-exposed to rabbit serum was characterized in the context of a typical ICIS-CE assay protocol. Figures C-3 to C-5 depict the results of one such study. In brief, 100 μL of magnetic particles were placed in two wells. One of the wells was spiked with 100 μL of rabbit serum and while 100 μL of 10 mM phosphate/0.015% casein pH 7.4 was placed in the other well. A 100 μL aliquot of phosphate/casein buffer was placed in a third. The plate was subjected to the usual incubation/wash cycles (as described in Chapters 3 and 4) and 200 μL of 10 nM fluo-bradykinin/10 nM fluo-neurotensin/5 nM BODIPY-FL/5 nM fluorescein in 10 mM phosphate/0.015% casein pH 7.4 was incubated in each well. These solutions were subsequently analyzed by CZE-LIF (in triplicate). As seen in the Figures C-3 and C-4, exceptional probe solution reproducibility was achieved for all incubation environments with respect to fluo-peptide/internal standard peak ratio values (CV < 4%). In addition, no significant peptide probe adsorption was detected when comparing wells with and without magnetic particles (pre-exposed and not pre-exposed to rabbit serum). Similar results were observed for the utilized internal standards, BODIPY-FL and fluorescein.

In summary, the inclusion of 0.015% casein within probe solutions containing fluo-neurotensin, fluo-bradykinin and fluo-substance P was found to minimize adsorption of these peptides to the various surfaces implicated in ICIS-CE assay procedure. Along with the use of an internal standard (either BODIPY-FL or fluorescein), a level of probe solution precision deemed satisfactory for reliable specific antibody detection via immunosubtraction is obtained.

Table C-1 Example of peak data precision from replicate CZE-LIF electropherograms of a 10 nM fluo-bradykinin (F-B)/10 nM fluo-neurotensin/5 nM BODIPY-FL (BDP) probe solution prepared in a glass sample vial with 10 mM phosphate/0.015% casein buffer at pH 7.4. Sequential separations were performed immediately following initial probe solution preparation with a 50 mM borate pH 9.5 run buffer under 25 kV of applied voltage.

		Compound			Ratio	
Parameter	Run	F-B	F-N	BDP	F-B/BDP	F-N/BP
	1	5.5123	9.4862	5.9128	0.9323	1.6044
	2	5.8428	9.5537	6.0200	0.9706	1.5870
	3	5.8494	9.6892	5.9246	0.9873	1.6354
Peak	4	5.8642	9.5558	5.9460	0.9862	1.6071
Height	5	5.6420	9.3695	5.9577	0.9470	1.5727
	6	5.5340	9.3510	5.9580	0.9781	1.6528
	Ave.	5.7070	9.5010	5.9030	0.9670	1.6100
	SD	0.165	0.127	0.126	0.022	0.030
	CV	2.88	1.34	2.13	2.32	1.85
	1	12.9670	8.5606	7.7818	1.6663	1.1001
	2	13.1980	8.4452	7.7168	1.7102	1.0944
	3	12.8730	8.4349	7.5497	1.7051	1.1172
Peak	4	13.2130	8.2887	7.5120	1.7590	1.1034
Area	5	12.6260	8.1987	7.5251	1.6778	1.0895
	6	12.1600	7.6680	6.918	1.7573	1.1084
	Ave.	12.8400	8.2660	7.501	1.7130	1.1020
	SD	0.399	0.319	0.306	0.039	0.010
	CV	3.11	3.87	4.08	2.27	0.90

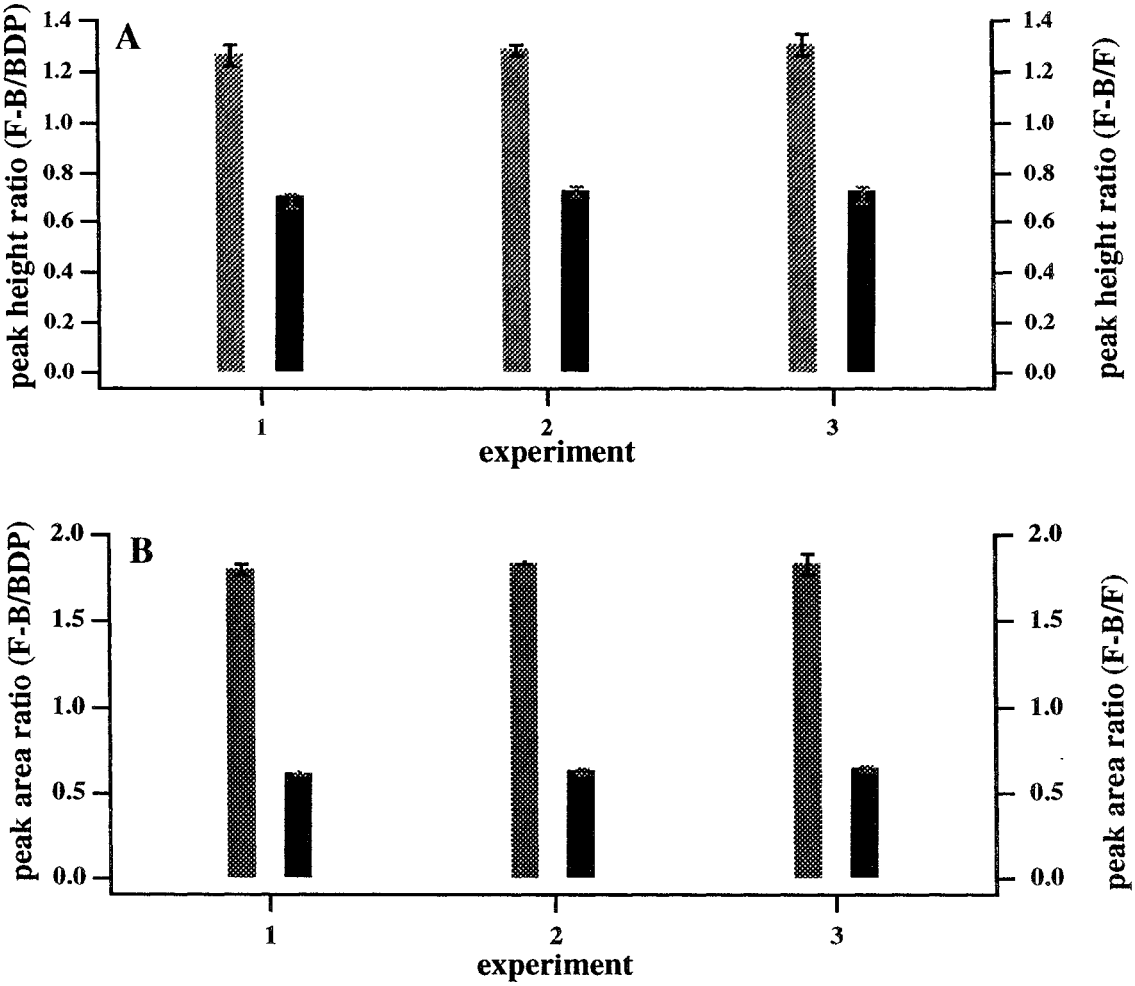

Figure C-3 Graphical comparison of signal ratio precision in terms of peak height (panel A) and peak area (panel B) for fluo-bradykinin (F-B) and the internal standards BODIPY-FL (BDP) and fluorescein (F) as represented by the gray and black bars, respectively. Data were obtained from aliquots of the same probe solution (prepared in 10 mM phosphate/0.015% casein pH 7.4 buffer) that was incubated in the following: a microtiter well with magnetic particles that was pre-exposed to rabbit serum, a microtiter well with magnetic particles, and a microtiter well (experiments 1 to 3, respectively). Error bars represent ± one standard deviation. See text for details.

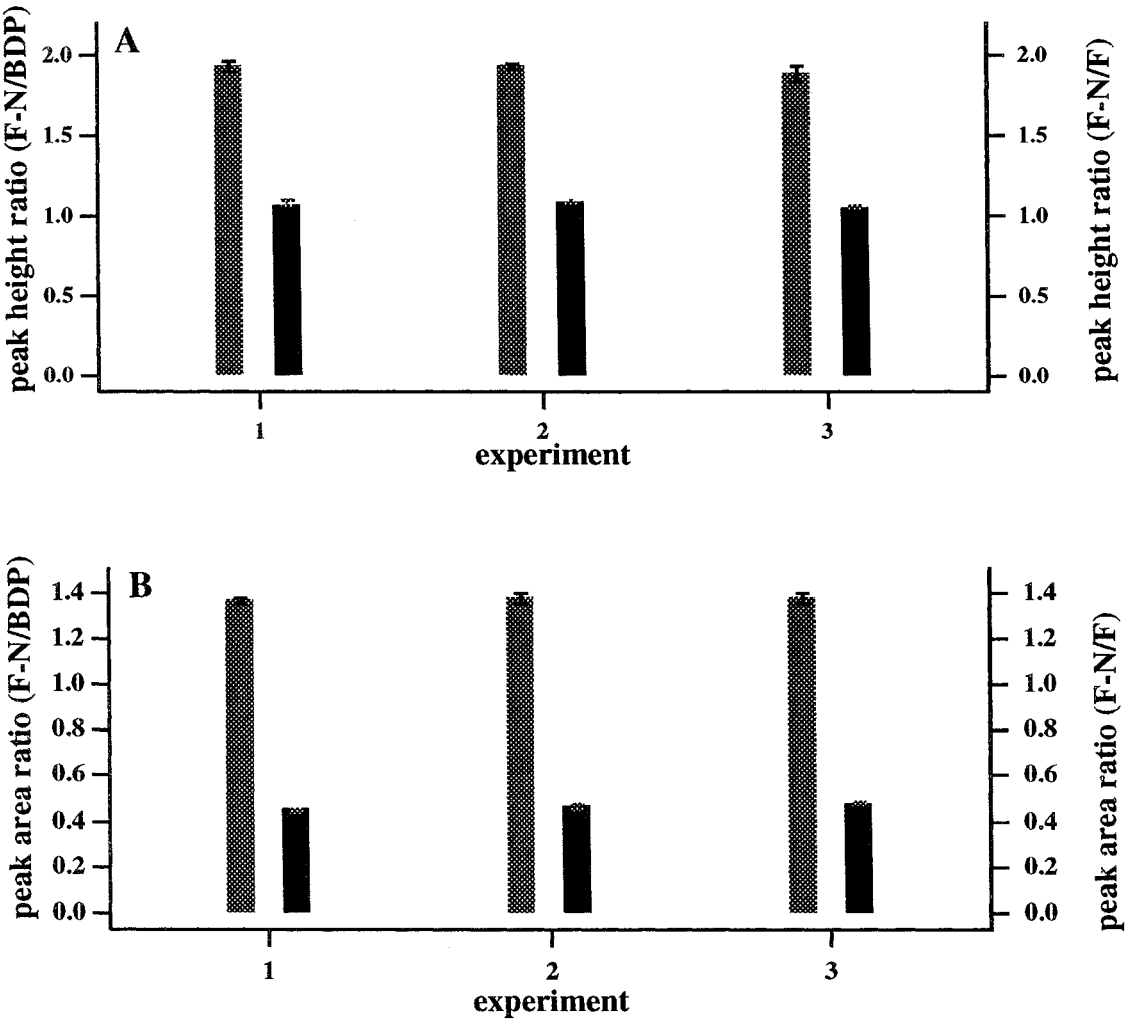

Figure C-4 Graphical comparison of signal ratio precision in terms of peak height (panel A) and peak area (panel B) for fluo-neurotensin (F-N) and the internal standards BODIPY-FL (BDP) and fluorescein (F) as represented by the gray and black bars, respectively. Same experimental conditions as described in Figure C-3.

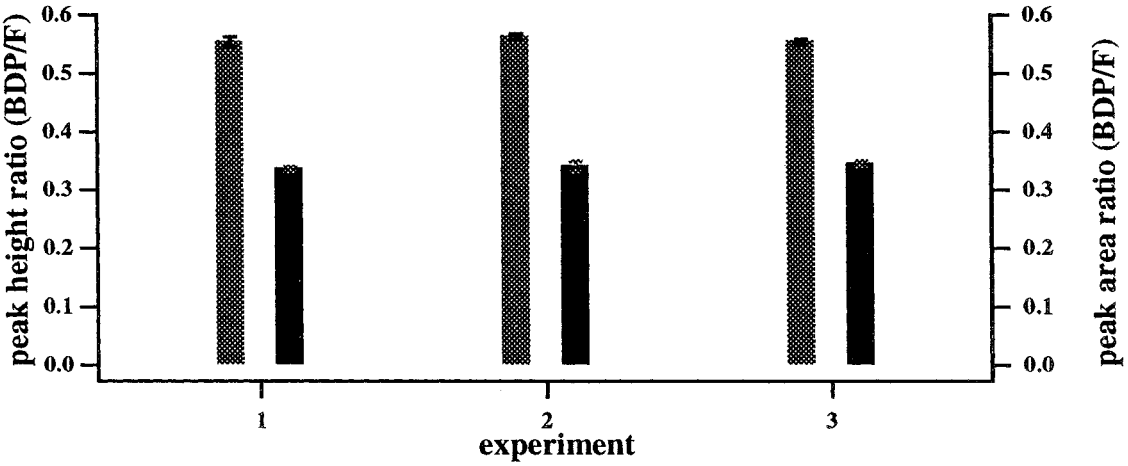

Figure C-5 Graphical comparison of signal ratio precision in terms of peak height (black bars) and peak area (gray bars) for BODIPY-FL (BDP) and fluorescein (F). Same experimental conditions as described in Figure C-3.

APPENDIX D

Pepscan Analysis

Pepscan Method and Peptide Probe Selection

The Pepscan method [1], involves the testing of overlapping nonapeptides (n + 8) covering the entire sequence of a protein for their reactivity towards polyclonal or monoclonal antibodies. Each synthetic peptide, still attached to the solid support, is incubated with the antibody containing sample of interest (e.g., ascites fluid or serum). Peptides recognized by the antibodies are subsequently detected through an ELISA assay format. The location of an epitope from a set of reactive overlapping peptides is identified by that peptide giving the strongest signal. For overlapping peptides that react equally, the amino acids shared by the reactive peptides are used to define the location and size of that epitope. Figure D-1 displays the location of the antigenic sites on the primary sequence of HIV-1 IIIB p24 as determined by Langedijk *et al.* [2]. The assayed antisera were obtained from two rabbits repeatedly immunized with purified p24 protein (Triton X-100 disrupted). Peptides producing an absorbance reading greater than threefold over the background signal were considered in their assessment. Similar antigenic amino acid sequences were reported by Devito *et al.* with an ELISA study involving partially overlapping 20-mer p24 peptides for rabbits immunized with virion HIV-1, baculovirus and *E. coli* recombinant p24 [3].

References

[1] Geysen, H. M., Meloen, R. H., Barteling, S. J., *Proceedings of the National Academy of Sciences, U.S.A.* 1984, 81, 3998-4002.

[2] Langedijk, J. P. M., Schalken, J. J., Termette, M., Huisman, J. G., Meloen, R. H., *Journal of General Virology* 1990, 71, 2609-2614.

[3] Devito, C., Levi, M., Broliden, K., Hinkula, J., *Journal of Immunological Methods* 2000, 238, 69-80.

1	Pro	Ile	Val	Gln	Asn	Leu	Gln	Gly	Gln	Met
11	Val	His	Gln	Ala	Ile	Ser	Pro	Arg	Thr	Leu
21	Asn	Ala	Trp	Val	Lys	Val	Val	Glu	Glu	Lys
31	Ala	Phe	Ser	Pro	Glu	Val	Ile	Pro	Met	Phe
41	Ser	Ala	Leu	Ser	Glu	Gly	Ala	Thr	Pro	Gln
51	Asp	Leu	Asn	Thr	Met	Leu	Asn	Thr	Val	Gly
61	Gly	His	Gln	Ala	Ala	Met	Gln	Met	Leu	Lys
71	Glu	Thr	Ile	Asn	Glu	Glu	Ala	Ala	Glu	Trp
81	Asp	Arg	Val	His	Pro	Val	His	Ala	Gly	Pro
91	Ile	Ala	Pro	Gly	Gln	Met	Arg	Glu	Pro	Arg
101	Gly	Ser	Asp	Ile	Ala	Gly	Thr	Thr	Ser	Thr
111	Leu	Gln	Glu	Gln	Ile	Gly	Trp	Met	Thr	Asn
121	Asn	Pro	Pro	Ile	Pro	Val	Gly	Glu	Ile	Val
131	Lys	Arg	Trp	Ile	Ile	Leu	Gly	Leu	Asn	Lys
141	Ile	Val	Arg	Met	Tyr	Ser	Pro	Thr	Ser	Ile
151	Leu	Asp	Ile	Arg	Gln	Gly	Pro	Lys	Glu	Pro
161	Phe	Arg	Asp	Tyr	Val	Asp	Arg	Phe	Tyr	Lys
171	Thr	Leu	Arg	Ala	Glu	Gln	Ala	Ser	Gln	Glu
181	Val	Lys	Asn	Trp	Met	Thr	Glu	Thr	Leu	Leu
191	Val	Gln	Asn	Ala	Asn	Pro	Asp	Cys	Lys	Thr
201	Ile	Leu	Lys	Ala	Leu	Gly	Pro	Ala	Ala	Thr
211	Leu	Glu	Glu	Met	Met	Thr	Ala	Cys	Gln	Gly
221	Val	Gly	Gly	Pro	Gly	His	Lys	Ala	Arg	Val

Figure D-1 Pepscan analysis results from rabbit antiserum to HIV p24. Amino acid sequences recognized by the immune system of rabbit #1 are in encased in a white box while those from rabbit #2 are in gray.

APPENDIX E

HIV Peptide Probe Solution Precision

Observations and Discussion

Preparation of peptide-based probe solutions using a 10 mM phosphate/0.015% casein pH 7.4 buffer was shown to limit peptide adsorption to the walls of glass and plastic sample vial containers, and ultimately provide for reliable probe solution precision for ICIS-CE detection of specific antibodies (Appendix C and Chapter 4). As such, the phosphate/casein buffer was adopted for probe solutions comprised of peptides representing various HIV protein epitopes. Table E-1 displays precision data for a probe solution consisting of fluorescein labeled-peptides related to epitopes on HIV p24, and fluorescein as the internal standard, that was incubated in a glass vial. These results actually represent elevated values for 13aa and 15aa peptide/fluorescein peak ratios (particularly for the latter) as most other investigations produced smaller standard deviations. Experiments paralleling those described in Appendix C involving microtiter plate well with magnetic particles (pre-exposed and not pre-exposed to rabbit serum) indicated minimal adsorption of these peptides to these surfaces and produced similar reproducibility data.

As depicted in Table E-2, relatively large variations in the gp120 peptide probe pep14 were observed despite the presence of the casein blocking agent. In this set of experiments, a probe solution containing 2.5 nM pep14/ 0.5 nM 15aa/0.5 nM fluorescein/2.5 nM pep13 was applied to detect particle immobilized anti-p24 by ICIS-CE. As described in Chapter 5, the reason for the pep14 probe imprecision is thought to stem from interaction between the negatively charged wall of the separation capillary and the peptide's arginine residues. Indeed, this fluo-peptide would presumably produce acceptable precision values as part of a probe solution separated under acidic conditions in an untreated fused-silica capillary.

Table E-1 Example of peak data precision from replicate CZE-LIF electropherograms of a 10 nM 13aa/3 nM 15aa/3 nM fluorescein (F) probe solution prepared in a glass sample vial with 10 mM phosphate/0.015% casein buffer at pH 7.4. Sequential separations were performed immediately following initial probe solution preparation with a 50 mM borate pH 9.25 run buffer under 25 kV of applied voltage.

		Compound			Ratio	
Signal	Run	13aa	15aa	F	13aa/F	15aa/F
	1	0.5652	4.8207	4.3687	0.1294	1.1035
	2	0.6170	4.9774	4.3996	0.1402	1.1313
	3	0.6470	5.0239	4.4013	0.1470	1.1415
Peak	4	0.6856	4.7078	4.5728	0.1499	1.0295
Height	5	0.6034	4.2190	4.1060	0.1470	1.0275
	6	0.6739	4.8173	4.4536	0.1513	1.0816
	7	0.6422	4.6754	4.5451	0.1413	1.0287
	8	0.6761	4.7716	4.3205	0.1565	1.0810
	Ave.	0.6388	4.7516	4.3959	0.1453	1.0810
	SD	0.0415	0.2467	0.1451	0.0083	0.0470
	CV	6.49	5.19	3.30	5.72	4.35
	1	2.3576	8.8095	6.3227	0.3729	1.3933
	2	2.2858	8.8729	6.5184	0.3507	1.3612
	3	2.3425	8.9627	6.3708	0.3677	1.4068
Peak	4	2.4846	8.3293	6.7589	0.3676	1.2323
Area	5	2.1256	7.1050	5.5265	0.3846	1.2998
	6	2.4666	8.3424	6.4184	0.3843	1.2998
	7	2.3139	8.2088	6.4236	0.3602	1.2779
	8	2.3078	8.2373	5.9983	0.3847	1.3733
	Ave.	2.3356	8.3585	6.2922	0.3716	1.3288
	SD	0.1119	0.5907	0.3745	0.0126	0.0631
	CV	4.79	7.07	5.95	3.38	4.75

Table E-2 Example of poor pep14/fluorescein (F) precision encountered with a pep14/15aa/fluorescein/pep13 probe solution (prepared in 10 mM phosphate/0.015% casein pH 7.4 buffer) used to detect specific antibodies to p24 by ICIS-CE. See text for details.

		Compound				Ratio		
Test	Run	pep14	15aa	F	pep13	pep14/F	15aa/F	pep13/F
Assay	1	0.6835	0.2538	0.4720	0.0989	0.1448	0.5376	0.2094
	2	0.0753	0.2529	0.4672	0.0945	0.1612	0.5413	0.2023
	3	0.0634	0.2553	0.4535	0.0949	0.1401	0.5644	0.2097
	Ave.	0.0690	0.2540	0.4639	0.0961	0.1487	0.5477	0.2072
	SD	0.0060	0.0012	0.0103	0.0024	0.0111	0.0145	0.0042
	CV	8.69	0.48	2.21	2.52	7.45	2.65	2.03
Blank	1	0.0719	0.3305	0.4606	0.0889	0.1561	0.7175	0.1931
	2	0.0816	0.3392	0.4472	0.0935	0.1825	0.7585	0.2091
	3	0.0852	0.3299	0.4312	0.0912	0.1976	0.7650	0.2116
	Ave.	0.0796	0.3332	0.4463	0.0912	0.1788	0.7470	0.2046
	SD	0.0069	0.0052	0.0147	0.0023	0.0210	0.0258	0.0101
	CV	8.66	1.56	3.29	2.51	11.76	3.45	4.91

APPENDIX F

ICIS-CE Method Validation Considerations

Discussion

ICIS-CE was developed primarily as a qualitative assay with an aim to function as a feasible alternative to the Western blot. The work presented in this thesis represents stepwise method development and preliminary characterization of the ICIS-CE assay as a means to detect specific antibodies within pseudo-clinical samples (rabbit serum model systems). As stated in the thesis conclusion, addition development is required before appropriate assessment of the assay in a true clinical setting can be made. Once at the point of clinical evaluation, demonstration that the assay is suitable for its intended purpose (i.e., a confirmatory assay for viral infection diagnosis) would need to be undertaken via a method validation process.

Strictly speaking, method validation is a process used primarily by the pharmaceutical industry in the analysis of drug related compounds and involves the evaluation/description of an assay's figures of merit including accuracy, precision, linearity, specificity, limit of detection, quantification limit, range, and robustness [1]. However, some of these parameters may not be necessarily relevant or applicable when defining the performance boundaries of certain clinical assays. Furthermore, parameter other than those mentioned above may be required in the validation process of a clinical test. For example, two main assay parameters that describe the performance of indirect ELISA and the Western blot for viral infection diagnosis are diagnostic sensitivity and diagnostic specificity. Diagnostic sensitivity refers a measure of an assays ability to produce a positive result when testing a sample from a patient positive for that disease, and diagnostic specificity refers a measure of an assays ability to produce a negative result when testing a sample from a patient negative for that disease [2]:

$$\text{sensitivity} = \frac{TP}{TP + FN} \qquad \textit{Equation F-1}$$

$$\text{specificity} = \frac{TN}{FP + TN} \qquad \textit{Equation F-2}$$

where TP is true positive, TN is true negative, FN is false negative and FP is false positive. Such calculations involve the comparison of results from the assay in question with that of a gold standard (e.g. viral genetic material detection through PCR analysis) and/or the known medical conditions of a panel of patients. Ultimately, it can be envisioned that ICIS-CE assay validation for use as a diagnostic tool would incorporate diagnostic sensitivity and specificity, the definition of an immunosubtraction threshold for that probe, as well as those parameters incorporating assay reliability such as precision and robustness.

With respect to preliminary ICIS-CE method development (to the extent outlined in this thesis), an approach based on parameters defined in large part by the pharmaceutical method validation process was used as a means to characterize and optimize the assay's protocol. A brief categorical summary of this work is presented below. Also included in these sections is a brief discussion relating to the importance, if applicable, of that parameter on the eventual method validation process of ICIS-CE as a diagnostic technique for viral infections.

Limits of Detection

The category of limit of detection (LOD) in relation to the ICIS-CE incorporates two separate considerations: that of the probe LOD, and the LOD of the intended antibodies to be detected. In regards to the former, several LOD experiments were performed in order to determine the CE-LIF instrument's detection capabilities with respect to the probes. As stated in Chapter 1, LODs for fluorescein at or below to pM level were typically obtained. Detection limits to 3σ for two peptide-based probes were also measured during the course of assay development: 5 pM for fluo-neurotensin and 7 pM for fluo-substance P (calculated from 50 nM concentrations, n = 3, CVs < 4%). Furthermore, all utilized peptide-based fluorescent probes were easily detected in the sub nM range (i.e., at or below the average K_d of most polyclonal antibody immuno-affinity reactions). As such, the LOD for the ICIS-CE assay was deemed to depend exclusively on the level of antigen-antibody interaction, and not the concentration of that probe.

An estimation of the ICIS-CE's LOD was made using known concentrations of anti-fluorescein polyclonal IgG reagent and the published concentration of IgG in rabbit serum (Chapter 3). Under the utilized assay conditions, it was surmised that as little as 45 femtomole of anti-fluorescein (i.e., 7 ng, n = 3, CV < 3%) could be easily detected. However, it was shown in Chapter 4 that increases in specific-antibody detectability could be readily attained by increasing the amount of solid-phase binding site, and/or decreasing the probe concentration. Although the absolute detection limits could not be determined for the neuropeptides and p24 rabbit antisera (Chapters 4 and 5) since concentration of the polyclonal antibody population for the corresponding probe were unknown, an LOD of at least 1-5 μg/mL of anti-neurotensin was calculated using the data

obtained for the experiments described in Figure 4.3, the reported binding capacity of the magnetic slurry, and an estimated antibody concentration of 10mg/mL in rabbit serum.

As with the Western blot and the indirect ELISA, the detection limits for the ICIS-CE assay are strongly attributed to the concentration and binding constant of the predominating polyclonal antibody population for that probe. Overall, the heterogeneity of a polyclonal antibody population and the differences that exist for such populations from one lot to another exempts this type of reagent from being used as an analytical standard. As such, from the point of view of a qualitative diagnostic assay for specific antibody detection, the importance of an absolute detection limit is overshadowed by a more relative term: antibody titer. Although the absolute ICIS-CE LOD warrants investigation, the analytical utility of this parameter in the diagnostic sense is inconsequential. In the end, it is the ultimate relative detection limits, as compared to the Western blot, for a panel of serum samples varying in antibody titer for a given probe that will need to be included in the ICIS-CE's method validation process (i.e., clinical definition of assay sensitivity)

Quantification Limit

The quantification limit is a parameter of an assay that defines the lowest point at which reliable measurement of low levels of compounds in sample matrices can be made. This parameter is used particularly in cases where the quantitative determination of impurities and or degradation products is required. Quantification limit was not investigated or used in the assay development procedure because ICIS-CE was designed strictly as a qualitative diagnostic tool. It can also be anticipated that this parameter

would not be included in any future method validation procedure as absolute quantification of specific antibodies is impractical.

Linearity

ICIS-CE assay linearity with respect to changes in antibody titer, which relates to specific antibody concentration, was demonstrated as part of the experimental data presented for each model system investigated. Figure 3.5 demonstrates that linearity for serum differing in anti-fluorescein titer was obtained with a correlation coefficient of 0.9959. Correlation coefficients of 0.9986 and 0.9961 were achieved for antisera to bradykinin and neurotensin (Figures 4.2A and 4.2B), respectively. Finally, assay linearity was also shown for the anti-p24 system ($r = 0.9928$, see Figure 5.2). The data used to generate each curves are exhibited in Tables F-1 to F-4, respectively.

The ability to obtain test results that are directly proportional to the concentration of specific antibodies within samples is inferred with the demonstrated linearity. As such, it is conceivable that the ICIS-CE assay could be used for the quantification of specific antibodies provided that a polyclonal antibody standard of known concentration for that antibody is known, and that this standard exhibits similar overall binding affinity towards that probe relative to those antibodies in the sample. Ultimately, the clinical practicality of ICIS-CE linearity rests not within the realm of absolute quantification, but as a means of measuring relative antibody titer over the course of an immune response towards, say, a viral infection. Analytical linearity, therefore, is not expected to be an important validation parameter when considering the proposed use of the ICIS-CE assay.

Table F-1 Data used to generate the anti-fluorescein calibration curve displayed in Figure 3.5.

		peak area signal		ratio
normal (μL)	run	BDP	F	F/BDP
300	1	2.2408	1.3164	0.5875
	2	1.9554	1.1457	0.5859
	3	1.5793	0.9013	0.5707
	average	1.9252	1.1211	0.5814
	SD	0.3318	0.2086	0.0093
	CV	17.23	18.61	1.59
435	1	2.2055	1.3342	0.6049
	2	1.6579	0.9492	0.5725
	3	1.5244	0.9424	0.6182
	average	1.7959	1.0753	0.5986
	SD	0.3609	0.2243	0.0235
	CV	20.10	20.86	3.93
600	1	2.2121	1.4818	0.6699
	2	2.0235	1.1865	0.5864
	3	1.7414	1.1084	0.6365
	average	1.9923	1.2589	0.6309
	SD	0.2369	0.1969	0.0420
	CV	11.89	15.64	6.66
800	1	2.4504	1.6192	0.6608
	2	1.6770	1.1502	0.6859
	3	2.0284	1.3244	0.6529
	average	2.0519	1.3646	0.6665
	SD	0.3872	0.2371	0.0172
	CV	18.87	17.37	2.58
1000	1	2.2578	1.4932	0.6614
	2	1.9069	1.2596	0.6605
	3	2.0437	1.5286	0.7480
	average	2.0695	1.4271	0.6900
	SD	0.1769	0.1462	0.0502
	CV	8.55	10.24	7.28

Table F-2 Data representing part of the anti-bradykinin calibration curve shown in Figure 4.2A.

		peak height signal		ratio
normal (μL)	run	F-B	BDP	F-B/BDP
2	1	1.0425	2.2830	0.4566
	2	1.0066	2.2240	0.4526
	3	0.9191	2.0037	0.4587
	average	0.9894	2.1702	0.4560
	SD	0.0635	0.1472	0.0031
	CV	6.41	6.78	0.68
3	1	1.0437	2.1946	0.4756
	2	0.9879	2.0782	0.4754
	3	0.9015	1.8862	0.4780
	average	0.9777	2.0530	0.4763
	SD	0.0716	0.1558	0.0014
	CV	7.33	7.59	0.30
4	1	1.1411	2.3349	0.4887
	2	1.0767	2.1812	0.4936
	3	0.9780	1.9189	0.5097
	average	1.0652	2.1450	0.4973
	SD	0.0822	0.2104	0.0110
	CV	7.71	9.81	2.20
5	1	1.0121	2.0480	0.4942
	2	0.9846	1.9786	0.4976
	3	0.8770	1.8250	0.4806
	average	0.9579	1.9505	0.4908
	SD	0.0714	0.1141	0.0090
	CV	7.45	5.85	1.84
6	1	1.1230	2.1390	0.5250
	2	1.0668	1.9971	0.5342
	3	0.9935	1.8936	0.5247
	average	1.0611	2.0099	0.5280
	SD	0.0650	0.1232	0.0054
	CV	6.12	6.13	1.02
7	1	1.1001	2.0388	0.5396
	2	1.0788	1.9523	0.5526
	3	0.9724	1.7618	0.5519
	average	1.0504	1.9176	0.5480
	SD	0.0684	0.1417	0.0073
	CV	6.51	7.39	1.34

Table F-3 Experimental values obtained and employed to produce the anti-neurotensin calibration curve presented in Figure 4.2B.

		peak height signal		ratio
normal (mL)	run	F-N	BDP	F-N/BDP
1	1	2087.42	4795.89	0.4353
	2	1922.47	4298.51	0.4472
	3	1882.00	4143.34	0.4542
	average	1963.96	4412.58	0.4456
	SD	108.81	340.90	0.0096
	CV	5.54	7.73	2.15
3	1	2281.94	4779.97	0.4774
	2	1806.00	3826.78	0.4719
	3	1992.00	4353.09	0.4576
	average	2026.65	4319.95	0.4690
	SD	239.85	477.46	0.0102
	CV	11.84	11.05	2.18
7	1	2649.74	5021.84	0.5276
	2	2305.51	4461.47	0.5168
	3	2340.98	4488.59	0.5215
	average	2432.08	4657.30	0.5220
	SD	189.33	315.99	0.0055
	CV	7.78	6.78	1.05
10	1	2661.00	4592.62	0.5794
	2	2465.32	4396.71	0.5607
	3	2135.00	3764.00	0.5672
	average	2420.44	4251.11	0.5691
	SD	265.86	433.07	0.0095
	CV	10.98	10.19	1.67
13	1	2356.00	4056.05	0.5809
	2	2410.51	4027.08	0.5986
	3	2557.81	4284.71	0.5970
	average	2441.44	4122.61	0.5921
	SD	104.40	141.13	0.0098
	CV	4.28	3.42	1.65

Table F-4 Data applied to construct the anti-p24 calibration curve presented in Figure 5.2.

		peak height signal		ratio
normal (μL)	run	15aa	F	15aa/F
1	1	0.181916	0.289151	0.629138
	2	0.192244	0.302421	0.635683
	3	0.19352	0.306931	0.6305
	average	0.189227	0.299501	0.631774
	SD	0.006363	0.009243	0.003453
	CV	3.36	3.09	0.55
2.5	1	0.180909	0.27308	0.662476
	2	0.189716	0.286262	0.662736
	3	0.195657	0.287867	0.679678
	average	0.188761	0.282403	0.668297
	SD	0.00742	0.008114	0.009858
	CV	3.93	2.87	1.48
4	1	0.191054	0.271495	0.703711
	2	0.197904	0.277664	0.712746
	3	0.202622	0.285748	0.709093
	average	0.197193	0.278302	0.708517
	SD	0.005817	0.007148	0.004545
	CV	2.95	2.57	0.64
5.5	1	0.214245	0.293737	0.729377
	2	0.209111	0.284506	0.734997
	3	0.213576	0.291174	0.7335
	average	0.212311	0.289806	0.732624
	SD	0.002791	0.004765	0.00291
	CV	1.31	1.64	0.40
7	1	0.17912	0.235544	0.760452
	2	0.205153	0.270824	0.757514
	3	0.214252	0.285258	0.751081
	average	0.199508	0.263875	0.756349
	SD	0.018234	0.025575	0.004793
	CV	9.14	9.69	0.63

Range

Investigations into the ICIS-CE assay range was not performed due to the associated cost and limited volume of antiserum for each probe involved in the assay's development process. Paradoxically, it is this reagent that will be supplied by patients as part of the sero-diagnosis protocol. Therefore, it is anticipated that the general titer range of the ICIS-CE assay would be determined in subsequent development stages within early feasibility studies in a clinical environment. However, as in the case of analytical assay linearity, the utility of analytical assay range is expected to be of limited use in the clinical sense.

Robustness

Although the capacity to remain unaffected by small, but deliberate variations in ICIS-CE method parameters was not examined, the effects of incubation time in relation to both immunocapture and immunosubtraction procedures were roughly explored as part of the assay optimization procedure (discussed in Chapter 3). Indeed, a substantial amount of work will be required in characterizing the robustness of the ICIS-CE assay once method development at the clinical stage has been completed. In addition to verifying the immunocapture and immunosubtraction robustness, all parameters involved in the CE analysis of a probe solution would need to be considered.

Specificity

The ability to indisputably detect a specific analyte in the presence of components that may be expected to be present is a method validation parameter that is of vital importance to the ICIS-CE's acceptance as a diagnostic tool. Although a majority of the

extraneous serum components are removed by the immunocapture step, a main concern is the cross reactivity of concomitant host antibodies immobilized to the surface of the magnetic particles. The specificity of the ICIS-CE assay is invariably determined by the uniqueness of the probe used to detect the presence of antibodies for a particular foreign agent. In demonstrating the feasibility of ICIS-CE, several real sample experiments were carried out in order to provide rudimentary examples of the specificity of a chosen probe (see Figures 4.5, 5.3, and 5.4). It is anticipated that assay specificity, in both the analytical and diagnostic sense, would be a major factor in validating the ICIS-CE for its intended use.

Accuracy

The closeness of agreement between an accepted reference value and an obtained value is an assay parameter that is of considerable importance with respect to absolute analyte quantification in the field of pharmaceutics (drug and drug impurity measurements). This parameter was not included as part method development process since the ICIS-CE was designed to be a qualitative assay for the detection of an immune response toward a particular foreign agent. Although a rough estimation of specific antibody level could be made by calculating the amount of probe that was immunosubtracted, the accurate determination of that antibody is of little significance in the realm of serological diagnosis an is not expected to be a factor in the ICIS-CE's method validation process.

Precision

Assay precision is comprised of three sub-categories: intra-assay precision, within-laboratory precision, precision between laboratories. Since the subject of the research performed dealt with ICIS-CE preliminary method development, within-day variability under the same operating conditions (intra-assay precision) was mainly focused upon as this parameter was essential in determining the reliability of positive or negative "calls" with respect to specific antibody detection. As shown in Tables F-1 to F-4 above, and Tables F-5 to F-8 below, which represent data from the various experiment presented throughout this thesis, probe solutions from both control and assay samples were analyzed by CE-LIF at least three times and produced, in general, CVs below 5% (also see Appendices B and E). Examples of attained overall assay precision involving immunosubtraction of the 15aa peptide probe by p24 antiserum include a 14.89 ± 4.6% and 33.9 ± 1.4% 15aa peak signal decrease when comparing the control and assay experiments, as represented by the overlaid electropherograms in Figures 5.3 and 5.4 (see Tables F-7 and F-8 for raw data). It is unfortunate that, due to an untimely CE-LIF instrument breakdown, the muliplexing data for Figure 4.5/Table 4.1 were obtained from duplicate probe solution analysis only. However, experience in the level of precision normally obtained with the ICIS-CE assay (as exhibited in Tables F-1 to F-8) projects that the CVs in Table 4.1 would be in the 5% or below range. Finally, as stated in Chapter 3, intra-assay precision with respect to repeat serum sample analysis (as opposed to repeat probe solution analysis) was performed once and produced a CV of < 1% (n = 4).

Investigations concerning within (different days, different analysts, different equipment, etc.) and between laboratory precision were not undertaken as these parameters clearly lay beyond the scope of the ICIS-CE's basic developmental stage. However, studies toward assay performance directed these parameters will certainly become necessary as part of ICIS-CE's continual evolution as a true clinical assay.

References

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[2] Henry, J.B., *Clinical Diagnosis and Management by Laboratory Methods*, 19th ed.; W.B. Saunders Company: Philadelphia, 1996

Table F-5 Data obtained from the investigation on the effects of fluo-neurotensin probe concentration on ICIS-CE assay sensitivity (see Figure 4.3).

		assay peak height		assay ratio	control peak height		control ratio	ratio of ratio
[F-N] (pM)	run	F-N	BDP	F-N/BDP	F-N	BDP	F-N/BDP	control/assay
50	1	0.0000	3.1122	--	0.0237	2.4977	0.0095	--
	2	0.0000	2.9005	--	0.0261	2.4563	0.0106	--
	3	0.0000	2.7100	--	0.0212	2.3203	0.0091	--
	average	--	2.9075	--	0.0237	2.4248	0.0098	--
	SD	--	0.2012	--	0.0025	0.0928	0.0008	--
	CV	--	6.92	--	10.39	3.83	8.06	--
100	1	0.0000	2.8818	--	0.0386	2.5138	0.0154	--
	2	0.0000	2.6900	--	0.0362	2.4097	0.0150	--
	3	0.0000	2.6273	--	0.0342	2.2716	0.0150	--
	average	--	2.7330	--	0.0363	2.3984	0.0151	--
	SD	--	0.1326	--	0.0023	0.1215	0.0002	--
	CV	--	4.85	--	6.20	5.07	1.34	--
500	1	0.0322	2.6825	0.0120	0.1606	2.5145	0.0639	5.3234
	2	0.0338	2.7821	0.0121	0.1534	2.3030	0.0666	5.4825
	3	0.0296	2.5767	0.0115	0.1482	2.3415	0.0633	5.5037
	average	0.0319	2.6804	0.0119	0.1541	2.3863	0.0646	5.4365
	SD	0.0021	0.1027	0.0003	0.0062	0.1127	0.0018	0.0985
	CV	6.57	3.83	2.83	4.03	4.72	2.72	1.81
750	1	0.0612	2.5133	0.0244	0.2467	2.4730	0.0997	4.0948
	2	0.0681	2.7041	0.0252	0.2310	2.3560	0.0980	3.8934
	3	0.0581	2.5244	0.0230	0.2307	2.3756	0.0971	4.2184
	average	0.0625	2.5806	0.0242	0.2361	2.4015	0.0983	4.0689
	SD	0.0051	0.1071	0.0011	0.0091	0.0626	0.0013	0.1640
	CV	8.17	4.15	4.50	3.87	2.61	1.36	4.03
1000	1	0.0998	2.5015	0.0399	0.3180	2.4918	0.1276	3.1982
	2	0.1069	2.8271	0.0378	0.3129	2.3984	0.1305	3.4517
	3	0.1020	2.5768	0.0396	0.2840	2.3507	0.1208	3.0537
	average	0.1029	2.6351	0.0391	0.3050	2.4136	0.1263	3.2345
	SD	0.0036	0.1704	0.0011	0.0183	0.0718	0.0049	0.2015
	CV	3.51	6.47	2.89	6.00	2.97	3.92	6.23
5000	1	1.1996	2.6268	0.4567	1.5998	2.4190	0.6613	1.4482
	2	1.2543	2.5842	0.4854	1.5588	2.3248	0.6705	1.3815
	3	1.1388	2.4630	0.4623	1.4763	2.2198	0.6651	1.4384
	average	1.1975	2.5580	0.4681	1.5450	2.3212	0.6656	1.4227
	SD	0.0578	0.0850	0.0152	0.0629	0.0997	0.0046	0.0360
	CV	4.82	3.32	3.25	4.07	4.29	0.69	2.53

Table F-6 Experimental values obtained from the investigation on the effects of particle slurry volume on ICIS-CE assay sensitivity (see Figure 4.4).

		assay peak height		assay ratio	control peak height		control ratio	ratio of ratio
slurry (μL)	run	F-N	BDP	F-N/BDP	F-N	BDP	F-N/BDP	control/assay
	1	1.3051	2.4731	0.5277	1.4788	2.0636	0.7166	1.3579
	2	1.1651	2.1302	0.5469	1.2701	1.8241	0.6963	1.2731
	3	1.0449	1.9825	0.5271	1.1012	1.5357	0.7171	1.3605
25	4	1.0653	2.0627	0.5164	--	--	--	--
	average	1.1451	2.1621	0.5295	1.2833	1.8078	0.7100	1.3305
	SD	0.1189	0.2159	0.0127	0.1891	0.2643	0.0119	0.0497
	CV	10.38	9.99	2.40	14.74	14.62	1.67	3.74
	1	0.8068	2.2962	0.3514	1.4006	2.0142	0.6954	1.9789
	2	0.7607	2.1115	0.3603	1.2737	1.8789	0.6779	1.8815
	3	0.7221	2.0243	0.3567	1.1869	1.7609	0.6741	1.8896
50	4	0.6420	1.8478	0.3474	1.0700	1.5449	0.6926	1.9937
	average	0.7329	2.0699	0.3540	1.2328	1.7997	0.6850	1.9359
	SD	0.0698	0.1865	0.0057	0.1396	0.1989	0.0106	0.0585
	CV	9.53	9.01	1.61	11.32	11.05	1.54	3.02
	1	0.4610	2.2423	0.2056	1.4028	2.0367	0.6888	3.3499
	2	0.4483	2.1230	0.2111	1.2557	1.8645	0.6735	3.1897
	3	0.4008	2.0126	0.1991	1.1577	1.7193	0.6733	3.3814
75	4	0.3738	1.8284	0.2044	1.0001	1.5145	0.6603	3.2301
	average	0.4210	2.0516	0.2051	1.2041	1.7837	0.6740	3.2878
	SD	0.0408	0.1759	0.0049	0.1693	0.2215	0.0116	0.0924
	CV	9.68	8.57	2.40	14.06	12.42	1.73	2.81
	1	0.2205	1.9568	0.1127	1.2611	1.8729	0.6734	5.9756
	2	0.2092	1.9114	0.1095	1.2016	1.7887	0.6718	6.1369
	3	0.1583	1.5545	0.1018	1.0703	1.6399	0.6526	6.4093
100	4	0.1651	1.5558	0.1061	0.9719	1.4276	0.6808	6.4133
	average	0.1883	1.7446	0.1075	1.1262	1.6823	0.6696	6.2338
	SD	0.0312	0.2196	0.0046	0.1302	0.1952	0.0120	0.2153
	CV	16.55	12.59	4.32	11.56	11.60	1.79	3.45

Table F-7 Experimental data obtained from the specific immunosubtraction of 15aa peptide probe by p24 antiserum in the presence of the fluo-bradykinin and fluo-neurotensin probes (see Figure 5.3).

		peak height signal					ratio
experiment	run	F-B	F-N	BP	15aa	F	15aa/F
control	1	0.7065	0.7073	1.5093	0.1561	0.6880	0.2269
	2	0.6670	0.6155	1.4400	0.1422	0.6172	0.2304
	3	0.6690	0.5510	1.3833	0.1538	0.6140	0.2505
	4	0.6800	0.5760	1.4102	0.1427	0.6052	0.2358
	average	0.6806	0.6125	1.4357	0.1487	0.6311	0.2359
	SD	0.0182	0.0686	0.0543	0.0073	0.0383	0.0104
	CV	2.67	11.20	3.78	4.90	6.06	4.41
assay	1	0.6914	0.6806	1.4764	0.1324	0.6698	0.1977
	2	0.6624	0.6406	1.4524	0.1293	0.6417	0.2015
	3	0.6589	0.5752	1.3838	0.1244	0.6185	0.2012
	4	0.6585	0.5150	1.3590	0.1268	0.6257	0.2027
	average	0.6678	0.6029	1.4179	0.1282	0.6389	0.2008
	SD	0.0158	0.0729	0.0555	0.0034	0.0228	0.0022
	CV	2.37	12.09	3.92	2.66	3.56	1.08

Table F-8 Experimental data obtained from the specific immunosubtraction of 15aa peptide probe by p24 antiserum in the presence of the pep14 and pep13 probes (see Figure 5.4).

		peak height signal				ratio
experiment	run	pep14	15aa	F	pep13	15aa/F
control	1	0.12491	0.3926	0.611	0.18928	0.642553
	2	0.08404	0.36973	0.56976	0.1759	0.648922
	3	0.0693	0.34598	0.54054	0.15604	0.640064
	average	0.0928	0.3694	0.5738	0.1737	0.6438
	SD	0.0288	0.0233	0.0354	0.0167	0.0046
	CV	31.06	6.31	6.17	9.63	0.71
assay	1	0.14652	0.53661	0.55414	0.17228	0.968365
	2	0.11362	0.5306	0.54917	0.18213	0.966185
	3	0.11425	0.51128	0.51763	0.16382	0.987733
	average	0.1248	0.5262	0.5403	0.1727	0.9741
	SD	0.0188	0.0132	0.0198	0.0092	0.0119
	CV	15.08	2.52	3.66	5.30	1.22

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