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IN VIVO MONITORING OF AMINO ACID NEUROTRANSMITTERS IN RAT BRAIN BY MICRODIALYSIS AND CAPILLARY ELECTROPHORESIS WITH LASER-INDUCED FLUORESCENCE DETECTION

BY

JIN WU

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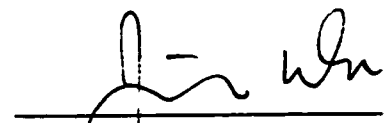

96-203 Guoliyuan Xincun
Nantong, CHINA 226001

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *In Vivo* Monitoring of Amino Acid Neurotransmitters in Rat Brain by Microdialysis and Capillary Electrophoresis with Laser-induced Fluorescence Detection submitted by Jin Wu in partial fulfillment of the requirements for the degree of Master of Science.

Dr. N. J. Dovichi

Dr. G. B. Baker

Dr. C. Lucy

Date: Nov. 28, 1999

ABSTRACT

This thesis presents the method of coupling microdialysis sampling to micellar electrokinetic chromatography with laser-induced fluorescence detection (MEKC-LIF) to monitor amino acids *in vivo* in rat brain.

The separation of amino acids in rat brain homogenate supernatants and microdialysates was improved by adding β-cyclodextrin (β-CD) to the buffer system. Rhodamine 6G was carefully selected as an internal standard to improve the precision dramatically.

The reaction rate and activation energy were investigated for five amino acids with the fluorogenic reagent 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ) in artificial cerebrospinal fluid (CSF). Detection limits for the labeled neurotransmitters were 0.6 – 9.5 pg/5μL, which correspond to 300 zeptomoles and 7 attomoles injected onto the capillary.

Finally, five amino acids in rat brain were continuously monitored by the combination of microdialysis sampling and β-CD aided MEKC-LIF. The administration of the antidepressant/antipanic drug phenelzine (PLZ) increased the extracellular levels of GABA and alanine, and decreased the glutamine concentration by 3-fold. Glutamic acid and taurine were not affected by PLZ.

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CHAPTER 1

AN INTRODUCTION TO NEUROTRANSMITTERS AND MICRODIALYSIS

1.1 Neurotransmitters

Neurotransmitters are endogenous signaling molecules that alter the behavior of neurons or effector cells [1]. Briefly, the traditional definition of a neurotransmitter states that the compound must be synthesized and released presynaptically; it must mimic the action of the endogenous compound that is released on nerve stimulation; and, where possible, a pharmacological identity is required where drugs that either potentiate or block postsynaptic responses to the endogenously released agent also act identically to the administered suspected neurotransmitter [2]. In its most general sense, neurotransmitters include not only messengers that act directly to regulate ion channels, but also those that act through second messenger systems, and those that act at a distance from their site of release. Included are neuromodulators, neuroregulators, neuromediators, and neurohumors, whether or not acting at synapses [1].

Many small molecules, including some short peptides, are neurotransmitters. Basically, neurotransmitters can be divided into three main groups: (a) amino acid neurotransmitters, (b) biogenic amine neurotransmitters, and (c) neuropeptides [2].

1.1.1 Amino acid neurotransmitters

Over the years several amino acids have gained recognition as major neurotransmitter candidates in the mammalian central nervous system (CNS) [3]. On the basis of neurophysiological studies, amino acids have been separated into two general classes: inhibitory amino acids (γ-aminobutyric acid (GABA), glycine, taurine, serine and β-alanine), which hyperpolarize mammalian neurons; and excitatory amino acids (glutamate, aspartate, cysteic acid and homocysteic acid), which depolarize neurons in the mammalian CNS [3]. Strictly from a quantitative standpoint, the amino acids are probably the major transmitters in the mammalian CNS, while the better-known monoamine transmitters (acetylcholine, dopamine, histamine, 5-hydroxytryptamine) probably account for transmission at only a small percentage of central synaptic sites [3].

GABA and glycine

GABA and glycine are the primary inhibitory transmitters. First synthesized in 1883, GABA was known for many years as a product of microbial and plant metabolism [4]. In recent years enough evidence has accumulated to allow the conclusion that GABA also functions as an inhibitory transmitter in the mammalian CNS [3]. GABA is considered an inhibitory transmitter even though it can induce both hyperpolarizing and depolarizing responses. Both actions are the result of GABA receptor-mediated change in chloride conductance [5]. GABA has been implicated in the pathogenesis of Huntington's disease, Parkinson's disease, senile dementia, depression as well as several other behavioral disorders [3].

Glycine is regarded as an inhibitory neurotransmitter in the vertebrate CNS, especially in the spinal cord; however, it also acts as a coagonist at the NMDA receptor, an excitatory amino acid receptor in the brain [3]. Like GABA, acting on the $GABA_A$ receptor, glycine inhibits neuronal firing by gating chloride channels [6].

Glutamate and aspartate

It has been recognized for many years that glutamate and aspartate occur in uniquely high concentrations in the brain and that they can exert very powerful stimulatory effects on neuronal activity [3]. They are the major excitatory neurotransmitters within the CNS [7]. They are responsible not only for normal synaptic function, but also play vital roles in many of the dysfunctional conditions of the CNS, as well as being implicated in neurodegeneration due to their ability to induce excitotoxicity under certain circumstances [8].

In addition to these major amino acid transmitters, there are several other important neuroactive amino acids.

Glutamine

Glutamine is the amino acid found in the highest concentrations both in cerebrospinal fluid and microdialysates [7]. In addition to having a general metabolic role in the brain, glutamine is also used as a metabolic pool for glutamate and GABA synthesis [9].

Alanine, serine and taurine

Alanine, serine and taurine are all inhibitory amino acids. However, they appear to have a weaker and relatively sluggish action on spinal neurons compared with that of GABA and glycine [3]. Alanine and serine in turn activate the glycine receptor [4].

1.1.2 Biogenic amine neurotransmitters

The term biogenic amine usually refers to the catecholamines (dopamine, norepinephrine and epinephrine), serotonin (5-hydroxytryptamine (5-HT)), histamine and tryptamine [2]. Acetylcholine (ACh) may also be included. Several biogenic amines are among the best characterized of all neurotransmitters. Table 1.1 shows dopamine, 5-HT, histamine and ACh with their precursors, synthetic enzymes, and their respective mechanisms of inactivation [10].

Table 1.1 Common Biogenic Amine Neurotransmitters

Neurotransmitter	Precursors	Synthetic enzymes	Inactivation
Dopamine	Tyrosine	Tyrosine hydroxylase	Reuptake
		Aromatic L-amino acid decarboxylase	Monoamine oxidase
			Catechol O-methyl transferase
5-HT	Tryptophan	Tryptophan 5-hydroxylase	Reuptake
		Aromatic L-amino acid decarboxylase	Monoamine oxidase
Histamine	Histidine	Histidine decarboxylase	Monoamine oxidase
			Histamine methyl transferase
Acetylcholine	Acetate, choline	Choline acetyl transferase	Acetylcholine esterase

1.1.3 Neuropeptides

Neuropeptides are gaining more and more attention from neuropharmacologists. Almost all peptides made by neurons will affect specific target cells of the central and peripheral nervous systems: neuronal, glial, smooth muscle, glandular, and vascular [11]. However, unlike the small molecule transmitters, peptides in central and peripheral neurons usually coexist with an amino acid or biogenic amine or even another neuropeptide. A neuron might then be able to transmit an enriched message by employing more than one type of transmitter for its fully refined signal content [12]. But as a minor, fractional percent of messenger-signaling capacity of the nerve terminal, the peptides might affect signals transmitted by the coexisting amino acid or biogenic amine [11].

The known neuroactive peptides mainly include the grand peptide families (oxytocin/vasopressin, the tachykinins, vasoactive intestinal polypeptide (VIP)-related peptides, opioid peptides, etc.), a few individual peptides (cholecystokinin (CCK), somatostatin, neurotensin, calcitonin-gene-related-peptide, etc.), and a new large family of neuroactive and "glia-active" peptides, the cytokines, which may figure prominently in pharmacological attacks on neurodegenerative disorders [11].

1.2 Microdialysis

Microdialysis is an *in vivo* sampling technique, which allows monitoring within a physiological environment with a minimum of disturbance to the animal [13]. It is generally thought of as a relatively recent technique, however the earliest example of a dialysis probe dates back to 1972 [14]. The development of the U-shaped probe by Zetterstrom and coworkers in the early 1980's [15] started the evolution and ever increasing use of the technique of microdialysis. Because of its relative ease of use, microdialysis has become the technique of choice for the *in vivo* analysis of neurotransmitters in the extracellular brain space.

1.2.1 Principles of microdialysis

Dialysis is the movement, primarily driven by concentration gradients, of small molecules and water through a semi-permeable membrane [16]. In *in vivo* microdialysis, a fine (0.2 – 0.5 mm diameter) dialysis probe fitted with a semi-permeable membrane is implanted into a discrete region of brain, systemic tissue or fluid of a living animal [13]. Using a microinfusion pump set at a low flow rate (usually 0.1 - 2 μL/min), the probe is perfused with a solution that mimics the ionic constituents of the brain's extracellular fluid. The endogenous substances, such as neurotransmitters, diffuse out of the extracellular fluid into the perfusion medium [17]. Alternatively, molecules can diffuse from the perfusate out of the probe into the extracellular space (a process known as reverse dialysis), thus providing a means of local administration of drugs [13].

The dialysis membrane constitutes a real barrier between the perfusion fluid and the interstitial space, and usually excludes the transport of larger molecules that may interfere with the substances of interest in the analytical procedure. Furthermore, enzymes that could cause a breakdown of the neuroactive compounds are also prevented from being picked up by the dialysate [16].

The level of a neurotransmitter in dialysate samples is the net result of the interaction between the process of release into and removal from the extracellular space. Consequently, *in vivo* microdialysis can only detect a neurotransmitter that is unaffected by clearing mechanisms. It does not provide information regarding intracellular levels of substances [17].

1.2.2 Dialysis probes

To date, several different types of dialysis probes have been used to study the extracellular compartment. The appropriate design for a particular experiment is important since it has been shown that differences in probe type can lead to conflicting results [16].

There are two main categories of dialysis probes: transcerebral and vertical probes (Fig. 1.1). The former provides a large area for dialysis and therefore can be implanted in large areas such as cortex, striatum and nucleus accumbens. The recovery of endogenous substances with such probes is high. However, the inaccessibility of deep brain structures and the need for intricate surgical procedure for implantation have led to a decline in the use of this type of probe. Vertical probes,

which can be subdivided further into concentric or I-shaped and loop or U-shaped (Fig. 1.1), do not have the above limitations and are thus more commonly used [16].

The body of the probe is usually constructed of stainless steel needle tubing, fused silica, or a combination of the two. The most common membrane materials are polycarbonate, polyacrylonitrile, and regenerated cellulose [18].

In vivo microdialysis experiments are not restricted to the use of one probe. Studies using two or more probes implanted in the animal present two distinct advantages. First, they permit a reduction in the number of animals used in a single experiment [16]. Secondly, they provide a powerful tool for examining the functional connections between different brain areas and the transmitter/receptors involved [19-21]. However, because of the complexity of this procedure, it is possible for an experiment to fail, e.g. incorrect placement or malfunction of one or both probes.

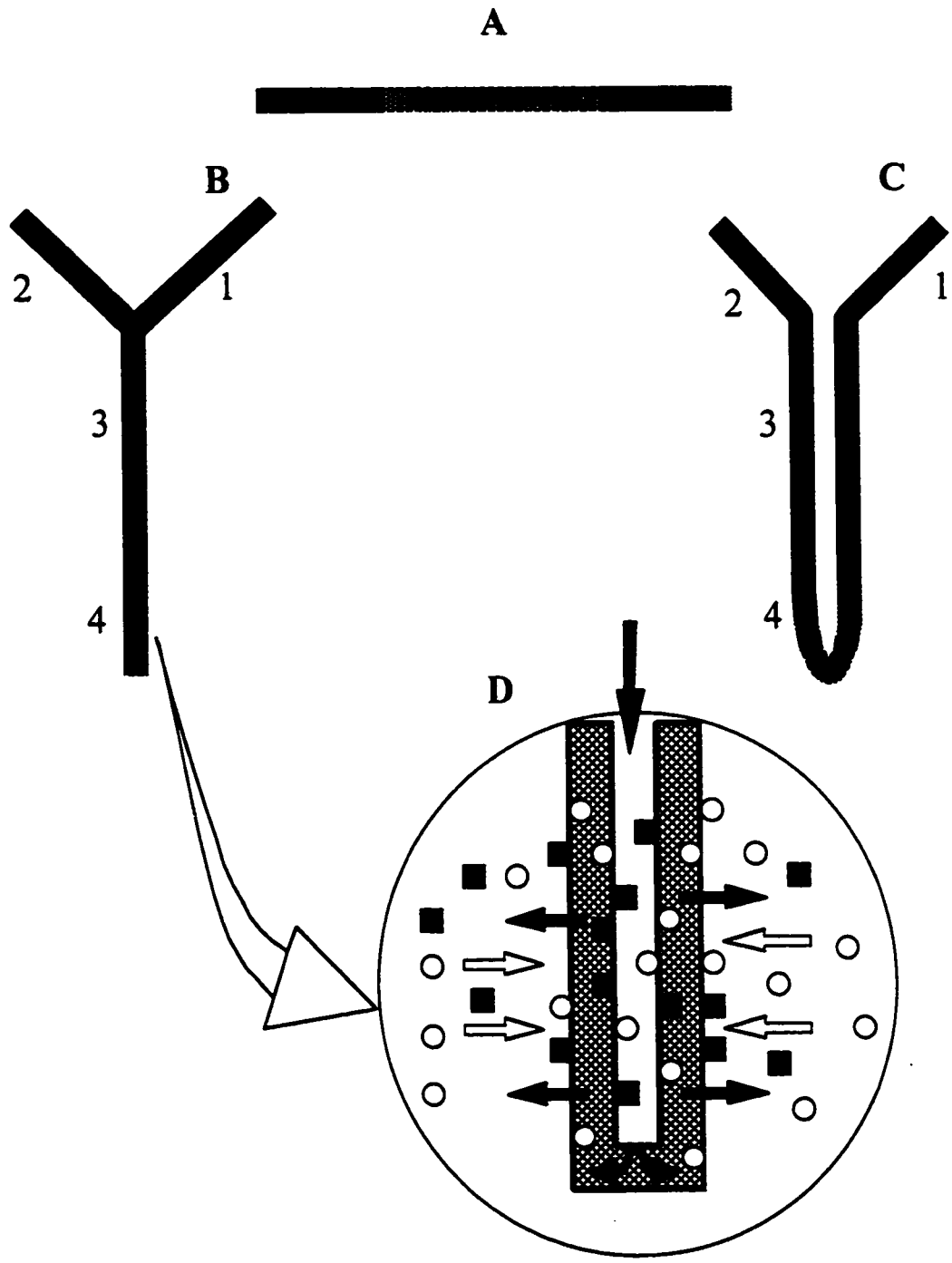

Figure 1.1 Schematic drawings of microdialysis probes. (A) transcerebral probe, (B) concentric or I-shaped vertical probe, (C) loop type of U-shaped vertical probe, (D) the membrane and the diffusion of molecules into (○) and out (■) of the probe.
1. Inlet, 2. outlet, 3. stainless steel shaft, 4. membrane.

1.2.3 Perfusion fluids

One of the crucial aspects in microdialysis studies is that the composition of the perfusion medium must be physiological, i.e., isotonic with respect to that of the interstitial space. With little variation, the fluids used to perfuse dialysis probes are those derived from Krebs-Ringer solutions or artificial cerebrospinal fluid (CSF). Typically the concentration of Ca^{2+} ions in the perfusion fluid may vary from 1.2 mM to 3.3 mM [16].

Other perfusion solutions contain glucose to prevent the depletion of this compound from the interstitial space produced by the continuous drainage of dialysate. Furthermore, the addition of glucose to the perfusion fluid provides the essential nutrient for neurons to cope with the cell damage and disruption of the blood-brain barrier caused by probe implantation. Nevertheless, the presence of glucose may favor bacterial growth in the perfusion fluid, thus altering the extraction of neurotransmitters [16].

One common problem inherent to most microdialysis studies is the very low concentration of analytes in the dialysate because of the efficient mechanisms of removal from the interstitial space. To circumvent this complication, an uptake blocker or an inhibitor of enzymatic breakdown are sometimes included the perfusion medium [16]. In the absence of such agents, the extracellular concentration of a transmitter reflects the balance between release and inactivation processes [22]. However, only the release component is measured in the presence of such agents [16]. The most common compounds added to the perfusion fluid are uptake inhibitors such as citalopram for 5-HT or nomifensine for dopamine, and enzyme inhibitors

such as physostygmine or neostygmine to block the enzymatic degradation of ACh [16].

The flow rate for the perfusion is an important practical point. Generally, low flow rates are preferred in order to approach ideal dialysis conditions and maximize the recovery of substances from the interstitial space. The relative recovery of neurochemical compounds through dialysis membrane increases as the flow rates declines. Flow rates ranging from 0.1 to 2 μL/min are typically used [16].

1.2.4 Advantages and limitations of microdialysis

Table 1.2 summarizes some of the advantages and limitations of the microdialysis technique [17].

Microdialysis offers a number of advantages for the analysis of extracellular fluid over conventional techniques such as push-pull perfusion and tissue homogenizing [16]. First, extracellular fluid can be sampled incrementally over long periods of time without fluid loss in order to observe chronological changes in chemical composition. Second, because the dialysis membrane is only permeable to relatively small molecules, macromolecules, such as proteins, that can interfere with separations are excluded, making sample purification unnecessary [23]. Third, post-sampling enzymatic reactions are eliminated since enzymes are prevented from crossing the dialysis membrane [24]. Fourth, molecules can be introduced into and removed from the brain of an animal that is awake and freely moving rather than anesthetized [23]. And lastly, it is possible to monitor multiple analytes by coupling

other techniques, such as liquid chromatography (LC), capillary electrophoresis (CE), mass spectrometry (MS), immunoassay or electrochemistry, to the microdialysis system [18].

The main limitations of microdialysis are the size of the probes and the tissue damage caused by the insertion of the probe. Also, the low amount of certain analytes in dialysates makes it necessary to collect samples every 20 or 30 minutes, a time scale that is far from that of neuronal events [16]. Recent advances in the detection of very low concentrations of certain transmitters with CE have permitted a considerable shortening of the sampling periods to several minutes or even as low as 45 seconds [24-26]. However, this is still far from the scale at which neuronal excitation or inhibition is associated with the release of a transmitter [16].

Table 1.2 Advantages and Limitations of the Microdialysis Technique [17]

Advantages
Easy to routinely use
No enzymatic degradation of transmitters in samples
Easily coupled with:
Local administration of drugs
Systemic drug administration
Stimulation and recording of electrical activity
Study of behavior in freely moving animals
Easy to couple with chemical methods of analysis: HPLC, MS, CE, etc
Dual probe approaches
Limitations
Invasive procedure: causes neuronal death and reactive gliosis
Limited temporal resolution
Limited spatial resolution
Difficult to detect some neurotransmitters
Low membrane recoveries with high molecular weight compounds

1.2.5 Applications of microdialysis

Microdialysis in the brain has been the most widely used application of this technique. Many of the small molecular weight endogenous neurotransmitters (i.e. dopamine [27], serotonin [28], ACh [29], the excitatory amino acids [30], GABA [31] and many of the other amino acids in the brain [32]) are routinely monitored, as well as some of the neuropeptides (e.g. substance P [33] and CCK [34]). The use of microdialysis within other tissues has been a more recent development, particularly for the study of drug pharmacokinetics [35] and, to a less degree, for the monitoring of drug metabolism [36].

The analysis of the microdialysis samples has conventionally used HPLC with various detection systems depending on the analyte(s) of interest. For instance, the amine neurotransmitters, dopamine and noradrenaline, were separated by reversed-phase HPLC with electrochemical detection (ECD), a highly sensitive and specific means of measurement [27-28]. LC with UV absorbance detection is routinely used for lactate, pyruvate, purines and some drugs [18].

These techniques have been used effectively for many years, but they do have certain disadvantages, namely long separation times and a requirement for relatively large injection volumes, usually several microliters [13]. CE is a superb analytical match for microdialysis because of its extremely low sample volume requirements and the excellent column efficiency obtained.

1.3 Capillary electrophoresis (CE)

Electrophoresis is the movement of a charged surface, such as dissolved or suspended materials, relative to a stationary liquid by an applied electric field [37]. Applying electrophoresis on capillaries has a published history that spans some 40 years [13]. However, the technique has only really been in the modern laboratory since the early 1980's [38] with many CE units now commercially available. The technique utilizes the same forces as those used in conventional electrophoretic separations, but they occur within a small-bore fused-silica capillary (typically 20-100 μm I.D.) [13]. This affords the CE technique with a number of advantages over its counterpart, LC. Firstly, higher column efficiency allows very high resolution (10^5 – 10^6 theoretical plates), which in some cases can result in rapid analysis. Secondly, CE has a very low sample volume requirement, typically less than 10 nL injection volume, compared to the μL quantities applied to a LC analysis [13]. In addition, CE provides very high sensitivity. Femtomole limits can easily be reached. Attomole or even zeptomole limits are also reported [39-40].

There is a range of detection systems available: UV detection, MS, ECD, laser-induced fluorescence (LIF), etc. LIF provides the greatest degree of sensitivity of any detector currently available for CE, with detection limits approaching the molecular level [40]. This high sensitivity makes LIF the ideal detector for CE when analyzing biological samples where absolute levels are very small, such as in microdialysis.

1.4 Applications of CE coupled to microdialysis sampling

1.4.1 Amino acids

CE is ideally suited for the analysis of small molecules such as amino acids due to their differing charge-to-mass characteristics. Probably some of the most commonly published analyses are those dealing with the excitatory amino acids (EAAs), glutamate and aspartate, as sampled from various brain regions [24, 41-44]. Hernandez and coworkers [41] used naphthalene 2,3-dicarboxyaldehyde (NDA) derivatization to gain temporal resolution of glutamate in 10 minutes, using the CZE-LIF. Stiller and coworkers [45] utilized micellar electrokinetic chromatography (MEKC)-LIF to resolve not only the EAAs but a number of amino acids with a temporal resolution of 15 min. This method employed 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA) derivatizations.

For further reduction to near-real-time analysis, Zhou et al. [46] and Lada et al. [26] have devised on-line derivatization and direct coupling of CE to microdialysis sampling. CE acts as a type of biosensor with temporal resolution now becoming a function of separation time [26]. Glutamate and aspartate were monitored with 120-second temporal resolution by derivatizing dialysates on-line with NDA and then automatically assaying the samples by CZE-LIF [46]. Lada and Kennedy [26] used *o*-phthaldialdehyde/β-mercaptoethanol (OPA/β-ME) for on-line derivatization of amino acids in the dialysate, using both CZE and MEKC separation modes. Temporal resolution was between 45 s and 3 min. It should be noted that a rapidly reacting derivatizing agent, such as NDA and OPA, is essential for on-line analysis [13]. The derivatization must be completed before analysis begins.

The major alternative for the LIF detection is ECD [24, 47-48]. NDA/CN can again be used as it also renders the product with electrochemical properties conducive to ECD. Recent preliminary data by Takada and coworkers [49] have demonstrated the use of MS detection for CE and its application to microdialysis. They identified endogenous GABA in rat striatum. Although detection was possible, accurate quantification was not. However, it demonstrated the feasibility of CE-MS for the measurement of underivatized amino acids in microdialysates.

1.4.2 Biogenic amines

Microdialysis sampling of biogenic amines, such as 5-HT, dopamine and noradrenaline, has been routinely performed and analyzed by HPLC with ECD or fluorimetric detection [17]. However, the application of CE to the analysis of these amines has not become as popular as for the amino acid transmitters. Bert and coworkers [50-51] reported the analysis of both dopamine and noradrenaline by CE-LIF. In this approach, on-line derivatization with NDA was performed, followed by two separate analyses, one to resolve EAAs and another for the amines.

1.4.3 Peptides

Peptides are extremely important biological molecules with huge diversity in function and structure. Many peptides can and have been measured by microdialysis [13]. Nevertheless, the combination of CE and microdialysis has not yet been extensively utilized. Advis and coworkers [52] reported the resolution and

measurement of luteinizing hormone-releasing hormone (LHRH) from the hypothalamus of the sheep using microdialysis and CE-UV analysis. Relatively large (20 nL) injection volumes were required, suggesting that sensitivity may be an issue for LHRH analysis. Using a more sensitive detector, i.e. LIF or ECD, could improve detection limits. However, peptides with electrochemical activity are rare and effective derivatization of a complex molecule can be difficult, particularly at the low concentrations present within a microdialysate.

1.4.4 Drugs – pharmacokinetic studies

Pharmacokinetic studies probably present the largest use of microdialysis next to the measurement of endogenous brain neurotransmitters. Once again CE can be utilized for the analysis of both original administered substances and also subsequent metabolic products.

Tellez et al. [39] used microdialysis and CE-UV analysis to determine the distribution of phenobarbital between the blood and brain by the simultaneous implantation of multiple probes into both the brain and the circulatory system of the rat. CE-ECD was employed for analyzing the drug L-DOPA and its metabolites sampled by intravenous microdialysis [53]. Using LIF detection, Paez and coworkers [54] demonstrated that amphetamine could be monitored in the brain up to 150 minutes after administration. Finally, microdialysis was directly coupled to CE-LIF for the continuous on-line pharmacokinetic evaluation of an antineoplastic drug, SR 4233, and its main metabolite [25].

1.5 CE-LIF instrumentation

The in-house constructed CE-LIF instrument is routinely used in our laboratory and has been described in detail elsewhere [55-56]. Fig. 1.2 A illustrates a schematic of the instrument. Basically, it contains four components: high voltage power supply with a platinum electrode, an injector, a separation capillary and an high-sensitivity LIF detector.

In our laboratory, we have achieved detection limits of one molecule using a LIF detector based on a sheath flow cuvette (Fig. 1.2 B) [55, 57-58]. Compared to on-capillary detection, the post-capillary detection using a sheath flow cuvette is advantageous. In on-capillary detection, the change in refractive index at the capillary/buffer interface produces a large amount of light scattering, which is responsible for the majority of the noise. The sheath flow cuvette eliminates this source of scattered light by surrounding the capillary elute with a sheath liquid of equal refractive index. The major source of noise is now light scattering from the solvent, which is significantly less than the light scattering found in on-capillary detection.

Of course, there is still a change in refractive index at the cuvette/sheath liquid interface. However, unlike on-capillary detection, this region of light scattering is removed from the analyte fluorescence and is easily discriminated from the fluorescence using the optics and a bandpass filter. The optical system is designed to collect as much sample fluorescence as possible and reject as much background light as possible. The bandpass filter allows only light over a small range of wavelengths to pass through and blocks most of other wavelengths.

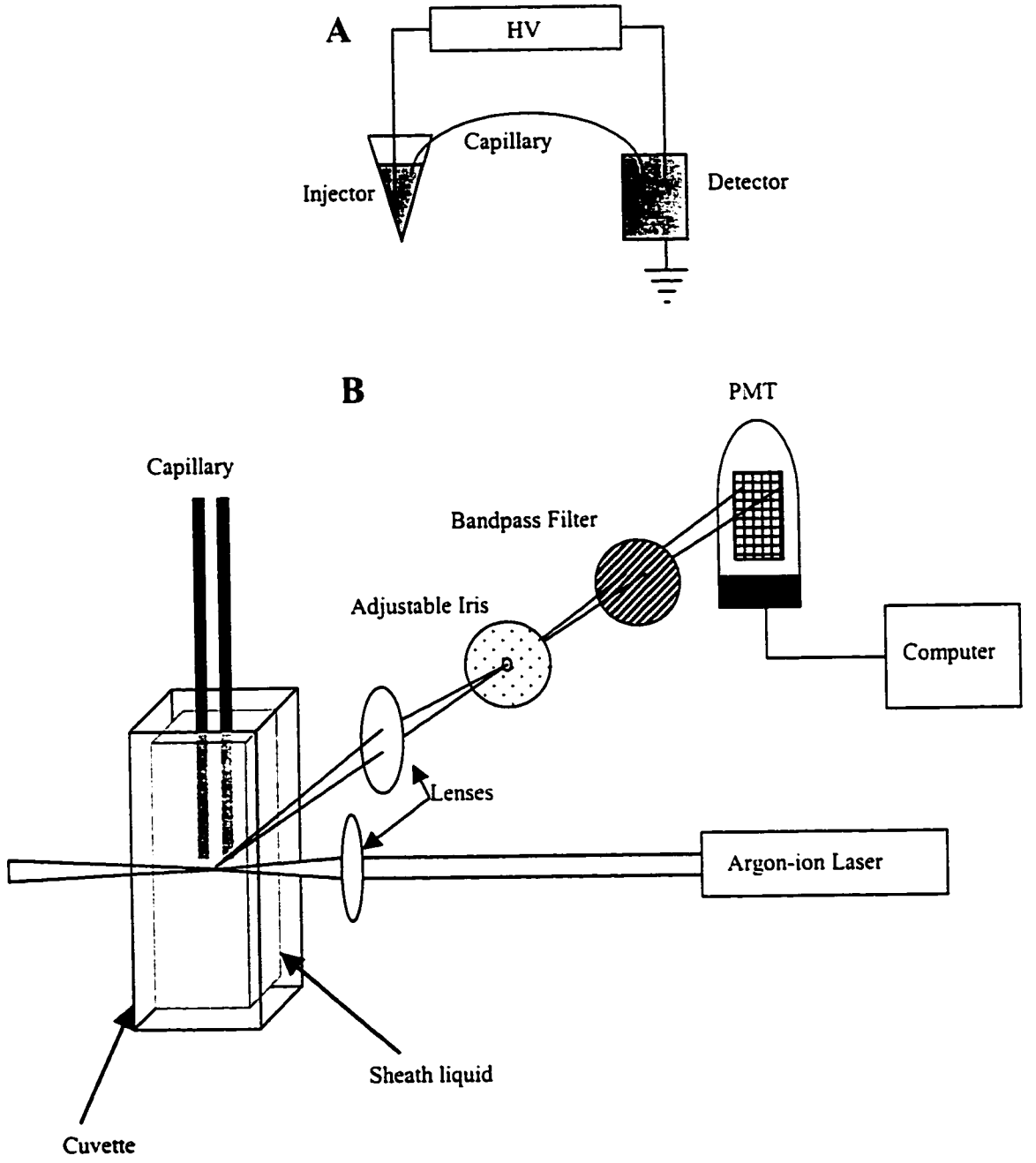

Figure 1.2 Schematic of (A) CE-LIF instrument, (B) high-sensitivity sheath flow assisted LIF detector.

1.6 Thesis summary

The application of CE to the analysis of a variety of molecules sampled by microdialysis has demonstrated the partnership between the two techniques. The next four chapters present the method of coupling microdialysis with MEKC-LIF to monitor amino acids in extracellular fluid *in vivo* in rat brain.

Chapter 2 presents a method for improving separation efficiency of amino acids in brain homogenate supernatants and microdialysates by adding β-cyclodextrin (β-CD) to the buffer system.

Chapter 3 describes the process of choosing a proper internal standard for precisely and accurately quantifying amino acid levels in brain microdialysates. Rhodamine 6G was determined to be a good candidate; it improved precision dramatically.

In Chapter 4, reaction rate and activation energy are investigated for five amino acid neurotransmitters with the fluorogenic reagent 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ). This study helped to better understand the labeling reaction and to choose the optimal reaction conditions.

Chapter 5 presents the method of continuously monitoring several important amino acids related to neurotransmission in rat brain by the combination of microdialysis sampling and MEKC-LIF detection. The effects of the antidepressant drug phenelzine on the levels of amino acids are discussed.

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CHAPTER 2

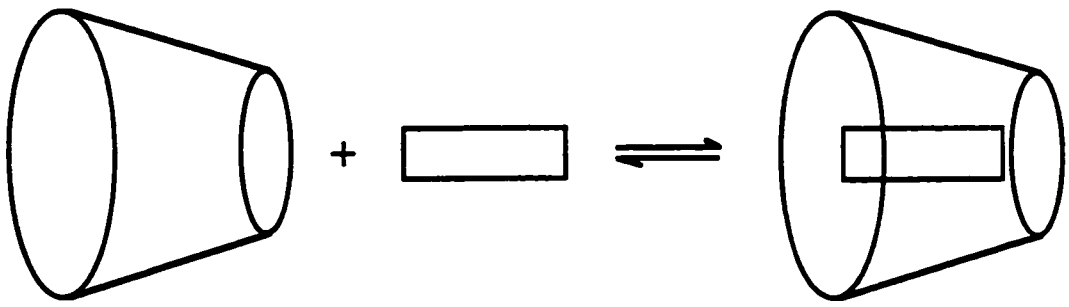
THE EFFECT OF β-CYCLODEXTRIN ON THE CE SEPARATION OF NEUROCHEMICALS

2.1 INTRODUCTION

2.1.1 Cyclodextrins

Cyclodextrins (CDs) are a homologous series of nonreducing cyclic oligosaccharides made up of six or more α-D-glucopyranose units linked together by α-1,4-glycoside bonds [1]. They were isolated as degradation products of starch by Villiers as long ago as 1891 [2], but it was not until 1904 that they were characterized by Schardinger as cyclic oligosaccharides (the "Schardinger dextrins") [3]. CDs with six, seven and eight glucose units are well known as α-, β- and γ-CDs respectively. So far, CDs with 6 to 19 D-glucose units have been purified and characterized [4]. However, only α-, β- and γ-CDs are currently commercially available. Many derivatives of these molecules have been produced, and usually show altered characteristics with regard to solubility, biocompatibility and complex formation [5-7].

The huge interest in CDs and their derivatives lies in their ability to act as host molecules to form inclusion complexes with a wide range of molecules (Figure 2.1). As a result of complex formation, the characteristics of the included substance, such as solubility, chemical reactivity, pK_a values and electrochemical properties, will be changed [8]. This unique property has led to a widespread utilization of cyclodextrins in pharmaceutical, food, chemical and other industrial areas [5]. In the pharmaceutical



Cyclodextrin Host Guest Inclusion Complex

Figure 2.1 Formation of inclusion complex.

industry, cyclodextrins and their derivatives have been used in drugs as solubilizers, diluents or tablet ingredients to improve the physical and chemical properties, or to enhance the bioavailability, of sparingly soluble drugs [5-6, 9-10]. Cyclodextrins have also been widely used for stabilizing flavors in food products and fragrances in cosmetics, for reducing foaming in foods and toiletries, and for eliminating undesired tastes and microbiological contamination [5, 11-13]. In the chemical industry, cyclodextrins and their derivatives are used as catalysts to improve the selectivity of reactions, as well as for the separation and purification of industrial-scale products [8].

Cyclodextrins and their derivatives have also received much attention in analytical chemistry, especially in the separation of structural, positional and stereoisomers [4]. CDs have been used as media modifiers in a diverse range of analytical techniques such as nuclear magnetic resonance (NMR), high-performance liquid chromatography (HPLC), gas chromatography (GC), thin layer chromatography (TLC) and capillary electrophoresis (CE) [4, 8, 14-16]. In the field of CE, CDs are considered the first choice as media modifiers for the separation of chiral compounds [17]. In addition to the importance of CDs in chiral separation, various forms of CE using CDs have been developed as among the most useful techniques for achiral separation and detection of small and neutral compounds [16].

This chapter describes the use of β-CD as a buffer modifier to improve the CE separation of 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ)-derivatized amino acids and primary amines in rat brain supernatants and microdialysates. This is an achiral separation for small molecules. Better separation was achieved than with micellar electrokinetic chromatography (MEKC).

2.1.2 Rat brain samples — supernatants and microdialysates

Many amino acids in mammalian brain function as neurotransmitters, including glutamic acid (or glutamate, Glu), aspartic acid (or aspartate, Asp), glycine (Gly) and γ-aminobutyric acid (GABA). Some are excitatory transmitters, such as glutamate and aspartate, while others are inhibitory transmitters, e.g. glycine and GABA [18]. These neurotransmitters have been implicated in many neurological and psychiatric disorders such as Huntington's disease, Parkinson's, epilepsy, schizophrenia and senile dementia [19]. Monitoring changes in the extracellular levels of these amino acids and biogenic amines in the brain is useful in determining their function in various neurological processes. Thus, there is great interest in developing methods that permit separation and quantitation of these amino acids in brain samples.

Of the various analytical methods currently in use, HPLC has been routinely employed with much success [18]. This methodology usually involves derivatization of the amino acids with *o*-phthaldialdehyde (OPA) prior to injection to produce compounds which can be detected by light absorption, fluorescence or electrochemical activity [18, 20, 21]. However, a problem with the OPA derivatives is that they are unstable and the reaction must be carefully timed to enable quantitation [22].

Derivatization of primary amines with 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ) in the presence of cyanide produces derivatives which are more stable than those of OPA. CE is an analytical counterpart to HPLC which offers a number of advantages, including higher column efficiency and smaller sample volume requirement. The high column efficiency allows the separation of the common amino acids labeled with FQ. Laser-induced fluorescence (LIF) has been the detection method of choice for

determining derivatized amino acids following separation by CE due to its high sensitivity [18]. CE has a very low sample volume requirement, typically < 10 nL; therefore, very good spatial resolution can be obtained in small samples of brain. Other advantages of CE over HPLC are the low solvent consumption and low equipment cost [18].

Most of the previous research on amino acid neurotransmitters using CE were based on naphthalene-2,3-dicarboxyaldehyde (NDA)/cyanide derivatization or OPA/β-mercaptoethanol (β-ME) derivatization. Weber and coworkers reported the use of NDA/CN derivatization for the separation and quantitation of six amino acids in rat brain by CE-LIF [18]. Church and coworkers used CE-LIF to determine glutamate and aspartate in rat brain dialysate following NDA/CN derivatization [23]. Lada and Kennedy monitored primary amines *in vivo*, by OPA/β-ME derivatization, using microdialysis coupled on-line with CE-LIF [24]. FQ was rarely used. However, many studies of amino acids, peptides and proteins using FQ/CN derivatization analyzed by CE-LIF have shown that FQ is a good fluorogenic reagent for primary amines [25-28].

In this chapter, amino acid standards as well as real rat brain samples were derivatized by FQ and detected by β-cyclodextrin aided MEKC-LIF. Amino acids and amines were separated and determined from rat brain samples: eight from rat brain supernatants and five from microdialysates (Table 2.1).

Name (1)	Name (2)	M.W.	Structure
Gln*	Glutamine	146.15	HOOC-CH (NH_2)-(CH_2)$_2$-CO-NH_2
Glu*	Glutamic acid	147.13	HOOC-CH (NH_2)-(CH_2)$_2$-COOH
Asp	Aspartic acid	133.10	HOOC-CH (NH_2)-CH_2-COOH
Asn	Asparagine	132.12	HOOC-CH (NH_2)-CH_2-CO-NH_2
IS*	O-methyl-*L*-threonine	133.10	HOOC-CH (NH_2)-CH (CH_3)-O-CH_3
Ala*	Alanine	89.09	HOOC-CH (NH_2)-CH_3
Taur*	Taurine	125.14	H_2N-CH_2-CH_2SO_3H
Ser	Serine	105.09	HOOC-CH (NH_2)-CH_2OH
GABA*	γ-Aminobutyric acid	103.12	HOOC-$CH_2CH_2CH_2$-NH_2

Table 2.1. Nomenclatures, molecular weights and structures of amino acids analyzed from rat brain supernatants (all) and microdialysates (*).

2.2 EXPERIMENTAL

2.2.1 Chemicals

All amino acids were purchased from Sigma (St. Louis, MO) and used as received. D-glucose, sodium carbonate, potassium hydroxide, potassium chloride, sodium dodecyl sulfate (SDS) and disodium tetraborate ($Na_2B_4O_7$) were supplied by BDH (Toronto, ON, Canada). Potassium cyanide and 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ) were obtained from Molecular Probes (Eugene, OR). HPLC grade methanol and β-cyclodextrin hydrate (β-CD) were from Aldrich (Milwaukee, WI). Magnesium chloride hexahydrate and concentrated hydrochloric acid were provided by Anachemia (Montreal, Quebec, Canada). Sodium chloride was supplied by ACP (Montreal, Quebec, Canada). Sodium phosphate dibasic (Na_2HPO_4) and calcium chloride were purchased from Fisher Scientific (Fair Lawn, NJ).

2.2.2 Stock solutions

A stock solution of FQ was prepared in pure methanol. Aliquots of the FQ stock solution (100 nmol) were dried in a SC110 Speed Vac (Savant Instruments, Farmingdale, NY) and stored at -20 °C to improve stability. A stock solution of KCN (25 mM) was prepared in 10 mM borate buffer (pH 9.2). The separation buffer consisted of 20 mM borate and 60 mM SDS (BS buffer). BS buffer with added CD was prepared by diluting appropriate amounts of β-CD in the above BS buffer. Stock solutions (1 mg/mL, ~0.01 M) of glutamine, glutamic acid, asparagine, aspartic acid, alanine, taurine, serine and GABA were prepared in deionized distilled water from a

Series 550 NANOpure water purification system (Barnstead, Dubuque, IA). A stock solution of 0.075 M O-methyl-*L*-threonine (IS) was prepared in deionized distilled water. All solutions and buffers were filtered with a 0.22 μm Millex-GS filter (Millipore, Bedford, MA).

A standard mixture of the brain supernatant was prepared in pure methanol. It consisted of 2.7×10^{-5} M glutamine, 3.3×10^{-5} M glutamic acid, 3.3×10^{-5} M aspartic acid, 2.0×10^{-5} M asparagine, 2.0×10^{-5} M alanine, 4.0×10^{-5} M taurine, 3.3×10^{-5} M serine and 3.3×10^{-5} M GABA.

A standard solution of the microdialysate was prepared in artificial CSF (see 2.2.3). It contained 3.2×10^{-5} M glutamine, 2.0×10^{-6} M glutamic acid, 6.0×10^{-5} M alanine, 4.0×10^{-6} M taurine and 2.0×10^{-6} M GABA.

2.2.3 Artificial cerebrospinal fluid (CSF)

Artificial cerebrospinal fluid (CSF) was prepared using double-distilled water and contained the following (in mM): NaCl (145), $CaCl_2$ (1.5), $MgCl_2$ (1.0) and dextrose (2.0). The pH value of the solution was adjusted to 7.3 with a mixture of Na_2HPO_4 and NaH_2PO_4 (final concentration approximately 4.0 mM). The solution was filtered and degassed before use (the recipe was provided by Dr. Glen Baker from Neurochemical Research Unit, Department of Psychiatry, University of Alberta).

2.2.4 Preparation of rat brain supernatants and microdialysates

Rat brain supernatants and microdialysates were kindly provided by Dr. Glen Baker from Neurochemical Research Unit, Department of Psychiatry, University of Alberta. The preparation procedures were described in detail in other references [29-31]. Briefly, they included the following steps:

2.2.4.1 Rat brain supernatants

Male Sprague-Dawley rats (200 – 250 g) were injected with physiological saline vehicle or with phenylethylidene hydrazine (30 mg/kg i.p. or 60 mg/kg i.p.). Groups of rats (n = 5 per group) were killed by cervical dislocation and decapitation at 6 h after drug administration. The brains were dissected out and homogenized in ice-cold HPLC-grade methanol. The homogenate was centrifuged to remove the protein precipitate and the supernatant was stored at - 80 °C until the time of analysis [29, 30].

2.2.4.2 Rat brain microdialysates

A microdialysis probe (2 mm membrane) was inserted into a guide cannula which was implanted into the rat brain four days before microdialysis. The probe was perfused at the rate of 1 μL/min with artificial cerebrospinal fluid. After a 2 h stabilization period, three 25-minute baseline samples were collected. Then, an antidepressant / antipanic drug phenelzine (30 mg/kg) was administered intraperitoneally (i.p.) and eleven 25-minute samples were collected. The samples were kept on dry ice during the collection process and then transferred to a –80 °C freezer for long-term storage [31].

2.2.5 Labeling reaction

2.2.5.1 Labeling rat brain supernatants

A 6-μL aliquot of sample or standard was added to a vial containing 100 nmol of previously dried FQ. To the above mixture, 4 μL of 0.1 M KOH, 1 μL of 0.075 M O-methyl-*L*-threonine (IS) and then 2 μL of 25 mM KCN prepared in 10 mM borate were added and mixed thoroughly to give a total volume of 13 μL. The pH of the reaction mixture was 9.5. The derivatized sample was allowed to react at 65 °C in a bath incubator (Fisher Scientific, Fair Lawn, NJ) for 30 minutes. Blanks were prepared using 6 μL pure MeOH instead of sample or standard. The reaction was quenched by diluting the mixture 25-fold with the artificial CSF and the diluted mixture was analyzed by MEKC-LIF.

2.2.5.2 Labeling microdialysates

To 100 nmol of dry FQ in a 500-μL vial, 2 μL of sample or standard and 1 μL of 1.5 mM IS were added. Next, 3 μL of 25 mM KCN prepared in 10 mM borate (pH 9.2) was added, mixed and allowed to react at 65 °C in the dark for 20 minutes. Blanks were prepared using 2 μL CSF instead of sample or standard. Samples were diluted 100-fold with the artificial CSF to quench the reaction. The diluted mixture was analyzed by MEKC-LIF.

2.2.6 CE instrumentation

Experiments were carried out using an in-house designed CE-LIF instrument with a detector based on a sheath-flow cuvette as previously described in Chapter 1 and elsewhere [32, 33]. Electrophoresis in the capillary was driven by a 0-30 kV dc power supply (Spellman, Plainview, NY) connected to platinum electrodes. The laser was a Model 2113-150MLYW argon-ion laser (Uniphase, San Jose, CA), which was operated at 12 mW output power at 488 nm. The laser beam was focused approximately 30 μm away from the tip of the capillary with a 6.3 x/0.2 NA microscope objective (Melles Griot, Nepean, ON, Canada). The emitted fluorescence was collected by a 60 x/0.7 NA objective (LWD-M Plan, Universe Kogaku, Japan) at right-angles to the incident beam, passed through a spatial filter to remove stray light and a 630DF30 bandpass filter (Omega Optical, Brattleboro, VT) to remove scattered light. A photomultiplier tube (R1477, Hamamatsu, Middlesex, NJ) biased at 900 V was used to detect fluorescence. The photocurrent passed through a current to voltage converter and a low-pass filter (RC= 47 ms) and then digitized with a 16-bit data acquisition board (NB-MIO-16XH-18, National Instruments, Austin, TX) connected to a Macintosh Centris 650 computer.

Electrophoresis was performed with a 38.2-cm long, 15-μm inner diameter and 145-μm outer diameter bare fused-silica capillary (Polymicro Technologies, Phoenix, AZ). Unless otherwise stated, sample introduction was accomplished by electrokinetic injection at 3.82 kV for 5 s. The separations were carried out under positive polarity at 15.28 kV (400 V/cm) or 19.1 kV (500 V/cm). A solution of 12.5 mM borate buffer was used as the sheath flow.

2.3 RESULTS AND DISCUSSION

2.3.1 The effect of β-CD on the separation of rat brain supernatants

Micellar electrokinetic chromatography (MEKC), which was first proposed by Terabe and coworkers [34], has been investigated by many researchers as a separation technique for both neutral and ionic compounds while retaining the high resolving power of CE [35]. In this separation mode, an ionic surfactant such as SDS is added to the buffer at concentrations exceeding the critical micellar concentration (CMC). The separation of the solutes occurs on the basis of the partition mechanism between an aqueous buffer and a micelle. This method was applied in this study. Buffers with various concentrations of borate and SDS were used to separate the FQ-labeled amino acids and amines. Among the different electrolytes, 20 mM borate and 60 mM SDS seemed to show the best separation (Figure 2.2 (a)). However, glutamine and glutamic acid co-eluted, aspartic acid and asparagine were suppressed, and the separation of alanine, taurine, serine and GABA was still incomplete.

A better approach was to add CDs to the MEKC system. This technique has already been reported by Terabe and coworkers for the separation of highly hydrophobic compounds [36]. The addition of CD to the micellar solution can change the migration selectivity for the solutes in MEKC by introducing another partition mechanism [35]. This method, named CD-MEKC by Terabe and coworkers [36], was applied to the separation of FQ-amino acids and amines in this study.

Figure 2.2 Effect of β-CD on the separation of rat brain supernatant standards. Peaks: a: Gln, b: Glu, c: Asp, d: Asn, e: Ala, f: Taur, g: Ser, h: GABA, IS: internal standard, O-methyl-*L*-threonine. Capillary: 38.2 cm x 145 μm o.d. x 15 μm i.d.; injection: 3.82 kV and 5 s; running voltage: 15.28 kV (400 V/cm). BS buffer: 20 mM borate, 60 mM SDS (pH 8.5). The electropherograms were obtained from BS buffer with (a) 0 mM (b) 0.5 mM, 1.0 mM and 5.0 mM β-CD.

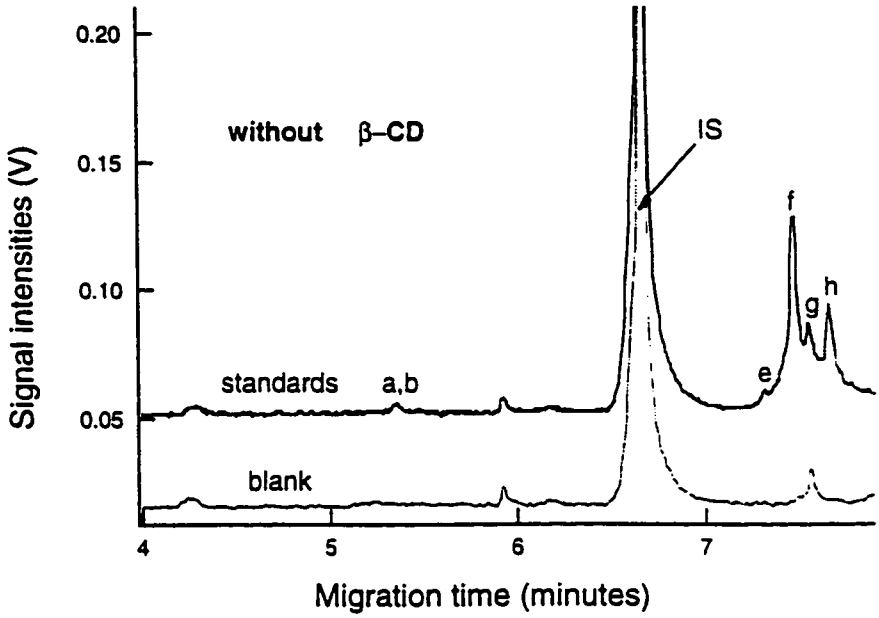


Figure 2.2 (a)

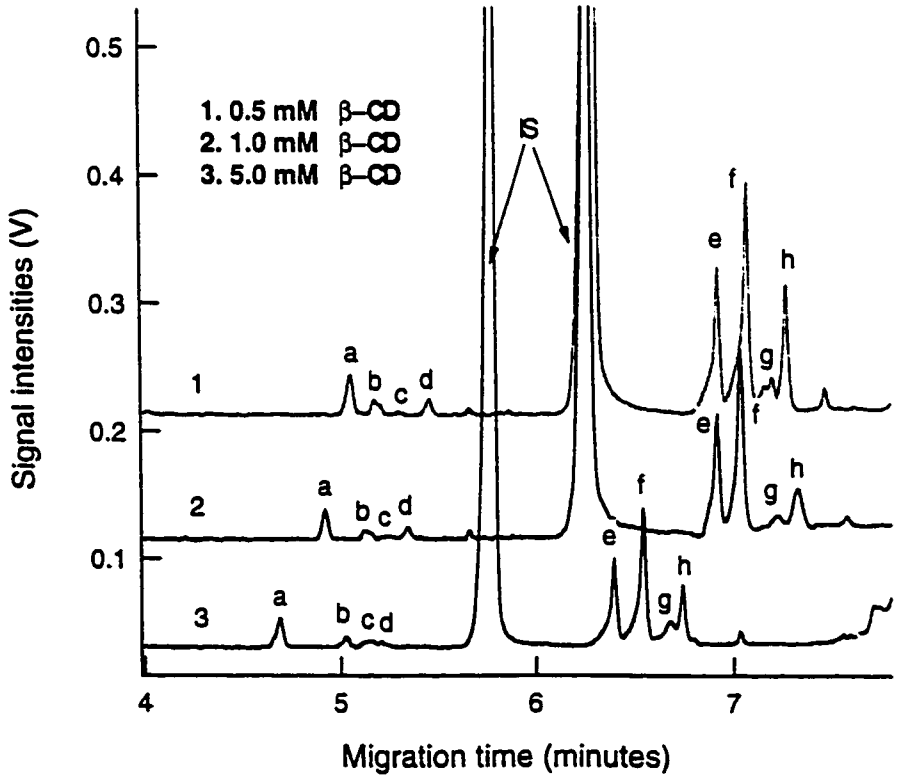


Figure 2.2 (b)

Figure 2.2 (b) shows the electropherograms of FQ-amino acids obtained by β-CD-MEKC with 0.5 mM, 1.0 mM and 5.0 mM of β-CD, respectively. Compared with Figure 2.2 (a), the separation of the amino acids and amines was dramatically improved by the addition of β-CD to the BS buffer. Glutamine and glutamic acid were well resolved in all cases. Alanine, taurine, serine and GABA were better separated. As the concentration of β-CD increased from 0.5 mM to 5.0 mM, the separation of glutamine and glutamic acid became better, but glutamic acid, aspartic acid and asparagine got closer, while alanine, taurine, serine and GABA were not affected much. Thus 0.5 mM β-CD was determined to be the optimum concentration for analyzing supernatant samples. Serine and GABA were still not well separated under these analytical conditions even at higher concentrations of β-CD.

Figure 2.2 also reflects the change in migration time of FQ-amino acids at different β-CD concentrations. All the FQ-amino acids and amines here migrated about 0.5 - 1 minute faster as the β-CD concentration increased from 0 to 5.0 mM, but the degree of change was different for different amines. A similar phenomenon was observed by Ueda and coworkers [35]. They demonstrated this reduced migration time with the use of β-CD in the determination of 14 NDA-CN-labeled amino acids by CE-LIF. Church and coworkers also reported the large decrease in migration time observed for NDA-CN-labeled glutamate and aspartate by using β-CD-aided CE-LIF [23]. The difference in migration time for an analyte is strongly dependent on the degree of complexation of the analyte with the β-CD [35]. Therefore, addition of β-CD to the micellar solution can change the migration selectivity so as to allow the separation of many analytes in MEKC.

α-CD and γ-CD were also tested as the buffer additives. However, α-CD had smaller effects on the separation than β- and γ-CD; γ-CD changed the migration order of amino acids that peaks had to be re-identified (data not shown). Therefore, β-CD was used as the buffer modifier.

MEKC was performed on nine FQ-labeled samples of rat brain supernatants using the protocol described in the Experimental Section. Figure 2.3 shows the electropherograms of two rat brain supernatants obtained when (a) BS buffer contained no β-CD or (b) BS buffer contained 0.5 mM β-CD. The separation of brain supernatants was greatly improved by the addition of β-CD. All peaks were identified by spiking the sample with standards. Use of the internal standard enabled quantification of these amino acids.

Figure 2.3 Effect of β-CD on the separation of rat brain supernatants. (a) BS buffer without β-CD. (b) BS buffer with 0.5 mM β-CD. Peaks and separation conditions are the same as in Figure 2.2.

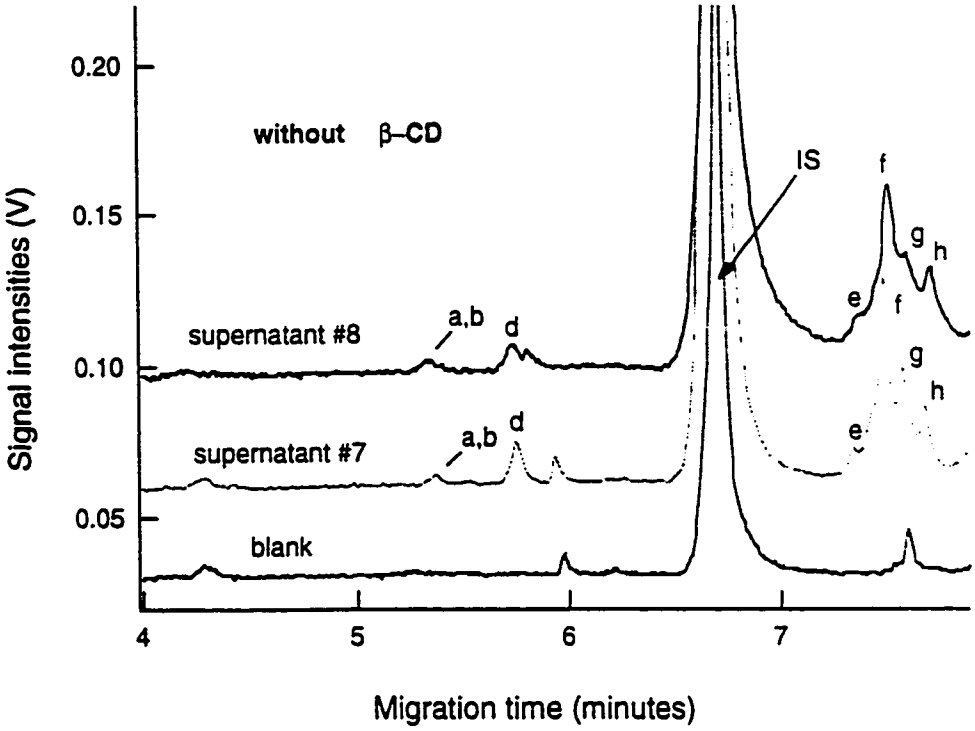


Figure 2.3 (a)

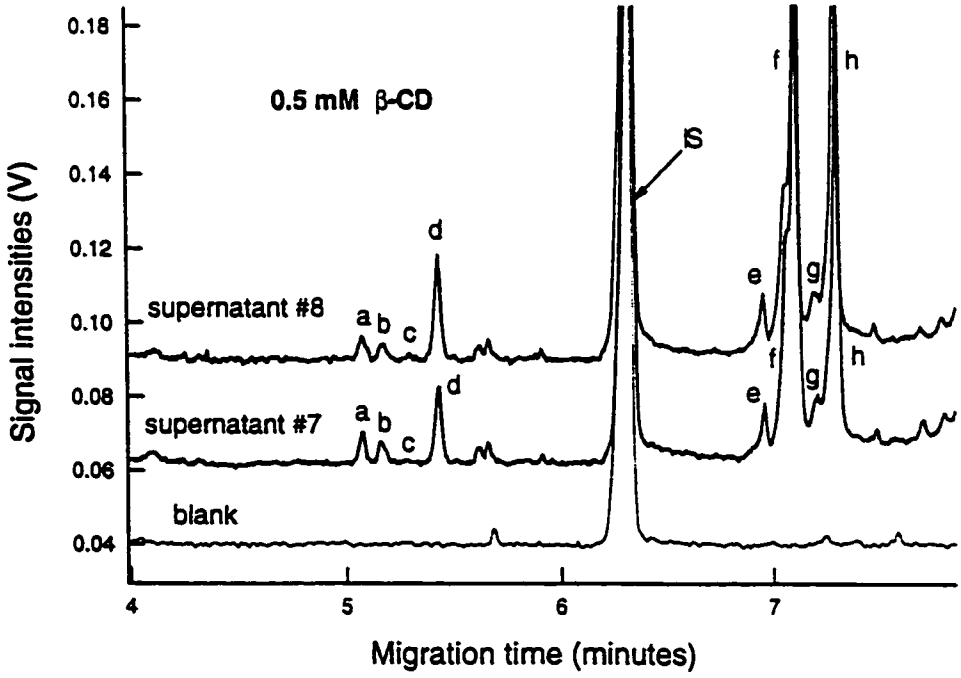


Figure 2.3 (b)

2.3.2 The effect of β-CD on the separation of rat brain microdialysates

The rat brain microdialysis samples were in cerebrospinal fluid (CSF) [31] while supernatant samples were diluted in methanol [29,30]. Two sets of standard mixtures were therefore prepared, one in artificial CSF and one in methanol (see Section 2.2.2) to mimic the real matrices for the two types of brain samples.

MEKC and β-CD-MEKC were both employed to standards and microdialysis samples. Figure 2.4 illustrates the electropherograms of the standard mixture in (a) BS buffer without β-CD and (b) BS buffers with 0.5 mM, 1.0 mM and 5.0 mM β-CD. A high separation voltage of 19.1 kV (500 V/cm) was applied without causing a large Joule heating problem due to the small inner diameter of the capillary (15 μm). No bubbles formed in the 15 μm-i.d. capillary under this high voltage whereas many bubbles were observed in the 50 μm-i.d. capillary even at a lower voltage, 15.28 kV (400 V/cm).

In MEKC, glutamine and glutamic acid co-eluted due to few differences between the two molecules (Figure 2.4 (a)). With the aid of β-CD, baseline separation of glutamine and glutamic acid was achieved. Better separation of glutamine and glutamic acid was obtained at higher concentrations of β-CD. The speed of migration of FQ-amino acids and amines was also improved by adding β-CD to the separation buffer. The internal standard showed a shoulder peak when β-CD was present, which might arise from the enantiomer of the internal standard or from an impurity in the internal standard.

Figure 2.4 Effect of β-CD on the separation of microdialysate standards. Peaks: a: Gln, b: Glu, c: Ala, d: Taur, e: Ser, f: GABA, IS: internal standard, O-methyl-*L*-threonine. Capillary: 38.2 cm x 145 μm o.d. x 15 μm i.d.; injection: 3.82 kV and 5 s; running voltage: 19.1 kV (500 V/cm). (a) BS buffer without β-CD, (b) BS buffers containing 0.5 mM, 1.0 mM and 5.0 mM β-CD.

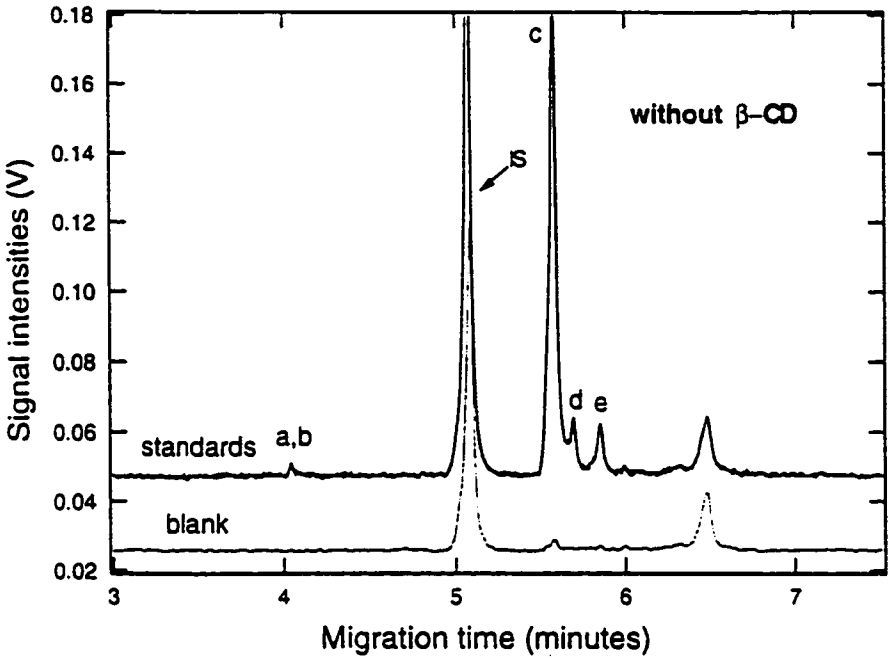


Figure 2.4 (a)

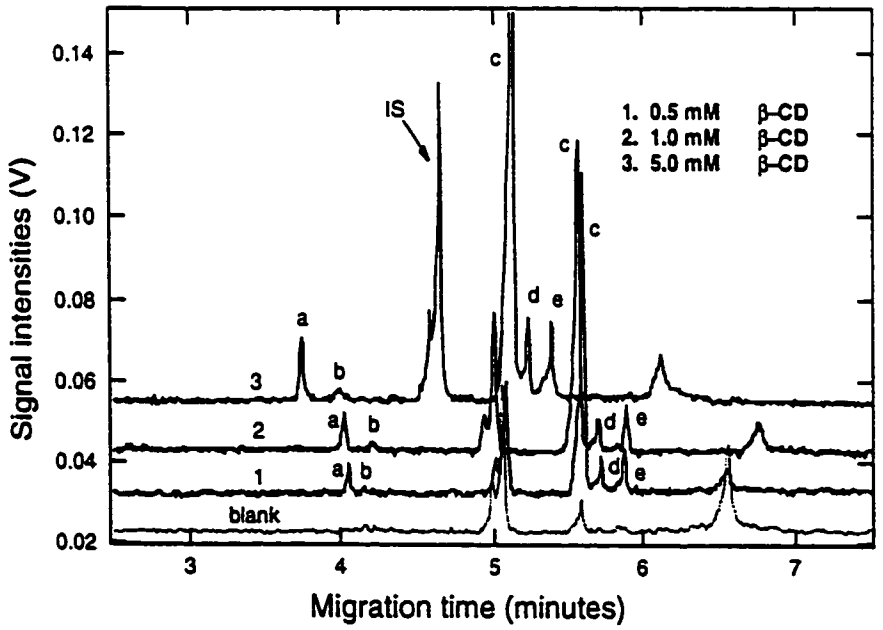


Figure 2.4 (b)

The electropherograms of two microdialysates are shown in Figure 2.5. Glutamine and glutamic acid were well resolved in the presence of 5.0 mM β-CD in BS buffer. Peaks were determined by spiking the sample with standards. The migration times could not be compared in this case since the electrophoresis was carried out under different voltages: (a) 19.1 kV, (b) 15.28 kV.

Figure 2.5 Effect of β-CD on the separation of rat brain microdialysates. Peaks: a: Gln, b: Glu, c: Ala, d: Taur, e: Ser, f: GABA, IS: internal standard, O-methyl-*L*-threonine. BS buffer: 20 mM borate, 60 mM SDS. Capillary: 38.2 cm x 145 μm o.d. x 15 μm i.d. (a) BS buffer without β-CD. Injection: 3.82 kV and 5 s; running voltage: 19.1 kV (500 V/cm). (b) BS buffer with 5.0 mM β-CD. Injection: 7.64 kV and 3 s; running voltage: 15.28 kV (400 V/cm).

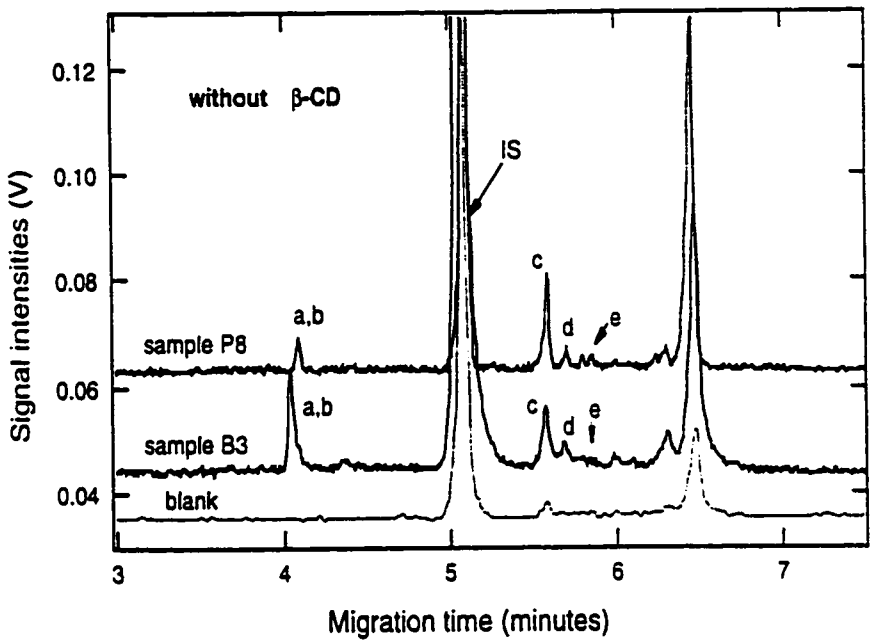


Figure 2.5 (a)

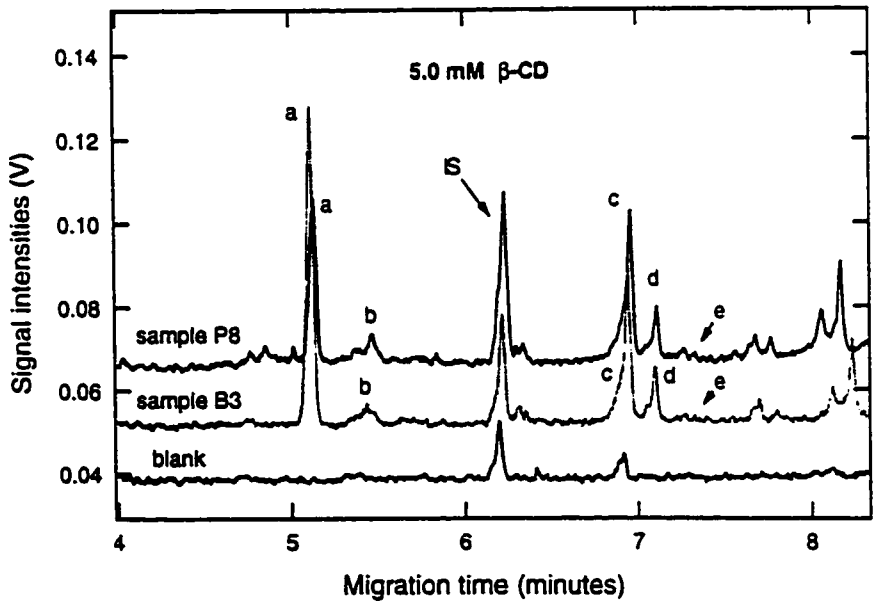


Figure 2.5 (b)

2.3.3 Detection limits

To assess the limits of detection (LOD) for the FQ-labeled amino acids, a 10^{-4} M solution of amines was labeled and diluted to construct the calibration curve. Table 2.2 lists the concentration detection limits (3σ) and mass detection limits for the amino acids and amines in rat brain supernatants. The results of detection limits for microdialysates are given in Table 2.3.

The concentration detection limits ranged from 6.9×10^{-9} M for alanine to 3.6×10^{-7} M for aspartic acid in rat brain supernatants. Better detection limits were obtained in microdialysates, ranging from 1.2×10^{-9} M for GABA to 1.9×10^{-8} M for glutamic acid. The difference is presumably due to the differences in sample matrices and labeling procedure. However, the concentration detection limits are slightly higher than the previously reported detection limits for amino acids, 10^{-10} M using CE-LIF [35]. Concentration detection limits scale inversely with the capillary cross-section. The detection limit suffered from the use of a 15-μm ID separation capillary; the use of a 50-μm ID capillary would have improved the concentration detection limit by about an order of magnitude.

The mass detection limit (3σ) is the product of the concentration detection limit and the injection volume. The mass detection limits ranged from 1 amol to 99 amol in supernatants and from 300 zmol to 7 amol in microdialysates. Alanine, taurine and GABA have better detection limits than other amino acids and amines, which may arise from higher reaction efficiency, better molar absorptivity or higher fluorescence quantum yield of the labeled molecules.

	Detection limit	
Amino acid	LOD_c (M)	LOD_m (amol)
Glutamine	3.3×10^{-8}	9.4
Glutamic acid	1.1×10^{-7}	29
Aspartic acid	3.6×10^{-7}	99
Asparagine	6.0×10^{-8}	16
Alanine	6.9×10^{-9}	1.4
Taurine	8.8×10^{-9}	1.8
Serine	4.3×10^{-8}	8.6
GABA	9.0×10^{-9}	1.6

Table2.2 Limits of detection for labeled amino acids in rat brain supernatants.

LOD_c - concentration limit of detection (3σ).

LOD_m - mass limit of detection (3σ). This value is the product of the concentration detection limit and the injection volume.

	Detection limit	
Amino acid	LOD_c (M)	LOD_m (amol)
Glutamine	1.4×10^{-8}	5.3
Glutamic acid	1.9×10^{-8}	6.7
Alanine	2.4×10^{-9}	0.5
Taurine	2.3×10^{-9}	0.6
GABA	1.2×10^{-9}	0.3

Table2.3 Limits of detection for labeled amino acids in rat brain microdialysates.

The terms LOD_c and LOD_m are described as in Table 2.2.

2.4 CONCLUSIONS

This chapter describes the use of β-CD in the separation of FQ-labeled amino acids. The addition of β-CD to the separation buffer improves the selectivity for FQ-labeled amino acids compared with MEKC, especially for glutamine and glutamic acid. β-CD also influences the overall migration time of the derivatized amino acids. Separation of peaks in two types of rat neural samples, brain supernatants and microdialysates, is improved by β-CD-MEKC. Detection limits are different for labeled amino acids in different matrices, ranging from 10^{-9} M to 10^{-7} M. The inherent small injection volume and short analysis time make this method ideal for the study of important amino acid neurotransmitters in the central nervous system.

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CHAPTER 3

SELECTION OF INTERNAL STANDARD FOR QUANTITATIVE ANALYSIS OF AMINO ACIDS IN BRAIN MICRODIALYSATES

3.1 INTRODUCTION

Standard curve, standard addition and internal standard methods are commonly used analytical methods for quantitative spectrochemical, electrochemical and chromatographic analyses [1-3]. The highest precision for quantitative chromatography is obtained by use of internal standards because the uncertainties introduced by sample injection are avoided [1]. The internal standard method is also widely used in capillary electrophoresis (CE) [4, 5]. In this method, a known concentration of a reference substance is added to all standards and samples, and the ratio of analyte to reference peak areas or peak heights serves as the analytical parameter. Known mixtures of internal standard and analyte are used to construct a calibration curve, in which the peak-area or peak-height ratio is plotted *vs.* the analyte concentration. The ratio for the unknown sample is then used to determine the analyte concentration in the sample [1, 3].

The internal standard method is particularly useful because it can minimize errors caused by sample preparation, the experimental apparatus and/or sample introduction. CE is an inherent microanalytical technique, requiring the use of narrow-bore capillaries. Total capillary volumes are in the microliter range, and sample loading volumes are usually nanoliters or subnanoliters [5, 6], whereas in HPLC, the sample volumes are in the microliter range [5]. Thus in CE, minute variations in sample

loading, voltage, temperature, electrolytes and capillary quality can cause considerable variation in measurements. These variations can be corrected for with the use of internal standards [5].

In vivo microdialysis, using dialysis probes inserted into discrete brain areas and subsequent analysis of neurotransmitters in the dialysates, is useful in yielding important information about the actions of psychotropic drugs and endogenous neurotransmitter systems [7]. HPLC is usually used for such analyses [7]. However, due to its high resolving power and small sample requirement, CE has also been explored as an alternative to HPLC for analysis of dialysates [8-10].

In order to accurately quantify amino acid neurotransmitters in dialysates, an internal standard must be carefully selected. The internal standard should not be a natural component of the dialysate, nor co-elute with any components normally found therein [11]. In addition, the internal standard should be reasonably stable, and its peak should appear close to the analyte peaks.

This chapter describes the selection of an internal standard for quantitative analysis of important amino acids, including glutamine (Gln), glutamic acid (Glu), alanine (Ala), taurine (Taur) and γ-aminobutyric acid (GABA) in brain microdialysates. Rhodamine 6G was determined to be a good choice. 3-(2-Furoyl)quinoline-2-carboxaldehyde/cyanide (FQ/CN) was used for derivatization of amino acid standards and dialysates. A β-cyclodextrin-assisted micellar electrokinetic chromatography (MEKC) separation mode was used.

3.2 EXPERIMENTAL

3.2.1 Materials

All amino acids were purchased from Sigma (St. Louis, MO). β-Cyclodextrin hydrate (β-CD), HPLC grade methanol, dichloromethane (CH_2Cl_2), rhodamine B and rhodamine 6G were provided by Aldrich (Milwaukee, WI). 5-Carboxytetramethyl-rhodamine succinimidyl ester (5-TAMRA-SE), tetramethylrhodamine-5-isothiocyanate (5-TRITC, isomer G), tetramethylrosamine chloride, FQ and potassium cyanide (KCN) were obtained from Molecular Probes (Eugene, OR). Disodium tetraborate and sodium dodecyl sulfate (SDS) were supplied by BDH (Toronto, ON, Canada). Sodium bicarbonate ($NaHCO_3$) was purchased from Anachemia (Montreal, Quebec, Canada). N, N-Dimethylformamide (DMF) and molecular sieve type 4A were provided by Caledon (Georgetown, ON, Canada). All chemicals were used as received except DMF.

Rat brain microdialysates were kindly provided by Dr. Glen Baker from the Neurochemical Research Unit, Department of Psychiatry, University of Alberta. The preparation procedures were described in 2.2.4 and elsewhere [7].

3.2.2 Stock solutions

A stock solution of 0.075 M O-methyl-*L*-threonine was prepared in deionized distilled water using a Series 550 NANOpure water purification system (Barnstead, Dubuque, IA). Stock solutions of 10^{-3} M rhodamine B, rhodamine 6G, 5-TAMRA-SE, 5-TRITC and tetramethylrosamine chloride were prepared in methanol and then diluted to 10^{-6} M in deionized distilled water. KCN was dissolved in 10 mM borate buffer (pH

9.2) at a concentration of 25 mM. For CE experiments, the running buffer was 45 mM SDS, 15 mM borate and 5 mM β-CD (pH 8.5). The sheath flow buffer was 10 mM borate (pH 9.2). All buffers were made with deionized distilled water and were filtered using a 0.2-μm filter. Stock solutions (1 mg/mL, ~0.01 M) of glutamine, glutamic acid, alanine, taurine and GABA were prepared in deionized distilled water.

A stock solution of 10 mM FQ was prepared in methanol. 10-μL aliquots were then placed into 500-μL microcentrifuge tubes and the solvent was removed under vacuum using a SC110 Speed Vac (Savant Instruments, Farmingdale, NY). The dried FQ aliquots were stored at -20 °C. These precautions are necessary since FQ degrades slowly in solution, even if the solutions are stored at -20 °C. A stock solution of 0.185 M $NaHCO_3$ (pH 8.3) was made in deionized distilled water.

A standard mixture of amino acids was prepared in artificial cerebrospinal fluid (CSF, pH 7.3, as described in Section 2.2.3) to mimic brain dialysates. It contained 4.8 x 10^{-5} M glutamine, 1.2 x 10^{-5} M glutamic acid, 2.4 x 10^{-5} M alanine, 6.0 x 10^{-6} M taurine and 6.0 x 10^{-6} M GABA.

3.2.3 FQ labeling reaction

3.2.3.1 Labeling amino acid standards

For the use of O-methyl-*L*-threonine as an internal standard, derivatization was typically performed by adding 4 μL standard mixture, 1 μL of 1 mM O-methyl-*L*-threonine and 4 μL of 25 mM KCN prepared in 10 mM borate (pH 9.2) to a vial containing 100 nmol of previously dried FQ. The reaction was carried out in a water

bath incubator (Fisher Scientific, Fair Lawn, NJ) at 65 °C for 20 minutes in the dark. The mixture was diluted 20-fold with the artificial CSF to quench the reaction.

When 5-TAMRA-SE, 5-TRITC, tetramethylrosamine chloride and 5-TAMRA-SE-labeled Nα-acetyl-*L*-lysine were tested as internal standards, 4 μL of 1 mM amino acid standard or artificial CSF, 2 μL of 10^{-6} M IS and 4 μL of 25 mM KCN were added to 100 nmol of dry FQ, mixed and allowed to react at 65 °C for 15 minutes in the dark. The mixture was then diluted 20-fold with the artificial CSF. Rhodamine B and rhodamine 6G were individually added to the diluted mixture to make a 1 x 10^{-8} M solution instead of incubating with amino acids.

3.2.3.2 Labeling brain microdialysates

4 μL dialysate and 4 μL of 25 mM KCN in 10 mM borate (pH 9.2) were added to 100 nmol of dry FQ, mixed and allowed to react at 65 °C for 50 minutes in the dark. Samples were diluted 25-fold with the artificial CSF. Rhodamine 6G was added to the diluted reaction mixture to make a 1 x 10^{-8} M solution.

3.2.4 Labeling reaction of 5-TAMRA-SE with Nα-acetyl-*L*-lysine

Succinimidyl esters are among the best reagents for modifying aliphatic amines since they form stable carboxamides and have good reactivity. During the conjugation, succinimidyl ester hydrolysis can compete as a side reaction, but this is usually slow below pH 9 [12].

The received DMF was distilled, sealed with Ar and stored with molecular sieves to protect the sample from humidity. 0.5 mM Nα-acetyl-*L*-lysine was made in 100 μL anhydrous DMF immediately before starting the reaction. 5-TAMRA-SE was dissolved in anhydrous DMF at a concentration of 2 mg/mL (3.8 mM). While stirring the amino acid solution, 100 μL 5-TAMRA-SE solution was slowly added. Then 100 μL of 0.185 M $NaHCO_3$ buffer (pH 8.3) was added to the mixture. The reaction was incubated at room temperature for 5 hours in the dark with continuous stirring. The blank was prepared using 100 μL anhydrous DMF instead of Nα-acetyl-*L*-lysine.

3.2.5 Separation of the labeled Nα-acetyl-*L*-lysine

Reversed phase chromatography was performed on the product mixture with a Sep-Pak plus C_{18} cartridge (Waters, Milford, MA). The cartridge was wetted with 30 mL methanol and then rinsed with 30 mL water. Next, the product mixture was loaded onto the cartridge carefully and washed with 30 mL water until the eluate became colorless. Then the cartridge was washed with methanol. The red-colored portion was collected and solvent removed under vacuum in a Speed Vac. The dried sample was then dissolved in 200 μL methanol and stored in a capped vial at -20 °C.

Thin layer chromatography (TLC) was carried out on the Sep-Pak separated sample with pre-coated TLC aluminum sheets polyamide 11 F_{254} (EM Science, Gibbstown, NJ), using MeOH/CH_2Cl_2 with different volume ratios as the eluent. Ion exchange TLC was performed on the Sep-Pak separated sample with pre-coated plastic sheets POLYGRAM IONEX-25 SB-Ac/UV_{254} (Brinkmann Instruments, Toronto, ON, Canada).

3.2.6 CE separation

A locally constructed CE-LIF instrument was used for the experiment [13]. An argon-ion laser provided excitation at 488 nm (Model 2113-150MLYW, Uniphase, San Jose, CA). A 630DF30 bandpass filter (Omega Optical, Brattleboro, VT) was chosen for the detector in most cases. A 580DF40 bandpass filter (Omega Optical) was used for the detection of 5-TAMRA-SE related compounds.

Separations were performed with 47.0 cm – 48.6 cm long, 15 μm i.d. and 145 μm o.d. fused-silica capillaries (Polymicro Technologies, Phoenix, AZ) at an electric field of 400 V/cm. To reduce the background signal, the polyimide coating was removed from the last few millimeters of the capillary using a gentle flame. Samples were introduced electrokinetically at 100 V/cm for 5 s or hydrodynamically at 7.6 kP_a (78 cm height) for 10 s. The running buffer was 45 mM SDS, 15 mM borate and 5 mM β-CD (pH 8.5). 10 mM borate buffer was used as the sheath flow.

3.3 RESULTS AND DISCUSSION

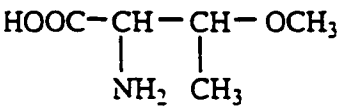
Seven chemicals have been tested as internal standards for analysis of amino acids (Table 3.1). The tested materials can be classified as: (1) reactive internal standards, which react with either FQ or amino acids, and (2) nonreactive internal standards, which react with neither amino acids nor FQ. Figure 3.1 shows the structures of these chemicals. All the tests were performed on standards. However, the purpose of this study was to find a good internal standard for quantitative analysis of amino acid neurotransmitters in real brain dialysates. The data for dialysates are also shown in this chapter.

3.3.1 O-Methyl-*L*-threonine

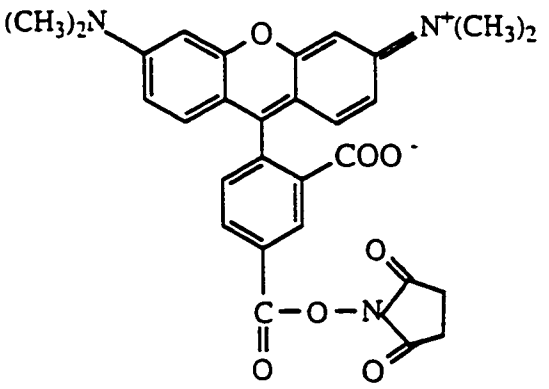
O-Methyl-*L*-threonine was first selected as an internal standard because it has reasonable reactivity with FQ and the resultant fluorescence peak does not overlap with other FQ-labeled amino acid peaks. We assumed it could correct for variations in both the injection volume and the labeling process. The ratio of the peak height for amino acids to that for the internal standard ($H_{amino\ acid}/H_{IS}$) was used to calculate the reproducibility. The reproducibility, or precision, was expressed as relative standard deviation (RSD), which is equal to (standard deviation / mean) x 100%. The intraassay reproducibility was determined by running the same sample three times, whereas the interassay reproducibility was determined by running three similar samples three times each for a total of nine runs. O-Methyl-*L*-threonine produced poor precision, especially for interassay variation (Table 3.2). Several parameters were considered as the source of the problem, including injection method, temperature, the amount of FQ and reaction time.

Type	Chemical
Reactive internal standard	O-Methyl-*L*-threonine
	5-Carboxytetramethylrhodamine succinimidyl ester (5-TAMRA-SE)
	Tetramethylrhodamine-5-isothiocyanate (5-TRITC)
Nonreactive internal standard	Rhodamine B
	Rhodamine 6G
	Tetramethylrosamine chloride
	5-TAMRA-SE-labeled Nα-acetyl-*L*-lysine

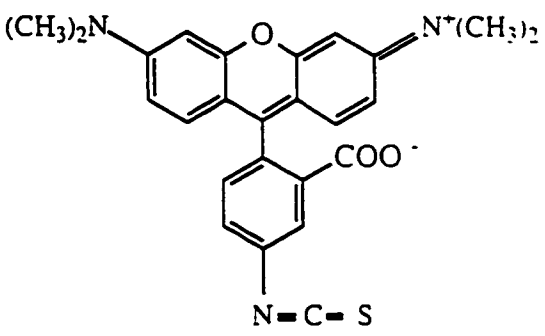
Table 3.1 Chemicals used as internal standards for quantitative analysis of amino acids.



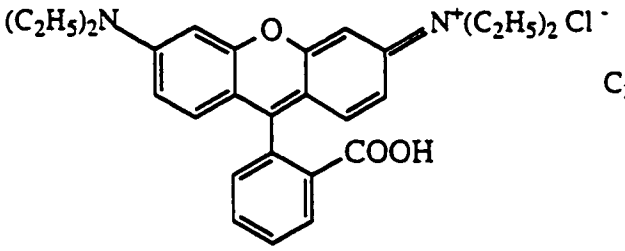
O-Methyl-*L*-threonine



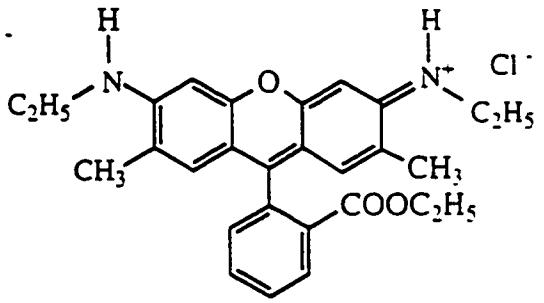
5-Carboxytetramethylrhodamine, succinimidyl ester (5-TAMRA-SE)



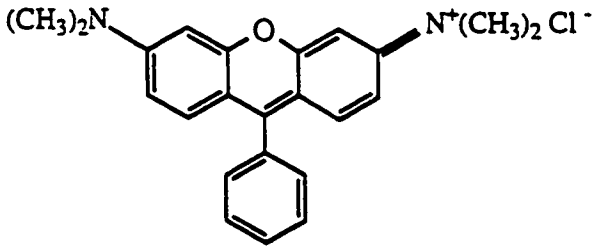
Tetramethylrhodamine-5-isothiocyanate (5-TRITC, isomer G)



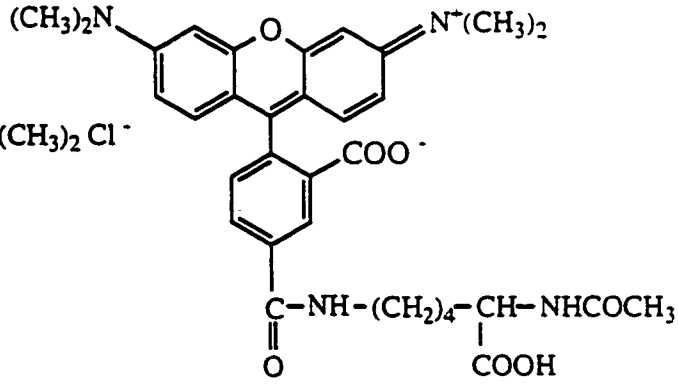
Rhodamine B



Rhodamine 6G



Tetramethylrosamine chloride



5-TAMRA-SE-labeled Nα-acetyl-*L*-lysine

Figure 3.1 Structures of the chemicals used as internal standards for quantitative analysis of amino acid neurotransmitters in brain microdialysates.

Amino Acid	RSD, %			
	Intraassay		Interassay	
	Electrokinetic	Hydrodynamic	Electrokinetic	Hydrodynamic
Gln	3.5	2.0	23.1	18.8
Glu	7.2	5.5	8.9	6.6
Ala	4.8	3.2	6.6	4.6
Taur	4.1	3.8	14.4	9.7
GABA	2.1	2.2	14.6	7.6

Table 3.2 Intra- (n = 3) and interassay reproducibility (n = 9) measured with use of O-methyl-*L*-threonine as an internal standard, using electrokinetic and hydrodynamic injection methods.

3.3.1.1 Injection method

Both electrokinetic injection and hydrodynamic injection methods were tested. Table 3.2 lists the intra- and interassay reproducibility for both injection methods. The hydrodynamic method produced 2.0-5.5% RSD for intraassay and 4.6-18.8% RSD for interassay, which were slightly better than for the electrokinetic method (2.1-7.2% RSD for intraassay and 6.6-23.1% RSD for interassay). This is presumably because with the electrokinetic method the injection volume varied for FQ-labeled amino acids due to different electrophoretic behaviors arising from different size/charge ratios. There is no such bias under the hydrodynamic injection method. However, injection method did not improve reproducibility much; therefore injection was not a major reason for the poor precision. The labeling reaction was then studied.

3.3.1.2 Reaction temperature

A Digi-Sense thermocouple thermometer (Cole-Parmer Instrument, Chicago, IL) was used to measure the temperature of the water bath in various wells of the incubator. The temperature ranged from 64.9 °C to 65.2 °C. Such minor differences in temperature should not cause much variation in the labeling reaction.

3.3.1.3 Amount of FQ

100 nmol, 200 nmol and 300 nmol FQ were used to label 4 μL amino acid standard mixture at 65 °C for 20 min respectively. The fluorescence signals as well as the peak-height ratios varied little for different amounts of FQ. FQ-labeled GABA is presented in Figure 3.2 as an example. This study demonstrates that 100 nmol FQ, present in nearly 70-fold excess, was far more than enough for the production of FQ-amino acids and would not affect the analysis.

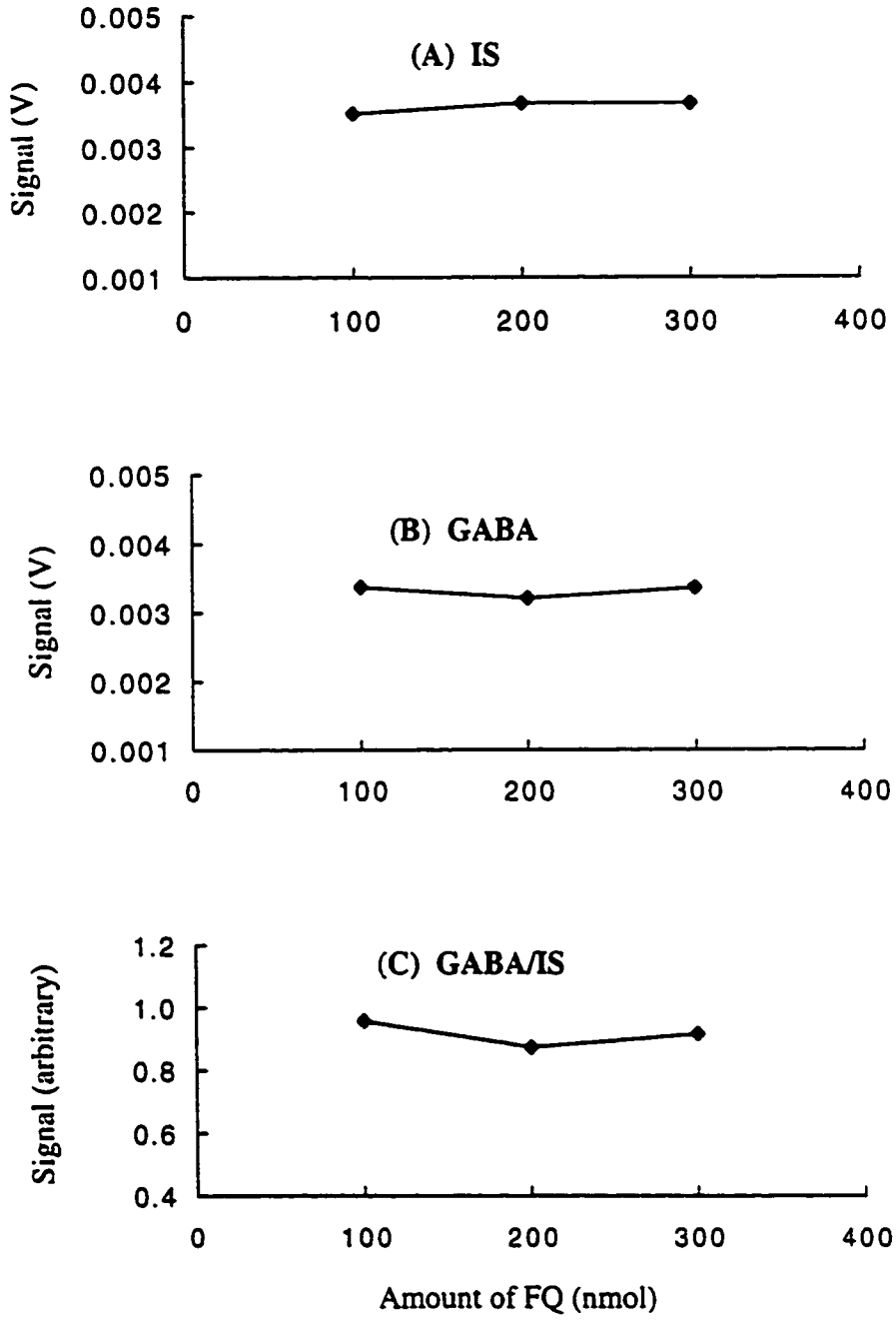


Figure 3.2 Effect of FQ amount on the fluorescence signals of (A) O-methyl-L - threonine (IS), (B) GABA and (C) peak-height ratios of GABA/IS.

3.3.1.4 Reaction time

4-μL amino acid standard mixture was derivatized by 100 nmol FQ at 65°C for a period ranging from 6 min to 60 min. Figure 3.3 shows the electropherograms of reaction products at 10, 20 and 30 min. All the fluorescence signals for FQ-labeled amino acids as well as for O-methyl-*L*-threonine increased with longer reaction times.

A typical diagram of fluorescence signal *vs.* reaction time for glutamine is shown in Figure 3.4. It is obvious that the labeling reaction did not go to completion at 20 min, which was usually employed in our previous studies on the labeling reaction, for O-methyl-*L*-threonine nor for glutamine nor other amino acids (Figure 3.4 A & B). However, the normalized signal (H_{Gln}/H_{IS}) did not show any obvious trend as reaction time increased, and even dropped after 50 min (Figure 3.4 C).

The reaction rate is different for O-methyl-*L*-threonine and other amino acids. As a result, minute variations in production of FQ-labeled O-methyl-*L*-threonine would affect quantification of amino acids only through small changes in the size of the FQ-labeled O-methyl-*L*-threonine peak.

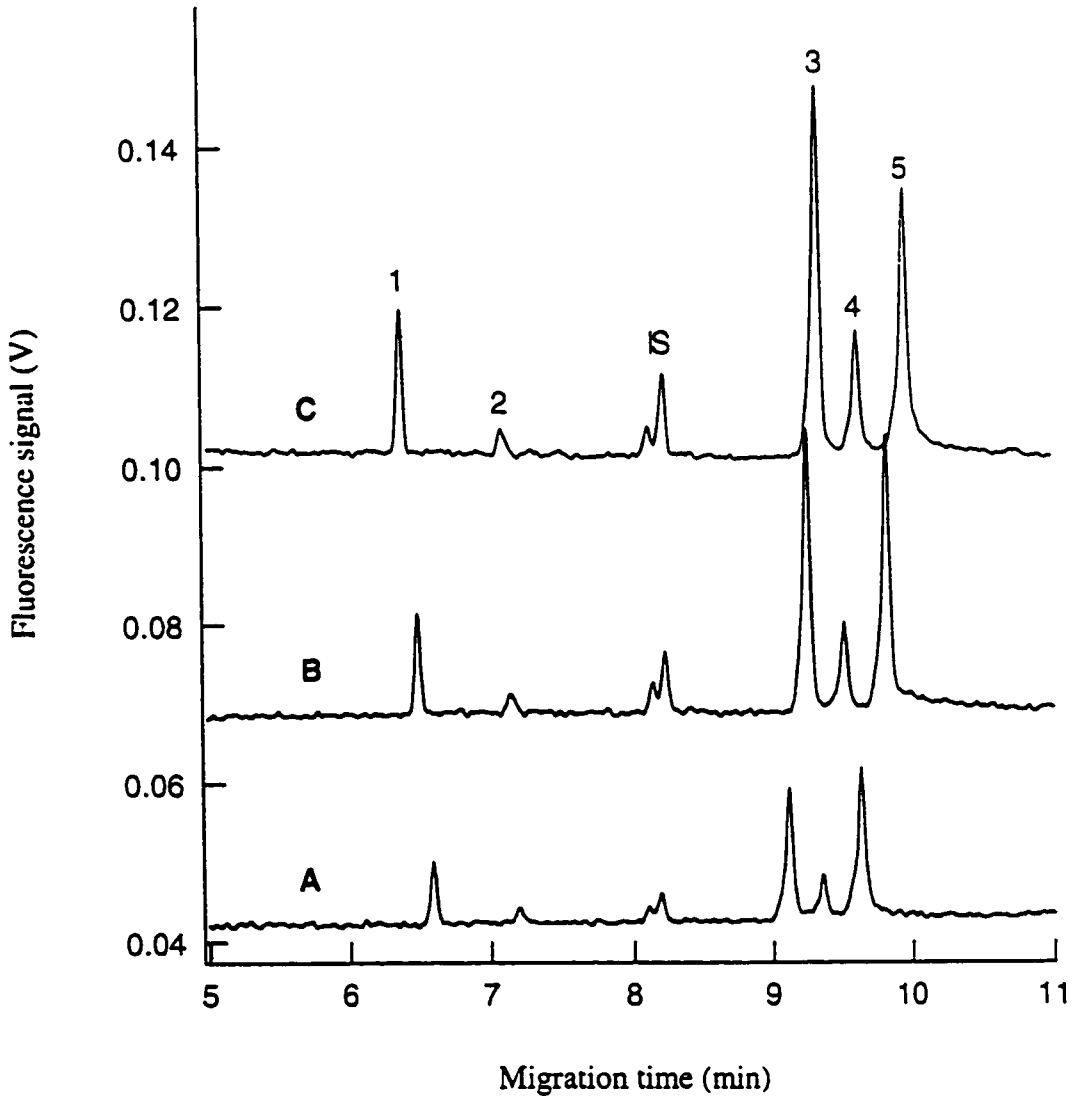


Figure 3.3 Electropherograms of FQ-labeled amino acids and amines at different reaction times. Peaks: 1. Gln, 2. Glu, 3. Ala, 4. Taur, 5. GABA, IS: O-methyl-*L*-threonine. Capillary: 47.1 cm x 15 μm i.d. x 145 μm o.d.; injection: 4.71 kV x 5 s; running voltage: 18.84 kV. Reaction time: A. 10 min, B. 20 min, C. 30 min.

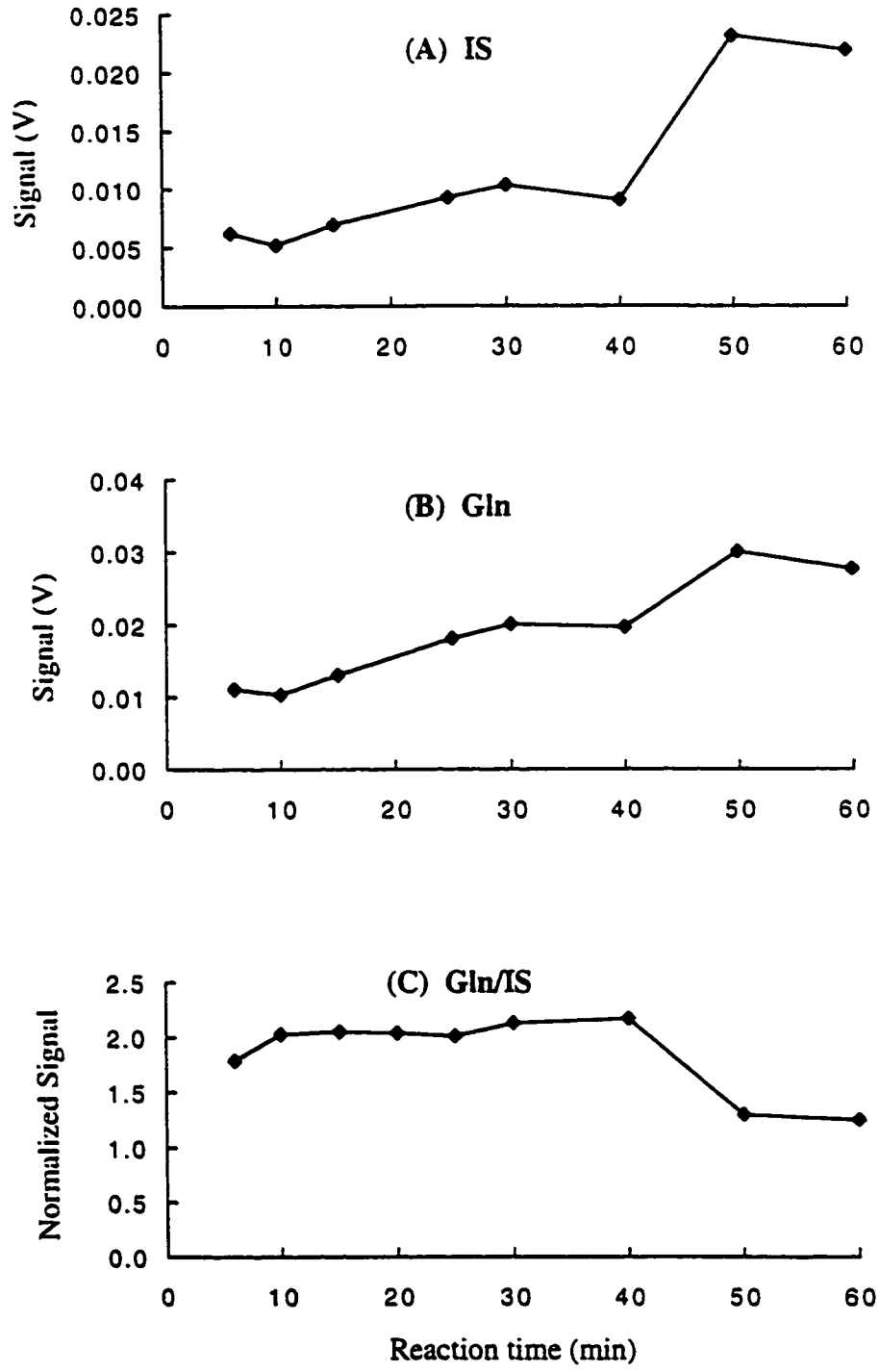


Figure 3.4 Effect of reaction time on the fluorescence signals of (A) O-methyl-*L* - threonine (IS), (B) glutamine and (C) peak-height ratios of glutamine/IS.

3.3.2 Fluorescent reagents

Since O-methyl-L-threonine was not a suitable internal standard, reagents that fluoresce but do not react with FQ were selected for further testing, including 5-TAMRA-SE, 5-TRITC, tetramethylrosamine chloride, the labeling product of Nα-acetyl-L-lysine with 5-TAMRA-SE, rhodamine B and rhodamine 6G. These reagents can be excited by the 488-nm line of the argon ion laser and have some emission at 630 nm that can be detected simultaneously with FQ-labeled amino acids.

3.3.2.1 5-TAMRA-SE and 5-TRITC

5-TAMRA-SE and 5-TRITC both have good reactivity with amines and are commonly used as amine-reactive probes [12]. They will cause interference with FQ-labeled amino acid peaks (data not shown) and are therefore not suitable internal standards.

3.3.2.2 Tetramethylrosamine chloride

Tetramethylrosamine chloride does not react with amines. However, it overlaps with a peak in the blank (Figure 3.5). Furthermore, it decomposes upon heating (Figure 3.5). Thus it was also rejected as an internal standard.

3.3.2.3 5-TAMRA-SE labeled Nα-acetyl-L-lysine

Succinimidyl esters are excellent reagents for amine modification, since the amide products formed are as stable as peptide bonds [12]. Succinimidyl esters will conjugate with aliphatic non-protonated amines. Consequently, the conjugation has to

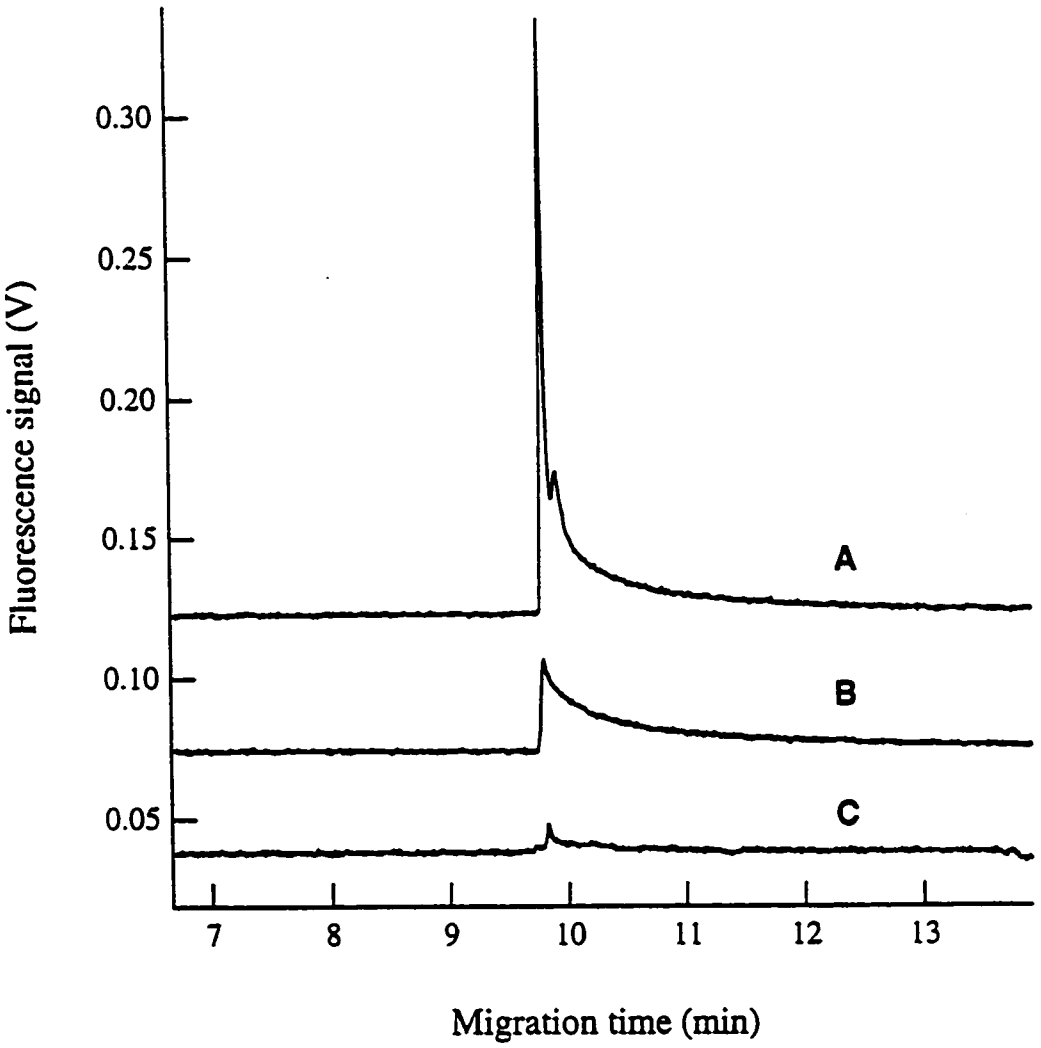


Figure 3.5 Effect of heating on fluorescence signal of 10^{-8} M tetramethylrosamine chloride. A. At room temperature, B. after 15 min heating at 65 °C, C. blank.

take place in a buffer with slightly basic pH. Molecular Probes recommended a 0.1 - 0.2 M $NaHCO_3$ buffer with a pH of approximately 8.3 [14]. We used a 0.185 M $NaHCO_3$ buffer with pH 8.3. During the conjugation, succinimidyl ester hydrolysis can compete as a side reaction, though usually slowly below pH 9 [12]; therefore the DMF solvent was carefully treated to remove any humidity.

Nα-acetyl-*L*-lysine was reacted with 5-TAMRA-SE. Figure 3.6 shows the labeling reaction. The labeled product was separated by a Sep-Pak cartridge and then analyzed by CE-LIF. An electropherogram of the product is shown in Figure 3.7. Since 5-TAMRA-SE was present in seven-fold excess, the product solution still contained plenty of 5-TAMRA-SE. The extra peak compared to the blank indicates production of the labeled compound. The compound does not react with FQ or amino acids and has good stability. However, in order to act as an internal standard, it has to be purified to remove any remaining 5-TAMRA-SE. TLC and ion exchange chromatography were performed on the product mixture. Unfortunately, separation was not satisfactory due to small structural differences between the product and 5-TAMRA-SE molecules.

3.3.2.4 Rhodamine B and rhodamine 6G

Rhodamine B and rhodamine 6G had similar properties. They did not co-elute with any FQ-labeled amino acids, but the fluorescence signals increased upon heating (Figure 3.8). Consequently, these two reagents were added to the diluted reaction mixture instead of incubating together with amino acids. Rhodamine 6G has a higher sensitivity to the instrument than rhodamine B, therefore it was selected as an internal standard to correct for variations in injection volume.

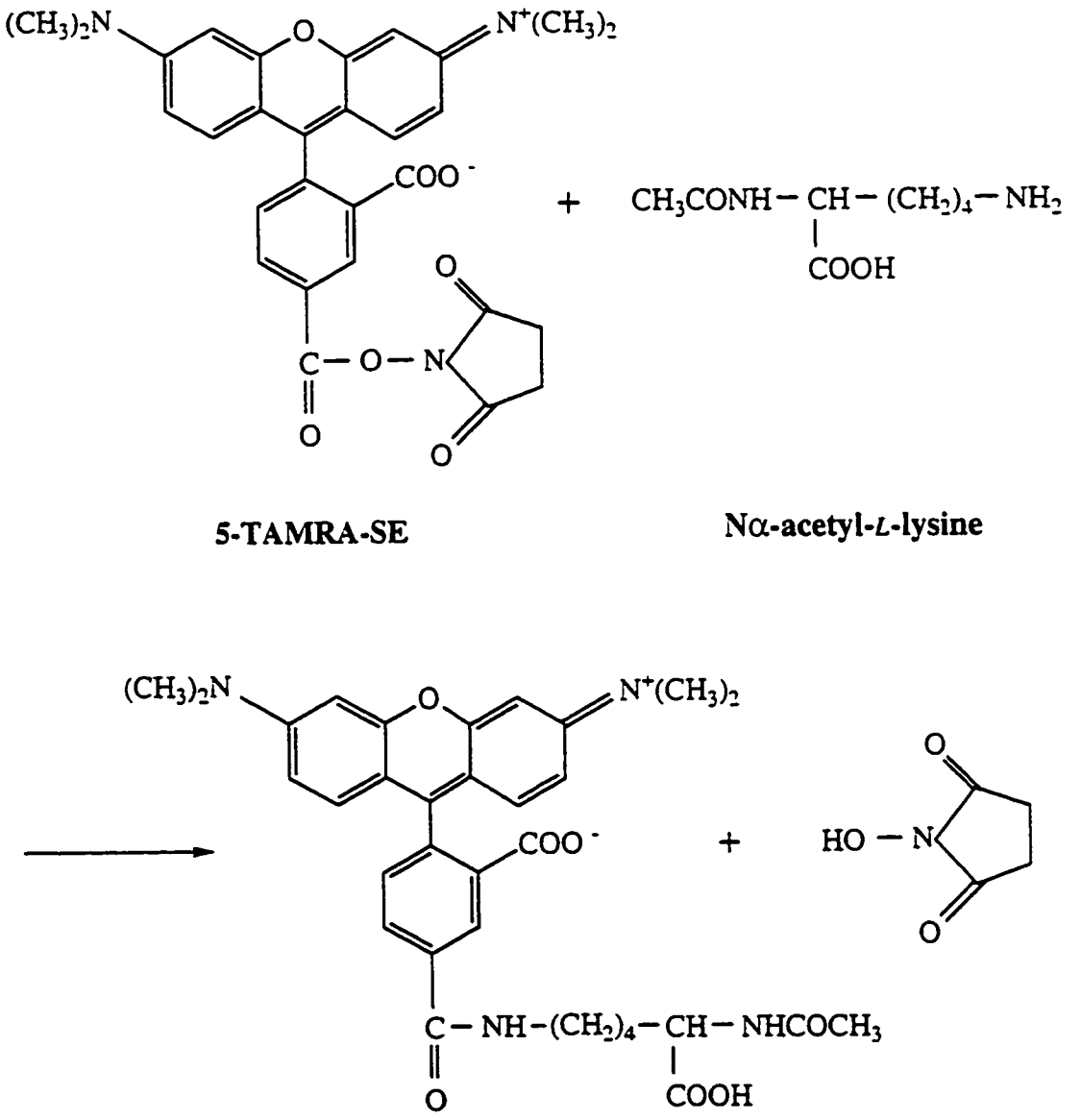


Figure 3.6 Labeling reaction of Nα-acetyl-*L*-lysine with 5-TAMRA-SE.

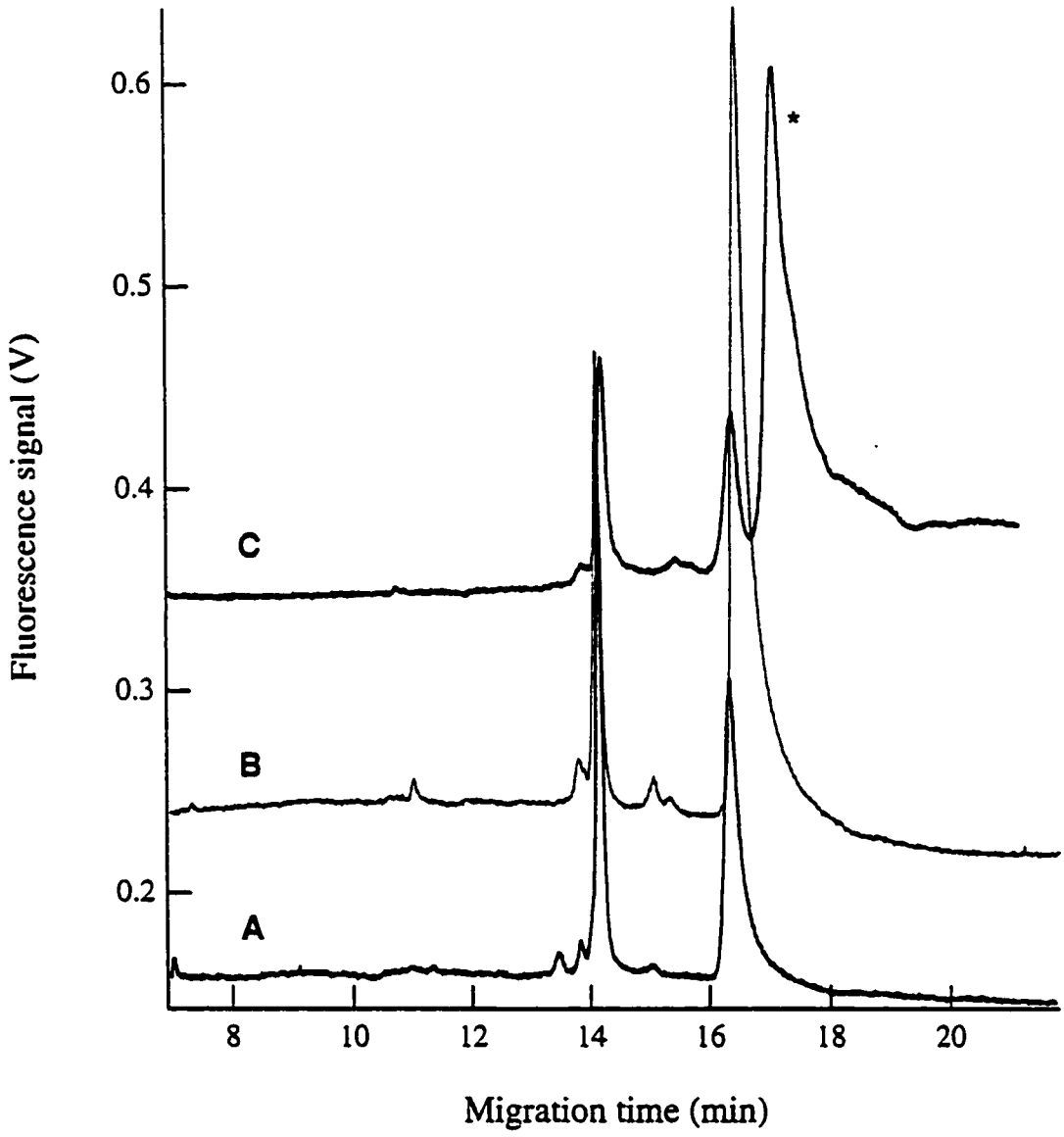


Figure 3.7 Electropherograms of 5-TAMRA-SE and 5-TAMRA-SE-labeled Nα-acetyl-*L*-lysine. Capillary: 48.6 cm x 15 μm i.d. x 145 μm o.d.; injection: 4.86 kV x 5 s; running voltage: 19.44 kV; filter: 580DF40. Peak * is the labeled product. A. 10^{-7} M 5-TAMRA-SE; B. Blank; C. Labeling product mixture.

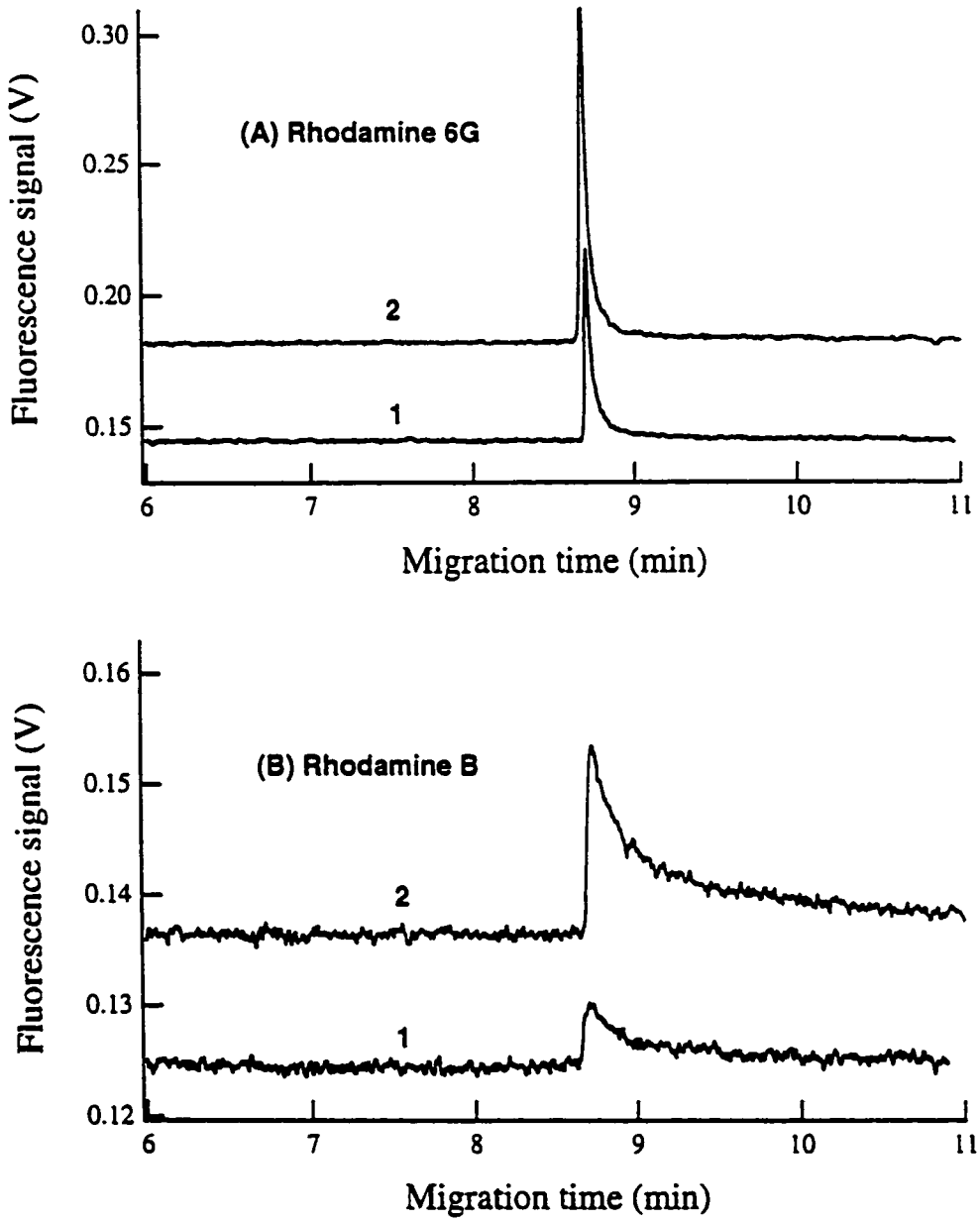


Figure 3.8 Effect of heating on (A) rhodamine 6G, (B) rhodamine B. Concentration: 10^{-7} M. 1. At room temperature, 2. after 15 min heating at 65 °C.

Figure 3.9 shows a typical electropherogram of an FQ-labeled amino acid mixture using rhodamine 6G as an internal standard. Intra- and interassay reproducibility is listed in Table 3.3. The precision drastically improved by introducing rhodamine 6G as an internal standard. Compared with O-methyl-*L*-threonine, rhodamine 6G produced much better precision, especially for interassay measurements. This arises from the fact that rhodamine 6G produces a stable signal independent on the reaction time but it is able to correct for variations in injection volume. Electrokinetic injection method was used because it was computer-controlled in our lab while hydrodynamic injection was manually controlled at the time.

3.3.3 Analysis of rat brain microdialysates using rhodamine 6G as an internal standard

Rhodamine 6G was selected as the internal standard for quantitation of amino acid neurotransmitters in rat brain microdialysates. The microdialysates were derivatized by FQ at 65 °C for 50 min and then detected by CE-LIF. A representative electropherogram is given in Figure 3.10. Over 15 peaks and shoulders were detected in a time window from 2 to 10 min. Of these peaks, five amino acids were identified and quantified: glutamine, glutamic acid, alanine, taurine and GABA. Given in Table 3.4 are the intra- and interassay precision data for the dialysates. The precision values were similar to those reported by Weber and coworkers [4], but our values for alanine (1.5% RSD intraassay) and GABA (2.2% RSD intraassay) were slightly better. Precision for glutamic acid (3.9% RSD intraassay and 5.9% RSD interassay) was not as good as those for the other amino acids, mainly due to its weak signal and relatively low concentration in the dialysates.

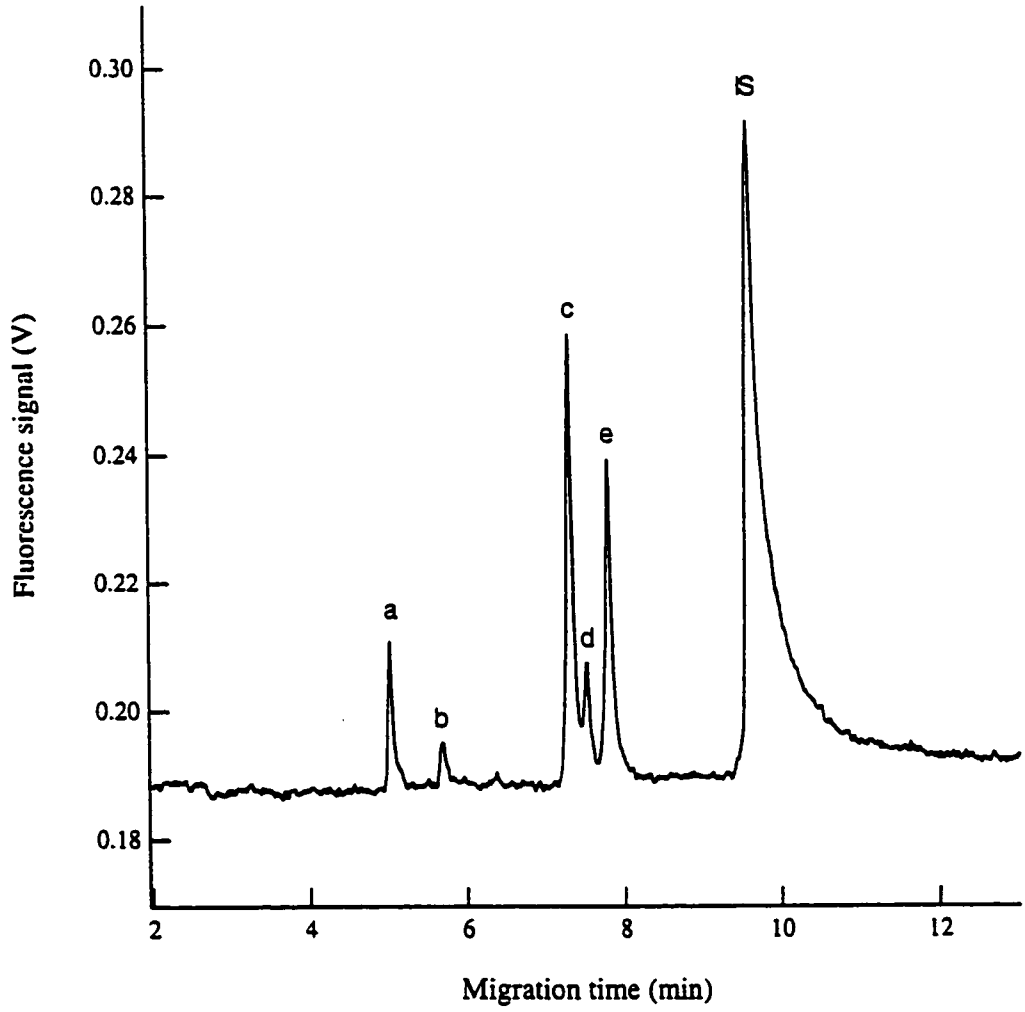


Figure 3.9 Electropherogram of FQ-labeled amino acids and amines using rhodamine 6G as an internal standard. Peaks: a. Gln, b. Glu, c. Ala, d. Taur, e. GABA, IS: rhodamine 6G. Capillary: 47.0 cm x 15 μm i.d. x 145 μm o.d.; injection: 4.7 kV x 3 s; running voltage: 18.8 kV. Separation buffer: 15 mM borate, 45 mM SDS and 5 mM β-CD (pH 8.5).

Amino Acid	RSD, %			
	Intraassay		Interassay	
	- IS	+ IS	- IS	+ IS
Gln	12.1	3.6	11.8	1.7
Glu	7.6	4.3	25.8	5.6
Ala	10.6	1.4	14.1	2.5
Taur	10.2	1.9	10.7	4.5
GABA	12.8	1.8	8.1	3.2

Table 3.3 Intra- (n = 3) and interassay reproducibility (n = 9) measured with and without use of rhodamine 6G as an internal standard. Injection method: electrokinetic injection. Peak height was used to do calculation.

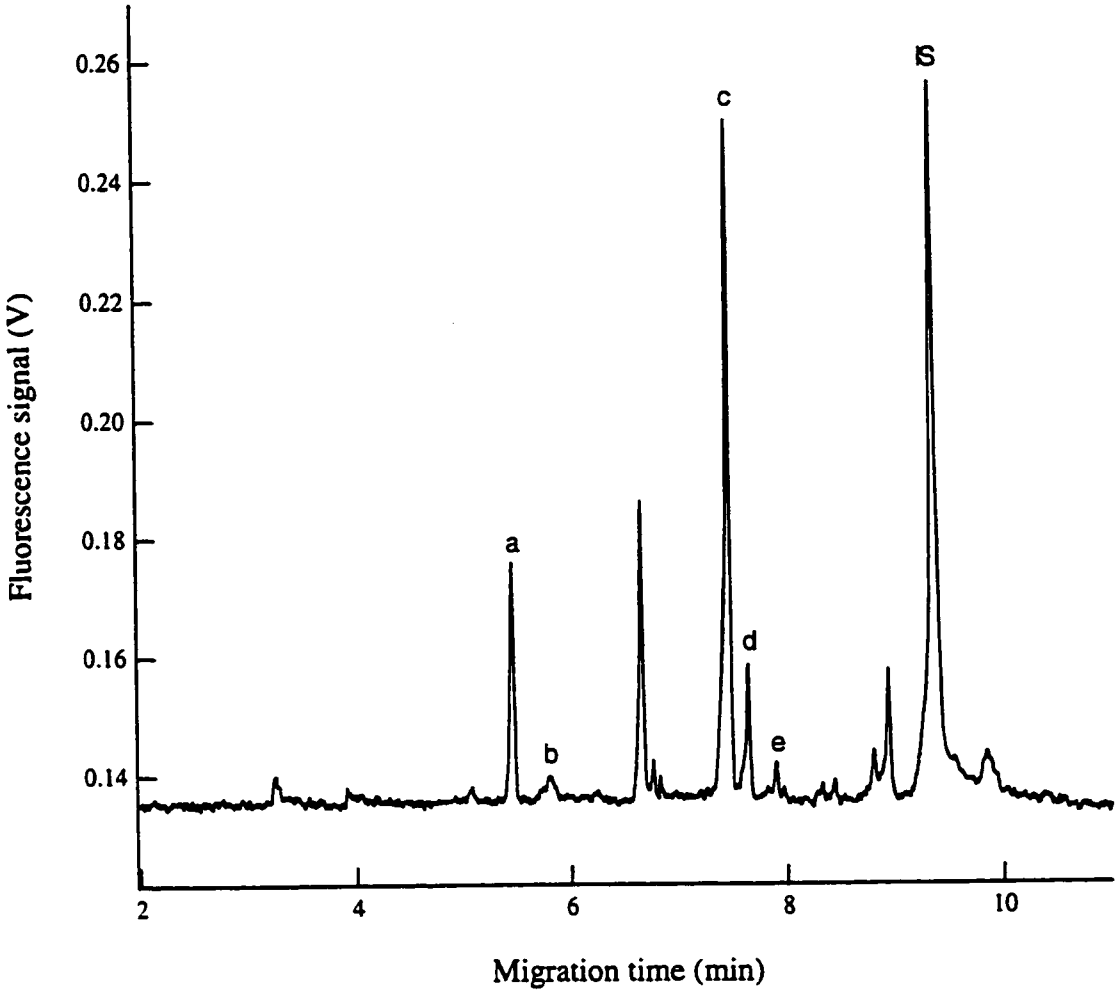


Figure 3.10 Electropherogram of an FQ-labeled brain microdialysate using rhodamine 6G as an internal standard. Peaks: a. Gln, b. Glu, c. Ala, d. Taur, e. GABA, IS: rhodamine 6G. Capillary: 47.0 cm x 15 μm i.d. x 145 μm o.d.; injection: 9.4 kV x 3 s; running voltage: 18.8 kV; separation buffer: 45 mM SDS, 15 mM borate and 5 mM β-CD (pH 8.5).

Amino Acid	RSD, %			
	Intraassay		Interassay	
	- IS	+ IS	- IS	+ IS
Gln	10.6	1.6	8.1	1.4
Glu	8.8	3.9	19.8	5.9
Ala	9.1	1.5	9.9	3.3
Taur	10.4	2.1	12.0	3.0
GABA	7.1	2.2	10.1	3.5

Table 3.4 Intra- (n = 3) and interassay reproducibility (n = 9) for brain microdialysates measured with and without use of rhodamine 6G as an internal standard. Injection method: electrokinetic injection.

3.4 CONCLUSIONS

Selection procedures for a suitable internal standard for quantitative analysis of amino acids were presented in this chapter. Rhodamine 6G was shown to be the best internal standard among the seven reagents tested. Introducing rhodamine 6G in the procedure dramatically improved the precision, demonstrating the importance of using a suitable internal standard for the correct measurement of amino acid neurotransmitters in brain microdialysates. However, since rhodamine 6G already fluoresces without derivatization, no control of the labeling procedure of the samples would be provided. Further study on the labeling reaction may help to circumvent this problem.

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CHAPTER 4

REACTION RATE, ACTIVATION ENERGY, AND DETECTION LIMIT FOR THE REACTION OF 3-(2-FUROYL)QUINOLINE-2-CARBOXALDEHYDE (FQ) WITH AMINO ACIDS IN ARTIFICIAL CEREBROSPINAL FLUID

4.1 INTRODUCTION

Capillary electrophoresis and laser-induced fluorescence (CE-LIF) have produced remarkable detection limits for highly fluorescent molecules. Single molecules of b-phycoerythrin have been detected by capillary electrophoresis with LIF detection and yoctomole detection limits have been reported for fluorescently labeled DNA sequencing fragments, amino acids, and sugars [1-5].

While outstanding detection limits have been produced for fluorescent molecules, non-fluorescent analytes must first be converted to a fluorescent derivative. Fluorogenic reagents have proven to be very valuable in the analysis of trace amounts of proteins and amines [6]. These reagents are non-fluorescent until they react with a primary amine, usually in the presence of a nucleophile. We are particularly interested in the reagent 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ). The fluorescent product generated by the reaction of FQ with a primary amine is excited by the 488-nm line of the argon ion laser. The emission has a large Stokes' shift, which makes it easy to spectrally filter the Raman scatter from the solvent. Proteins at extremely low concentration (10^{-13} M) have been labeled with the reagent and analyzed by CE-LIF [7-8].

The investigation of amino acids is of great importance because several of them are involved in neurotransmission and metabolism in the mammalian central neural system [9]. The CE-LIF technique has been increasingly explored in this research area. Hernandez and coworkers monitored glutamate in the brain by CE-LIF [10]. Aspartate and glutamate were continuously monitored using microdialysis sampling with on-line derivatization and CE-LIF detection by Zhou and coworkers [11]. Lada and Kennedy monitored glutamate and aspartate in vivo using microdialysis coupled on-line with CE-LIF [12]. For LIF detection of amino acids, chemical derivatization is generally employed because these compounds lack properties for direct determination. Separation and measurement are both based on derivatized products. To what degree the derivatization reaction proceeds will affect sensitivity as well as accuracy of the measurement. Therefore, understanding of the derivatizing reaction is very important, especially for application of quantitative analyses.

In this chapter, we consider the reaction rate and activation energy for the reaction between FQ and several neurotransmitters in artificial cerebrospinal fluid (CSF). This analyte system mimics both cerebrospinal fluid and brain dialysates. By studying the reaction in some detail, we were able to optimize the reaction conditions for rapid and high-sensitivity analysis of these compounds. Our goal was to minimize the sample manipulations, so the cerebrospinal fluid sample was analyzed without modification.

4.2 EXPERIMENTAL

4.2.1 Reagents

HPLC grade methanol, β-cyclodextrin hydrate (β-CD) and rhodamine 6G were obtained from Aldrich (Milwaukee, WI). The derivatizing reagents, 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ) and potassium cyanide (KCN), were provided by Molecular Probes (Eugene, OR). D-Glucose, sodium carbonate, potassium hydroxide, potassium chloride, sodium dodecyl sulfate (SDS) and disodium tetraborate ($Na_2B_4O_7$) were supplied by BDH (Toronto, ON, Canada). All amino acids were purchased from Sigma (St. Louis, MO) and used as received. Magnesium chloride hexahydrate and concentrated hydrochloric acid were from Anachemia (Montreal, Quebec, Canada). Sodium chloride was supplied by ACP (Montreal, Quebec, Canada). Sodium phosphate dibasic (Na_2HPO_4) and calcium chloride were purchased from Fisher Scientific (Fair Lawn, NJ).

A stock solution of 10 mM FQ was prepared in methanol. Aliquots of the FQ stock solution (100 nmol) were dried in a SC110 Speed Vac (Savant Instruments, Farmingdale, NY) and stored at –20 °C. 25 mM KCN was prepared in 10 mM borate buffer (pH 9.2). A stock solution of 10^{-3} M rhodamine 6G was prepared in methanol and then diluted to 10^{-6} M in deionized distilled water. All buffers were made with Milli-Q deionized water and were filtered using a 0.2-μm filter.

4.2.2 Artificial cerebrospinal fluid

An alkaline stock solution was prepared by dissolving 23.6 g NaCl, 9.2 g Na_2CO_3, 1.2 g KCl, and 0.32 g Na_2HPO_4 in 100 mL of deionized water. An acidic stock solution was prepared by dissolving 2.4 g $CaCl_2$ and 1.2 g $MgCl_2 \cdot 6H_2O$ in 30 mL of concentrated HCl in a 45 °C water bath. A glucose stock solution was prepared fresh daily by dissolving 0.8 g of glucose in 50 mL of deionized water. To make the working solution, 2.5 mL of the alkaline stock solution was diluted to 100 mL using deionized water. Next, 5 mL of the glucose stock solution was added. The pH was adjusted to 7.14 using the acidic stock solution.

4.2.3 Labeling reaction

A standard mixture of amino acids was prepared in the artificial cerebrospinal fluid, with 3×10^{-4} M glutamine, 3×10^{-4} M glutamic acid, 1.2×10^{-4} M alanine, 6×10^{-5} M taurine, and 3×10^{-5} M GABA. A 2-μL aliquot of this standard neurotransmitter mixture was mixed with 4 μL of 25 mM KCN prepared in 10 mM borate buffer (pH 9.2) and with 100 nmol dry FQ reagent. The reaction mixtures were held in a thermostated chamber (Fisher Scientific, Fair Lawn, NJ) at 65 °C or 40 °C. Samples were diluted 100-fold with the artificial cerebrospinal fluid to quench the reaction. Rhodamine 6G was used as the internal standard. It was added to the diluted reaction mixture to make a 1×10^{-8} M solution.

4.2.4 Capillary electrophoresis conditions

A locally constructed capillary electrophoresis instrument with laser-induced fluorescence detection was used for this experiment [3]. Electrophoresis was performed with a 47-cm long, 15-μm inner diameter, and 145-μm outer diameter fused-silica capillary (Polymicro Technologies, Phoenix, AZ). Samples were injected at 5.64 kV for 3 s. Separation was performed at 18,800 V. The separation buffer was 15 mM borate, 45 mM SDS, and 5 mM β-cyclodextrin (pH 8.5). 10 mM borate was used as the sheath flow buffer. A 10-mW argon-ion laser beam at 488 nm (Model 2113-150MLYW, Uniphase, San Jose, CA) was used for excitation. Emission was detected at 630 nm.

4.3 RESULTS AND DISCUSSION

The amino acid mixture was derivatized by FQ, followed by CE-LIF detection. A typical electropherogram of FQ-labeled neurotransmitters is given in Figure 4.1.

4.3.1 Reaction rate

Figure 4.2 presents a typical kinetic curve obtained for GABA in artificial cerebrospinal fluid. The smooth curve is the least-squares fit of a first-order kinetic curve to the data

$$\text{signal} = \alpha(1-e^{-k\,t}) \qquad (4.1)$$

where α is a scale factor that is related to the sensitivity of the instrument, k is the reaction rate in s^{-1}, and t is reaction time in s. The precision of the data was improved by adding rhodamine 6G as an internal standard to the reaction products; this standard corrects for variations in injection volume and improves the precision dramatically (see Table 3.3).

The reaction rate constant was also determined for GABA that was prepared in a mixture with alanine, taurine, glutamine, and glutamic acid; the rate constant for the reaction of GABA with FQ was identical to the value obtained from GABA alone, 0.00350 s^{-1} at 65 °C. This demonstrates that the labeling reaction for each amino acid does not interfere with each other while FQ is present in 60-fold excess. The reaction rate constant was determined for the other four amino acids from the electrophoresis data generated on the mixture.

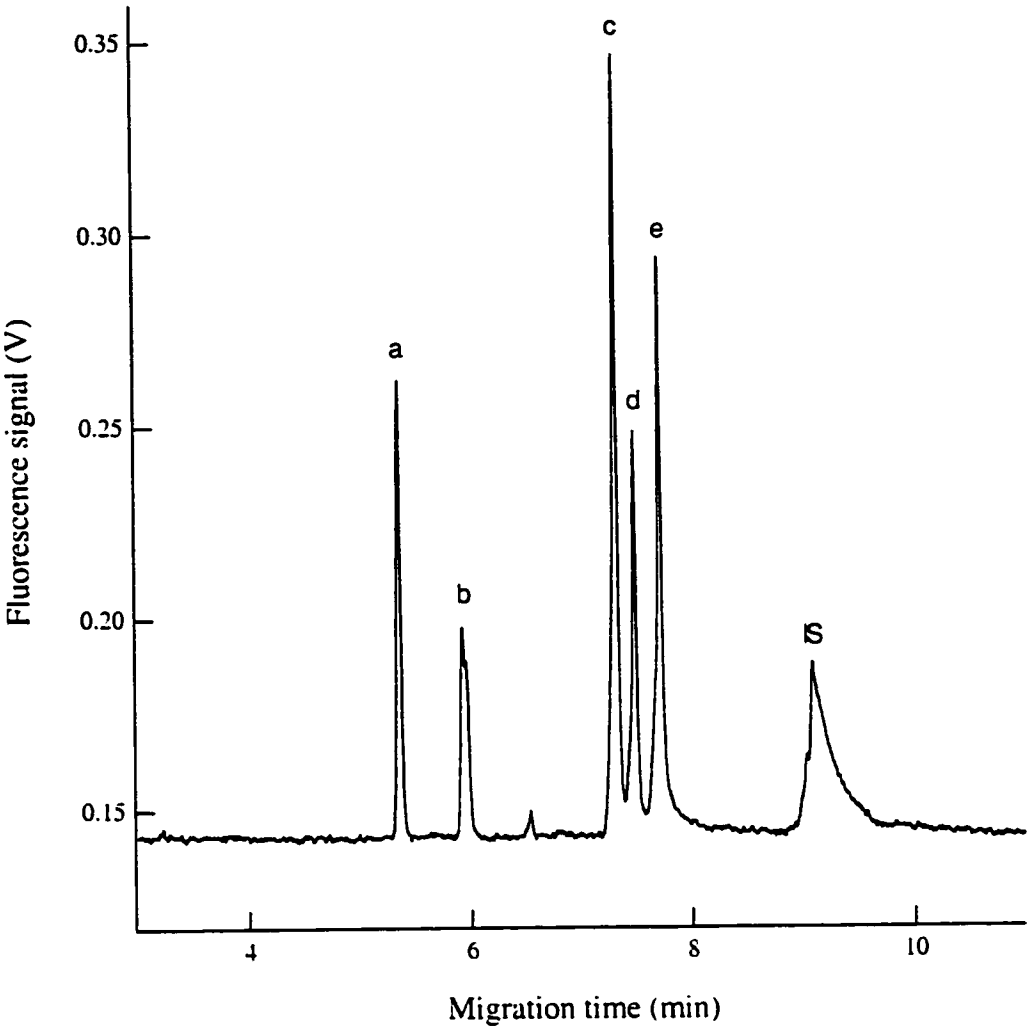


Figure 4.1 Electropherogram of FQ-labeled amino acids using rhodamine 6G as an internal standard. Peaks: a. Gln, b. Glu, c. Ala, d. Taur, e. GABA, IS: rhodamine 6G. Concentrations are given in Experimental section. Capillary: 47.0 cm x 15 μm i.d. x 145 μm o.d.; injection: 5.64 kV x 3 s; running voltage: 18.8 kV. Separation buffer: 15 mM borate, 45 mM SDS and 5 mM β-CD (pH 8.5).

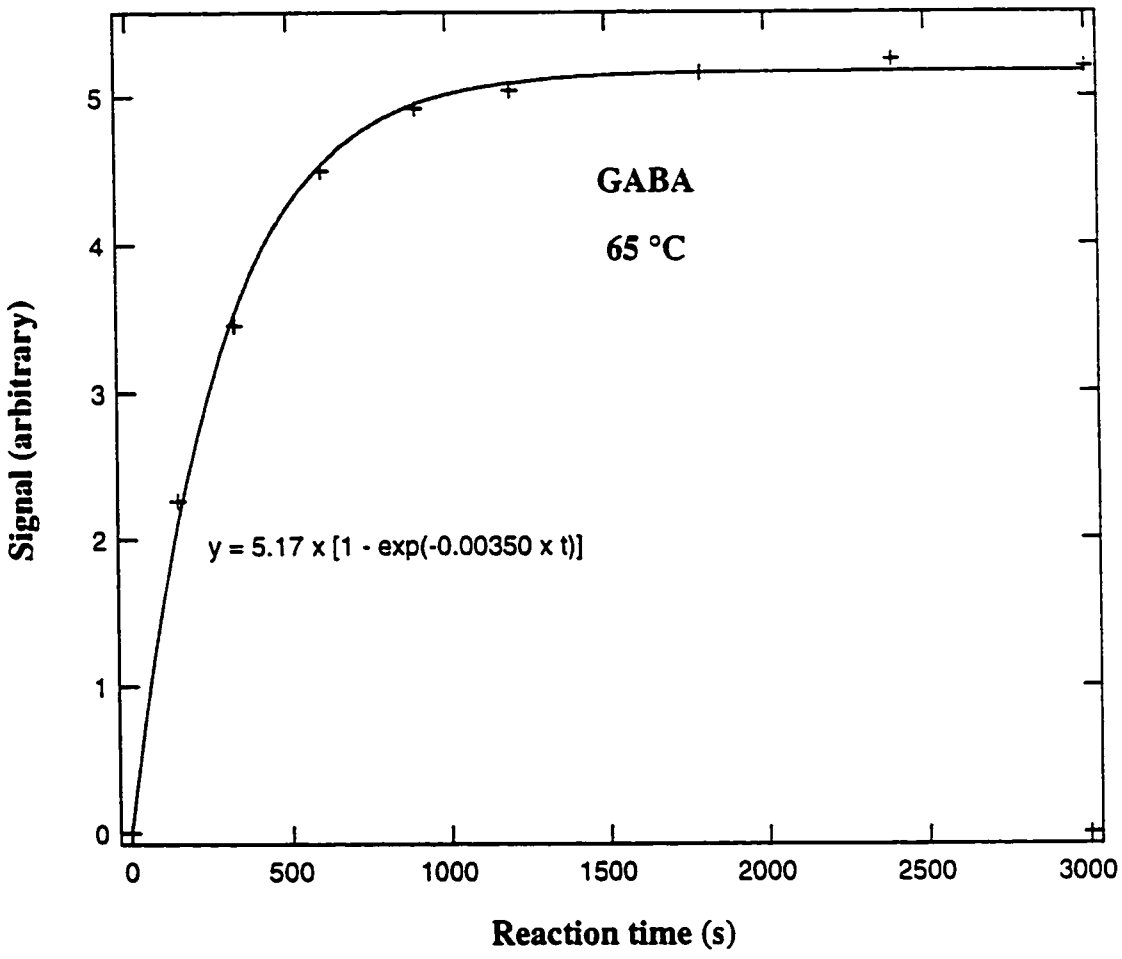


Figure 4.2 Kinetic curve for the reaction of GABA with FQ in artificial cerebrospinal fluid at 65 °C. The crosses represent the signals measured by capillary electrophoresis. The smooth curve is the least-squares fit of equation 4.1 to the data.

The reaction rate was determined at 40 °C and 65 °C, Table 4.1. Figure 4.3 shows kinetic curves obtained for alanine, taurine, glutamine and glutamic acid at 65 °C. The reaction requires 25 minutes (1500 s) for GABA (Figure 4.2) and about 40 minutes (2400 s) for the other four neurotransmitters to go to completion at 65 °C. Kinetic curves at 40 °C are presented in Figure 4.4. Complete reaction requires much longer time at this lower temperature: around 60 minutes (3600 s) for alanine and taurine, 40 minutes (2400 s) for GABA,and 100 minutes (6000 s) for glutamine and glutamic acid. Interestingly, the fluorescence signal of FQ-labeled GABA decreased after a 90-minute reaction at 40 °C, which is presumably due to the decomposition of the labeled product. This phenomenon was not found for other amino acids or for GABA itself after a 50-minute reaction at 65 °C.

4.3.2 Activation energy

The Arrhenius equation was used to estimate the activation energy for the reaction

$$k = A \exp(-E_a/RT) \quad (4.2)$$

where A is the pre-exponential term, E_a is the activation energy in kJ mol^{-1}, R is the gas constant, and T is the absolute temperature in K.

Using reaction rate constants obtained at 40 °C and 65 °C, we can derive another equation:

$$\ln(k_2/k_1) = E_a/R \times (1/T_1 - 1/T_2) \quad (4.3)$$

The activation energy is then estimated by the following equation:

$$E_a = R \times \ln(k_2/k_1)/(1/T_1 - 1/T_2) \quad (4.4)$$

Table 4.1

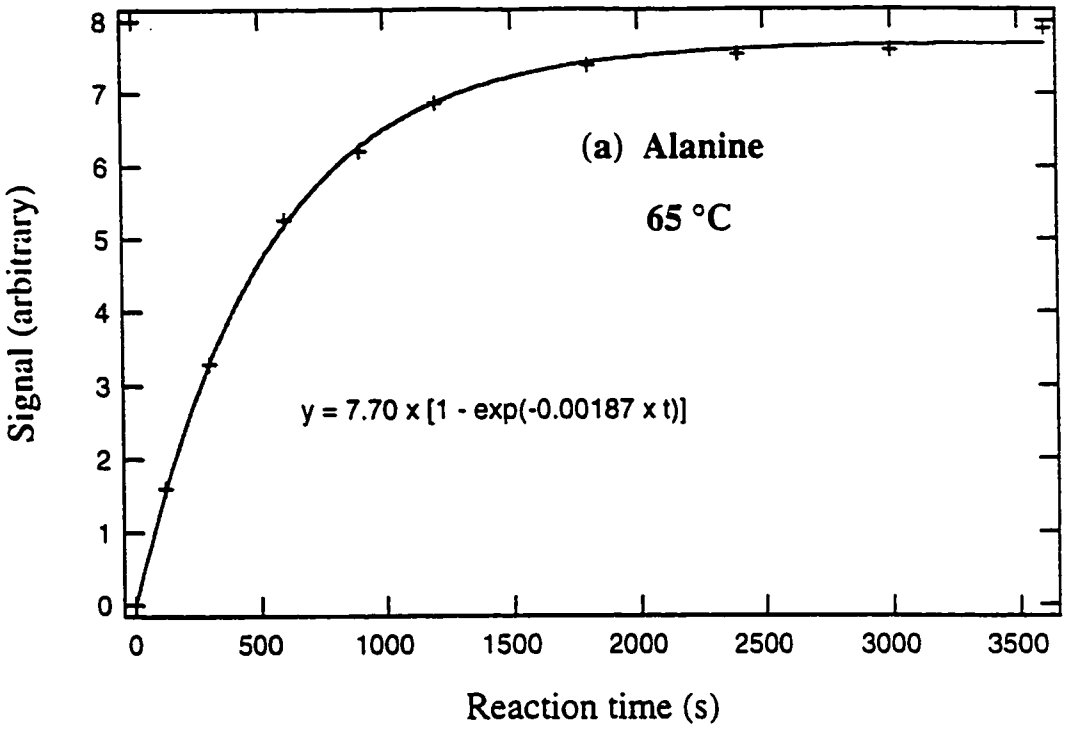
Kinetic data and detection limit for FQ-labeled amino acids

Neurotransmitters	E_a (kJ mol^{-1})	rate (s^{-1}) at 40 °C	rate (s^{-1}) at 65 °C	LOD_c (M)	LOD_m (amol)
Alanine	10.6	0.00138	0.00187	2.4×10^{-9}	0.6
Taurine	10.7	0.00129	0.00175	2.3×10^{-9}	0.5
GABA	22.0	0.00179	0.00350	1.2×10^{-9}	0.3
Glutamine	31.4	0.00081	0.00198	14×10^{-9}	5.3
Glutamic acid	34.4	0.00071	0.00190	19×10^{-9}	6.7

LOD_c – concentration limit of detection (3 σ) determined for the fluorescent products generated by a 3000 s reaction at 65 °C.

LOD_m – mass limit of detection (3 σ) for the fluorescent products generated by a 3000 s reaction at 65 °C. This value is the product of the concentration detection limit and the injection volume.

Figure 4.3 Kinetic curves for the reaction of (a) alanine, (b) taurine, (c) glutamine and (d) glutamic acid with FQ in artificial cerebrospinal fluid at 65 °C. The meanings of the symbols are the same as in Figure 4.2.


(a) Alanine
65 °C
y = 7.70 x [1 - exp(-0.00187 x t)]
Signal (arbitrary)
Reaction time (s)
0
1
2
3
4
5
6
7
8
0
500
1000
1500
2000
2500
3000
3500

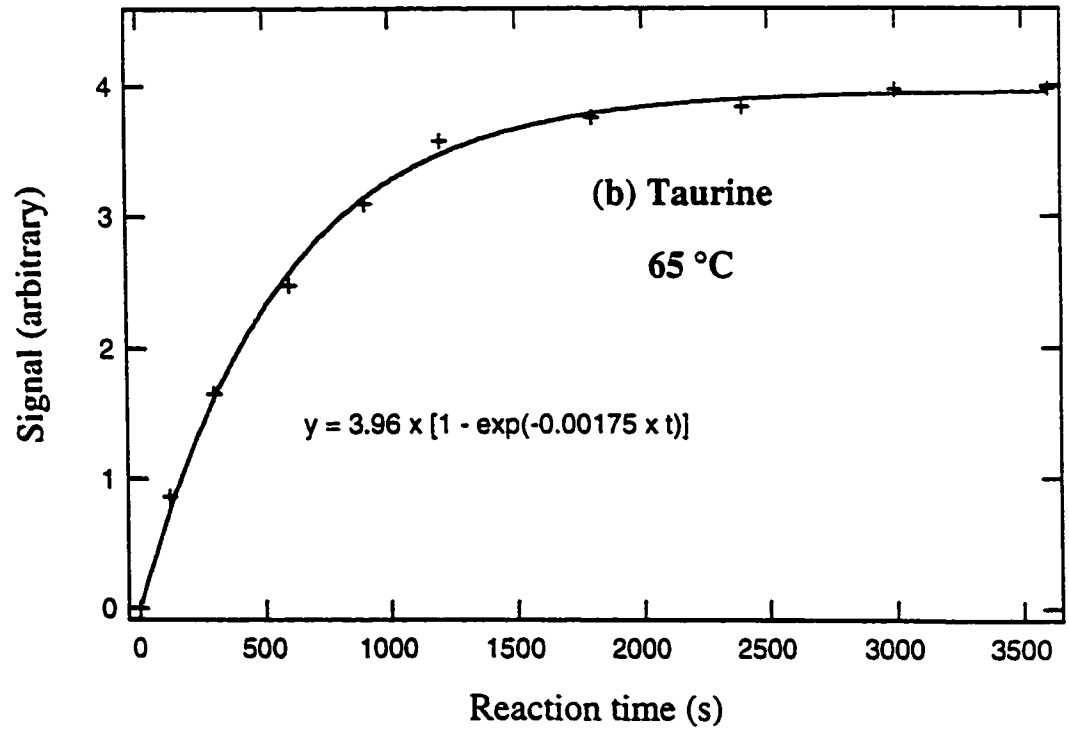
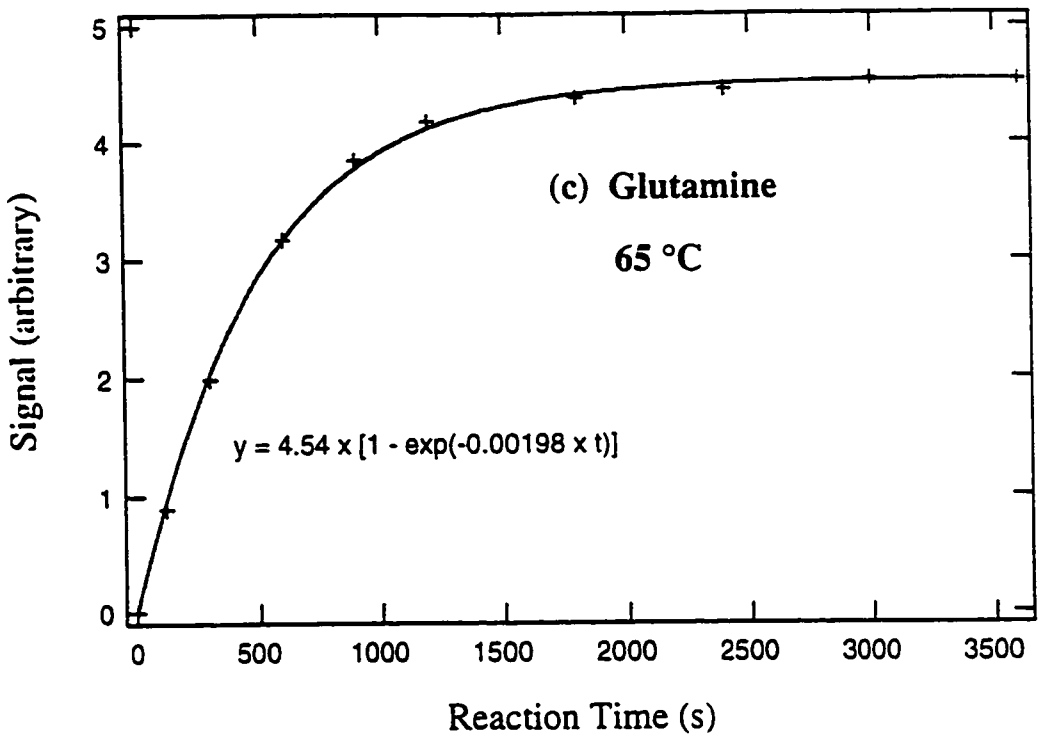

(b) Taurine
65 °C
y = 3.96 x [1 - exp(-0.00175 x t)]
Signal (arbitrary)
Reaction time (s)
0
1
2
3
4
0
500
1000
1500
2000
2500
3000
3500

Figure 4.3



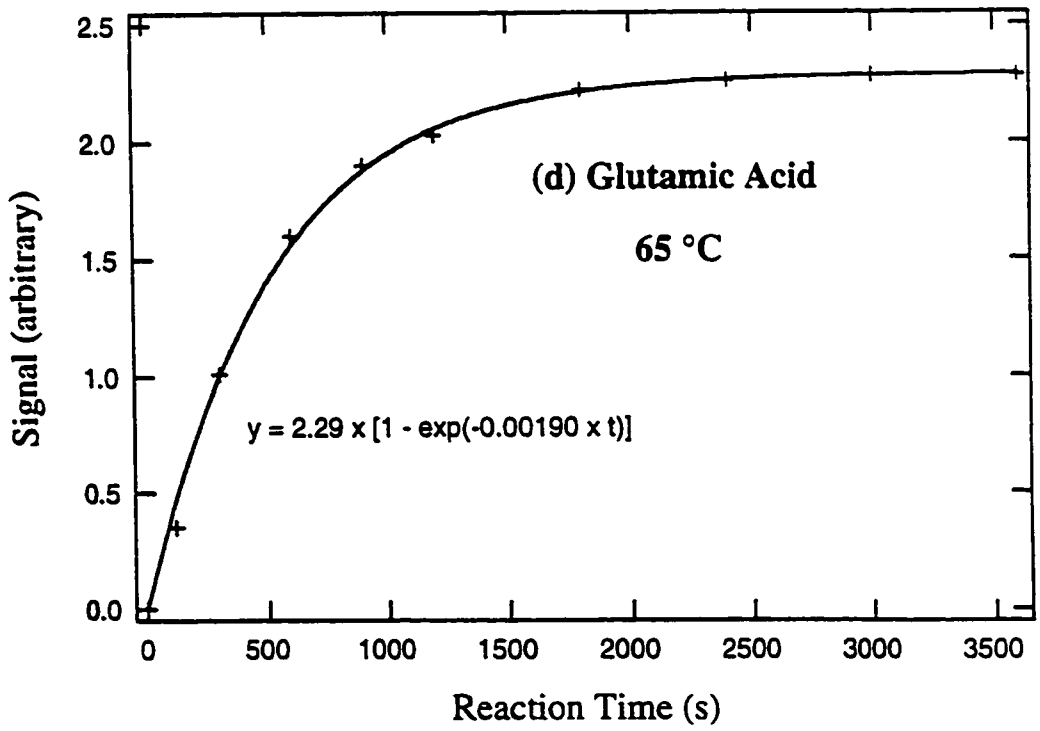


Figure 4.3 (cont'd)

Figure 4.4 Kinetic curves for the reaction of (a) alanine, (b) taurine, (c) GABA, (d) glutamine and (e) glutamic acid with FQ in artificial cerebrospinal fluid at 40 °C. The meanings of the symbols are the same as in Figure 4.2.

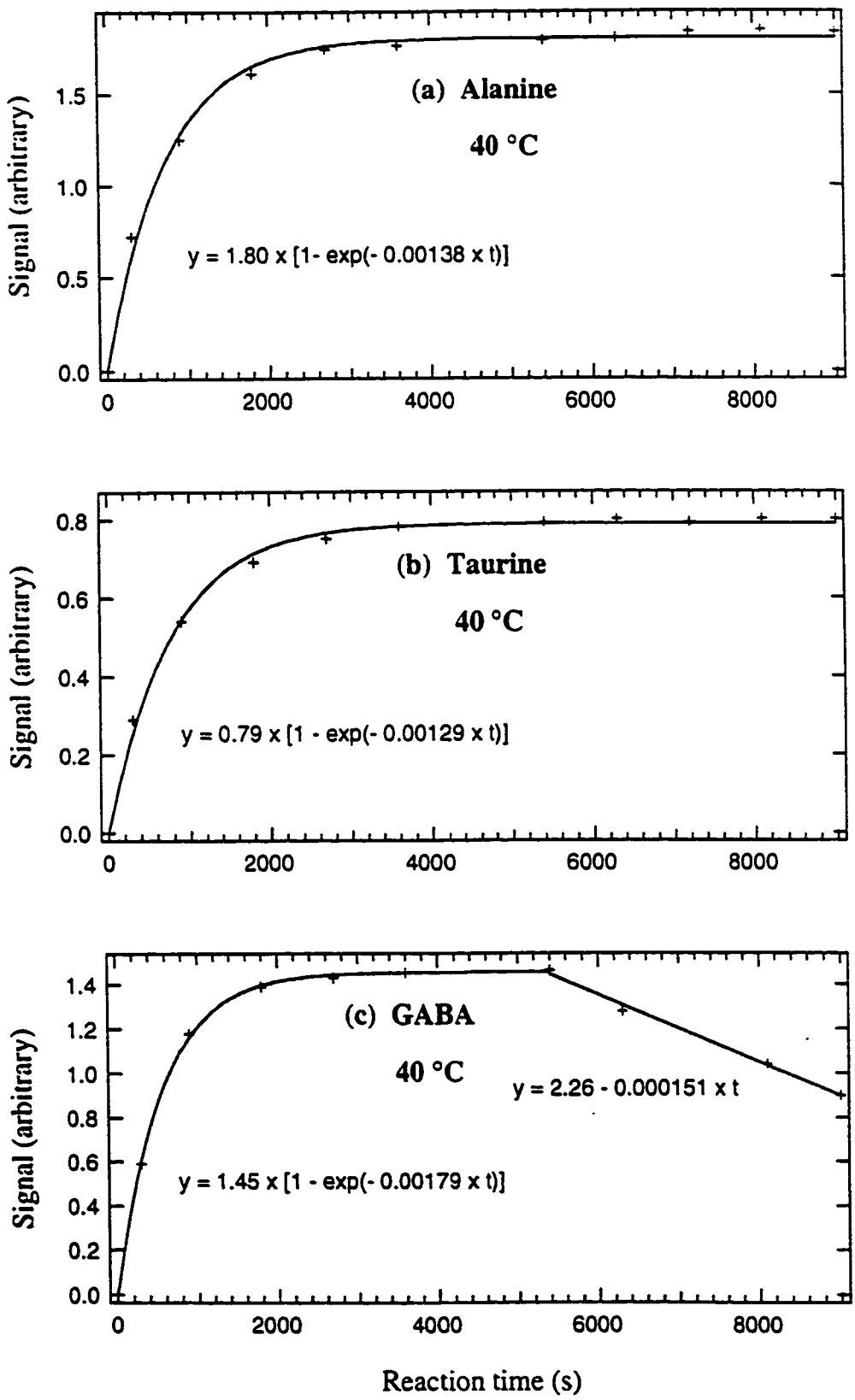
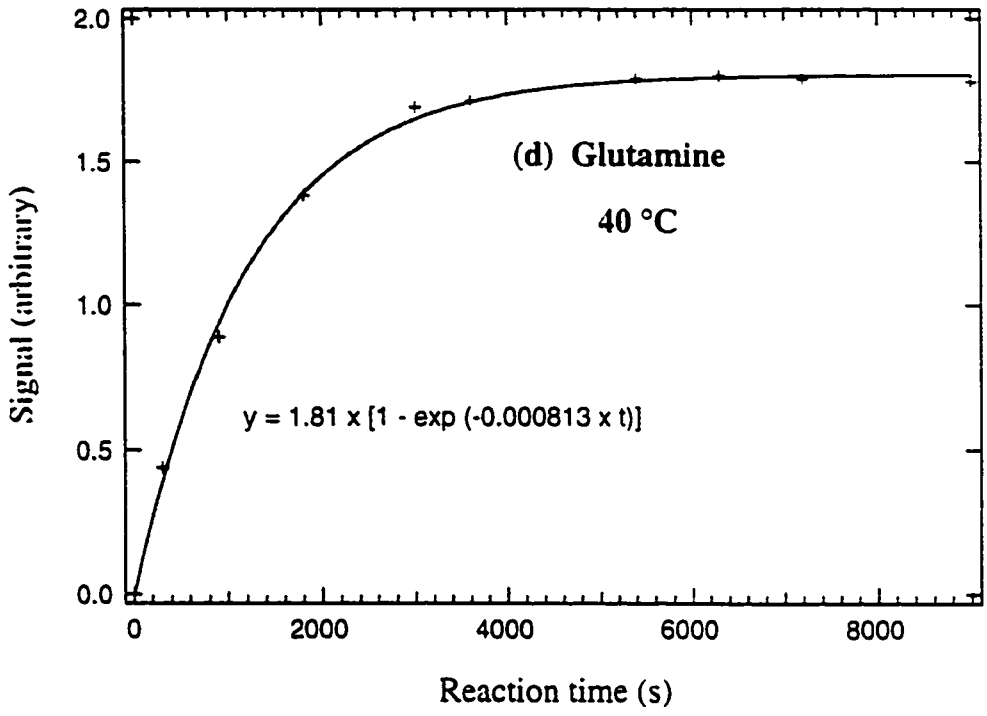

(a) Alanine
40 °C
y = 1.80 x [1- exp(- 0.00138 x t)]
Signal (arbitrary)
(b) Taurine
40 °C
y = 0.79 x [1 - exp(- 0.00129 x t)]
Signal (arbitrary)
(c) GABA
40 °C
y = 2.26 - 0.000151 x t
y = 1.45 x [1 - exp(- 0.00179 x t)]
Signal (arbitrary)
Reaction time (s)

Figure 4.4



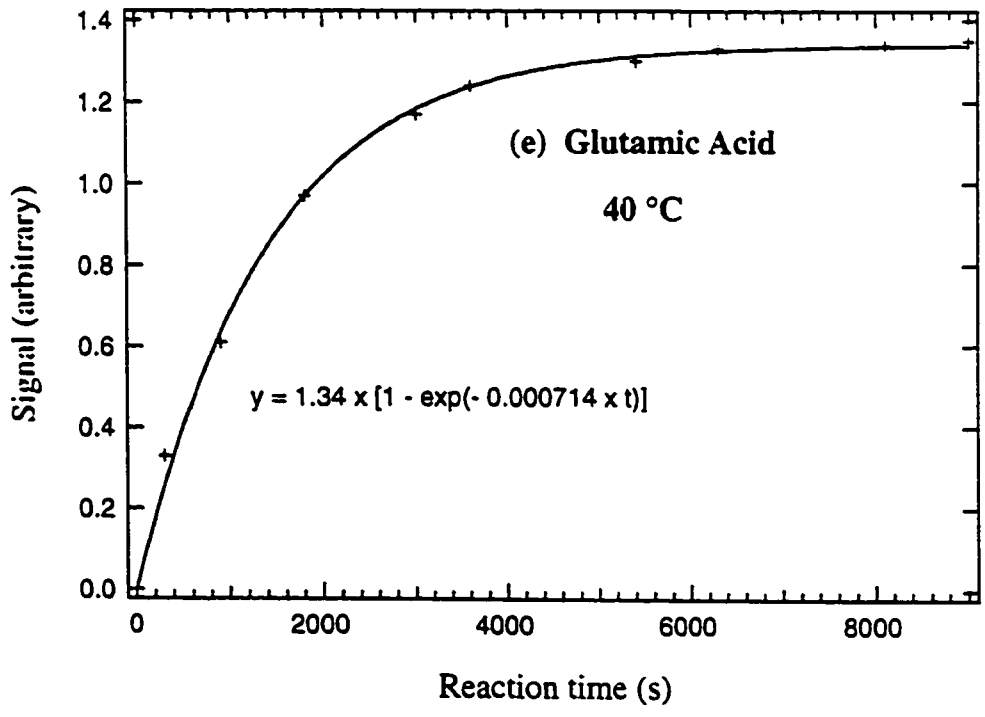


Figure 4.4 (cont'd)

The activation energy is presented in Table 4.1 for the five amino acids, and values ranged from 11 to 34 kJ mol^{-1}.

Although the activation energy is relatively low for these reactions, the rate constant is not particularly high. The rate constant at 65 °C is ~0.002 s^{-1} for most of the amino acids, although the reaction rate is roughly twice as fast for GABA. This sluggish reaction requires about one hour to quantitatively convert the amino acids to their fluorescent products, even at 65 °C. This reaction rate is much slower than the labeling reaction of proteins by this reagent [8], similar to the result obtained from FQ labeling of Nα-acetyl-L-lysine (unpublished data in our lab).

4.3.3 Limits of Detection (LOD)

Concentration detection limits (3σ) were also determined for the fluorescently labeled amines (Table 4.1). A 10^{-4} M solution of amines was labeled and diluted to construct the calibration curve. Similar data were obtained by labeling a 10^{-6} M solution of amines. Detection limits ranged from 1.2 x 10^{-9} M for GABA to 1.9 x 10^{-8} M for glutamic acid. Concentration detection limits scale inversely with the capillary cross-section. The detection limit suffered from the use of a 15-μm ID separation capillary; the use of a 50-μm ID capillary would have improved the concentration detection limit by about an order of magnitude. The mass detection limit ranged from 300 zmol for GABA to 7 amol for glutamic acid injected onto the capillary.

The best detection limit was obtained for GABA, which also had the fastest reaction rate of the five amino acids. Interestingly, the detection limit ranged by an order of magnitude for the other amino acids, even though they had a very similar

reaction rate. This variation in detection limit does not reflect the kinetics of the reaction but instead may arise from differences in spectral properties of the product molecules, such as molar absorptivity, emission spectrum, and fluorescence quantum yield.

4.4 CONCLUSIONS

In this chapter, we monitored the reaction rate between the fluorogenic reagent FQ and five amino acids at 40 °C and at 65 °C in artificial cerebrospinal fluid. The reactions followed pseudo-first order kinetics. The activation energy for the reaction of FQ was 10.6, 10.7, 22.0, 31.4, and 34.4 kJ mol^{-1} for alanine, taurine, GABA, glutamine, and glutamic acid, respectively. At 65 °C, the reaction rate was quite similar for alanine, taurine, glutamine, and glutamic acid (1.8×10^{-3} s^{-1}) but was twice as fast for GABA. Nearly one hour reaction time was required to quantitatively convert these amino acids to their fluorescent products at 65 °C. Detection limits for the labeled amino acids were 10^{-9} to 10^{-8} M, which corresponded to 300 zmol to 7 amol injected onto the capillary.

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CHAPTER 5

IN VIVO MONITORING OF AMINO ACIDS IN RAT BRAIN BY MICRODIALYSIS AND CAPILLARY ELECTROPHORESIS WITH LASER-INDUCED FLUORESCENCE (CE-LIF) DETECTION

5.1 INTRODUCTION

Microdialysis sampling of extracellular fluids has emerged as an important technique for biomedical, pharmaceutical and neurochemical applications [1-3]. Microdialysis is accomplished by implanting a small, semipermeable probe into the tissue of interest. The probe is slowly perfused with a sampling solution. Small molecules in the extracellular space diffuse into the probe and are swept away to be collected for analysis [3]. Microdialysis probes can be implanted in many tissues with minimal discomfort to the experimental animal. The introduction of microdialysis sampling has provided a technique which can continuously monitor chemical reactions *in vivo* [3]. Advantages of microdialysis relative to other sampling approaches include less tissue damage and production of samples free of high molecular weight components, such as proteins, so as to allow direct injection into the chromatographic systems [4].

High-performance liquid chromatography (HPLC) with electrochemical or fluorescence detection is the technique most commonly employed for the analysis of

microdialysis samples [5]. However, while microdialysis is a continuous sampling method, HPLC requires discrete samples. The dialysate must, therefore, be collected over a fixed time interval to provide the required sample volume, and each sample represents an average concentration value obtained over this time interval. Thus, the temporal resolution becomes dependent upon the sample requirements of the chromatographic system [5]. To minimize the sample volume required, thereby increasing the temporal resolution, capillary electrophoresis (CE) has been explored as an alternative to HPLC for analysis of dialysates [3-10].

Laser-induced fluorescence (LIF) has been shown to be an extremely sensitive method for use with CE [11-13]. The combination of a high-resolution separation technique with a high-sensitivity detector results in a powerful analytical tool. The small sample volume requirement of CE, typically several nanoliters, is an added advantage when coupled to microdialysis sampling, in which collected volumes are usually a few microliters [6]. Such volume-limited samples and the ability to easily reduce the volume to achieve greater temporal resolution using CE should prove to be an ideal combination of both techniques [6].

Phenelzine (2-phenylethylhydrazine, $PhCH_2CH_2NHNH_2$, PLZ) is the most commonly used of the monoamine oxidase (MAO)-inhibiting antidepressants [14]. It is also used extensively in the treatment of panic disorders such as bulimia, social phobia and post-traumatic stress disorder (PTSD) [15]. In addition to being a potent MAO inhibitor, PLZ also causes marked increases in brain levels of the amino acids

γ-aminobutyric acid (GABA) [14, 16] and alanine [17]. Studies have also been carried out on the effects of PLZ on glutamine [18-19], an important substance whose formation is known to be involved in GABA metabolism [20].

In this chapter, the combination of microdialysis sampling and CE-LIF is demonstrated for continuous monitoring of amino acids in the brain. The microdialysis samples were pre-column labeled by the fluorogenic reagent 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ) and then separated with CE-LIF based on a β-cyclodextrin assisted micellar electrokinetic chromatography (MEKC) buffer system. The accuracy, precision and linearity of the technique are reported. The effects of the antidepressant/antipanic drug PLZ on GABA, glutamine (Gln), glutamic acid (Glu), alanine (Ala) and taurine (Taur) in rat brain are illustrated. The sulfate salt of PLZ was utilized in these studies. To the best of our knowledge, this is the first report about the levels of these amino acid neurotransmitters after administration of the drug PLZ using the combined microdialysis/CE technique. The results are similar to those previously found using HPLC with fluorescence detection [21]. Minimal work-up procedures are involved and only small quantities of samples are required for analysis.

5.2 EXPERIMENTAL

5.2.1 Chemicals

All amino acids were purchased from Sigma (St. Louis, MO) and used as received. Disodium tetraborate ($Na_2B_4O_7$) and sodium dodecyl sulfate (SDS) were supplied by BDH (Toronto, ON, Canada). Derivatization reagents, 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ) and potassium cyanide (KCN) were obtained from Molecular Probes (Eugene, OR). β-Cyclodextrin hydrate (β-CD) and rhodamine 6G were provided by Aldrich (Milwaukee, WI).

5.2.2 Stock solutions

A stock solution of 10 mM FQ was prepared in methanol. 10-μL aliquots were then placed into 500-μL microcentrifuge tubes and the solvent was removed under vacuum using a SC110 Speed Vac (Savant Instruments, Farmingdale, NY). The dried FQ aliquots were stored at -20 °C. A stock solution of 25 mM KCN was made in 10 mM borate buffer (pH 9.2). The separation buffer was 15 mM borate, 45 mM SDS and 5 mM β-CD (pH 8.5). All buffers were prepared in deionized water and filtered using a 0.2-μm filter. Rhodamine 6G was dissolved in methanol at a concentration of 10^{-3} M and then diluted to 10^{-6} M in deionized water. Stock solutions (1 mg/mL) of GABA, glutamine, glutamic acid, alanine and taurine were prepared in deionized water.

Artificial cerebrospinal fluid (CSF) was prepared using double-distilled water and contained the following (in mM): NaCl (145), $CaCl_2$ (1.5), $MgCl_2$ (1.0) and dextrose (2.0). The pH value of the solution was adjusted to 7.3 with a mixture of Na_2HPO_4 and NaH_2PO_4 (final concentration approximately 4.0 mM). The solution was filtered and degassed before use (Section 2.2.3).

5.2.3 Microdialysis experiment

The microdialysis experiment was described in detail elsewhere [21]. Briefly, it included the following steps:

5.2.3.1 Surgical procedure

Male Sprague-Dawley rats (250g-350g) were individually housed with water and food *ad libitum* and maintained on a 12 h light-dark cycle.

The rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally [i.p.]) and placed in a stereotaxic instrument. A permanent intracerebral guide cannula (Bioanalytical Systems [BAS], West Lafayette, IN) was implanted into the region immediately dorsal to the septum (0.5 mm anterior to bregma; 4.4 mm ventral to dura). The cannula was secured to the skull by jeweller's screws and cranioplastic cement. A wire loop was additionally embedded in the cement to be later used to attach

the rat to a tether during the microdialysis procedures. A dummy cannula was inserted into the guide cannula to keep it free of debris. The skin was sutured rostral and caudal to the cemented area and the incision was sealed with an adhesive. Following surgery, the rats were kept in a warm, temperature-controlled environment until recovery from anesthesia.

5.2.3.2 Microdialysis procedure

After at least 4 days of recovery, the rats were ready for microdialysis. The rats was placed in a round Plexiglas bowl (BAS) that contains a mixture of clean bedding and bedding from the rat's cage. After 5 min, the rat was attached to the tether (BAS). Following a 1 h habituation period, a microdialysis probe (2 mm membrane, BAS) was inserted into the guide cannula. The probe was perfused with artificial CSF at the rate of 1 μL/min. After a 2 h stabilization period, three 25-minute baseline samples were collected. Then, PLZ (30 mg/kg) was administered intraperitoneally and eleven 25-minute samples were collected following the injection. The samples were kept on dry ice during the experiment and then transferred to a - 80 °C freezer for long-term storage.

5.2.4 Derivatization procedure

4-μL dialysate sample and 4 μL 25 mM KCN (pH 9.2) were mixed with 100 nmol of dry FQ reagent. The reaction was allowed to proceed in a water bath incubator

(Fisher Scientific, Fair Lawn, NJ) at 65 °C for 50 min in the dark. A blank solution of 4 μL artificial CSF or 4 μL standard mixtures were derivatized with the same protocol. Then 192 μL artificial CSF was added to the mixture to quench the reaction. 2 μL of 10^{-6} M rhodamine 6G was added to the diluted mixture to make a 1 x 10^{-8} M solution.

5.2.5 Capillary electrophoresis system

A sheath flow assisted capillary electrophoresis instrument with LIF detection was used for this study [22]. The samples and standards were injected onto a 47.0 cm long, 15 μm i.d. and 145 μm o.d. fused-silica capillary (Polymicro Technologies, Phoenix, AZ). Post-column LIF detection was accomplished by using the 488 nm line of a 10 mW Ar^{+} laser (Model 2113-150MLYW, Uniphase, San Jose, CA). The fluorescence was filtered by a bandpass filter (Omega Optical,Brattleboro, VT) centered at 630 nm and detected by a photomultiplier tube (R1477, Hamamatsu, Middlesex, NJ) operated at 1000 V. Data collection was achieved using a LabVIEW hardware/software package (National Instruments, Austin, TX) and all the data were analyzed by IGOR Pro (WaveMetrics, Lake Oswego, OR). Sampling rate was 5 Hz.

5.2.6 Fluorescence measurements

The samples, blank and standards were injected at 200 V/cm or 120 V/cm for 3 s. Separations were performed under positive polarity at 18.8 kV (400 V/cm). The running buffer was 15 mM sodium borate, 45 mM SDS and 5 mM β-CD (pH 8.5) and the sheath flow buffer was 10 mM sodium borate.

Three experiments were conducted. In the first experiment, a series of standard mixtures of five neurotransmitters with 5 different concentrations were derivatized by FQ and then injected to determine the linearity of the derivatization procedure. The concentrations before derivatization ranged from 0.0156 - 1.0 ng/5μL for GABA, 0.125 - 8.0 ng/5μL for glutamine, alanine and taurine, and 0.188 - 4.0 ng/5μL for glutamic acid. The final concentrations were diluted 50-fold.

In the second experiment, four concentrations of standard mixtures (n = 5 for each concentration) provided by Dr. Glen Baker (Neurochemical Research Unit, Department of Psychiatry, University of Alberta) were quantified using the CE-LIF method, and the results were compared to the original concentrations to evaluate the accuracy and precision of the method.

In the third experiment, the actual concentrations of GABA, glutamine, glutamic acid, alanine and taurine in brain dialysates before and after PLZ injections were measured.

5.3 RESULTS AND DISCUSSION

5.3.1 Optimization of separation, derivatization and detection parameters

As discussed in Chapter 2, BS buffer composed of 20 mM borate and 60 mM SDS was initially used as the separation buffer for brain dialysates. However, separation was not satisfactory. Glutamine and glutamic acid could not be resolved. 5mM β-CD was then added to the buffer and much better results were obtained (Figure 2.5). Therefore, 15 mM borate, 45 mM SDS and 5 mM β-CD (pH8.5) were chosen for efficient separation of amino acid standards as well as the microdialysis samples.

In order to accurately quantify concentration levels of amino acids in brain dialysates, a suitable internal standard is crucial to correct for variations in derivatization and injection processes. As described in Chapter 3, rhodamine 6G was shown to be a good internal standard. It corrected for variations in injection volume and improved precision dramatically (Table 3.3 & Table 3.4).

Nevertheless, correction for the labeling process is not so crucial for a complete labeling reaction. For this purpose, reaction rate and activation energy were investigated for the five amino acids (Chapter 4). Reaction at 65 °C was much faster than at 40 °C (Figure 4.3 & Figure 4.4), however, nearly one hour reaction time was still required to quantitatively convert these neurotransmitters to their fluorescent products at 65 °C.

Therefore, 50 min was employed to achieve complete labeling reaction at 65 °C for subsequent experiments.

5.3.2 Validation of the CE-LIF method for quantitative analysis of amino acids

For validation of the CE-LIF method, linearity, sensitivity, accuracy and precision were evaluated.

5.3.2.1 Linearity

Linearity was demonstrated over a concentration range from 0.0156 to 1.0 ng/5μL of GABA, from 0.125 to 8.0 ng/5μL of glutamine, alanine and taurine, and from 0.188 to 4.0 ng/5μL of glutamic acid. The calibration ranges were selected according to the predicted concentrations in the dialysate samples to be determined. Peak-height ratios of amino acid compared to internal standard were plotted against the concentrations. Calibration graphs were obtained with six points and each concentration was measured by three replicate injections of standard mixtures. The linear equations and correlation coefficients (r^2) for the five neurotransmitters are indicated in Table 5.1.

Table 5.1 Linearity and detection limits of five amino acids

Amino acid	LR	r^2	LOD_c (ng/5μL)
GABA	y = 0.548 x + 0.013	0.996	0.6×10^{-3}
Glutamine	y = 0.038 x + 0.009	0.994	7.0×10^{-3}
Glutamic acid	y = 0.015 x + 0.007	0.988	9.5×10^{-3}
Alanine	y = 0.217 x + 0.031	0.992	1.2×10^{-3}
Taurine	y = 0.218 x + 0.020	0.996	1.2×10^{-3}

Note: LR is linear regression; y is peak-height ratio of amino acid / IS; x is concentration (ng/5 μL); r^2 is correlation coefficient; LOD_c is concentration limit of detection.

5.3.2.2 Sensitivity

The CE method showed a reasonable sensitivity. As reported in Chapter 4 (Table 4.1), detection limits for the five amino acids at a signal-to-noise ratio of 3 were 0.6×10^{-3} ng/5μL (1.2×10^{-9} M) for GABA, 7.0×10^{-3} ng/5μL (1.4×10^{-8} M) for glutamine, 9.5×10^{-3} ng/5μL (1.9×10^{-8} M) for glutamic acid, 1.2×10^{-3} ng/5μL (2.4×10^{-9} M) for alanine and 1.2×10^{-3} ng/5μL (2.3×10^{-9} M) for taurine (Table 5.1). This ensures the quantitation of these amino acids in brain dialysates.

5.3.2.3 Accuracy and precision

Table 5.2 gives the accuracy and precision of the CE method expressed as relative standard deviation (RSD). Twenty standard mixtures at four concentrations (n = 5 for each concentration) were derivatized by FQ at 65 °C for 50 min, followed by CE-LIF detection. The accuracy was determined by comparing quantitative results from CE-LIF with the real concentrations of amino acid standards. The experimental values are expressed as mean ± standard deviation (SD), and the accuracy is experimental mean/real value x 100%. The accuracy ranged from 90% for glutamic acid to 106% for alanine.

The interassay precision was determined by measuring five derivatized samples at the same concentration with three replicate injections for each sample. The precision based on concentrations ranged from 1.5% for glutamine to 5.6% for glutamic acid and

Amino Acid	c (ng/5μL)	Real c	Experimental c	Accuracy, %	RSD, %
	c_1	1.0	1.0 ± 0.05	100	5.0
GABA	c_2	0.25	0.25 ± 0.01	100	4.0
	c_3	0.0625	0.0623 ± 0.0029	99	4.7
	c_4	0.0156	0.0155 ± 0.0008	99	5.2
	c_1	8.0	8.1 ± 0.2	101	2.5
Glutamine	c_2	2.0	2.0 ± 0.03	100	1.5
	c_3	0.50	0.49 ± 0.01	98	2.0
	c_4	0.125	0.125 ± 0.004	100	3.2
	c_1	4.0	4.0 ± 0.1	100	2.5
Glutamic acid	c_2	1.0	0.9 ± 0.05	90	5.6
	c_3	0.25	0.24 ± 0.01	96	4.2
	c_4	0.188	0.186 ± 0.005	99	2.7
	c_1	8.0	8.0 ± 0.3	100	3.8
Alanine	c_2	2.0	2.0 ± 0.05	100	2.5
	c_3	0.50	0.53 ± 0.02	106	3.8
	c_4	0.125	0.127 ± 0.007	102	5.5
	c_1	8.0	8.0 ± 0.2	100	2.5
Taurine	c_2	2.0	2.1 ± 0.09	105	4.3
	c_3	0.50	0.49 ± 0.01	98	2.0
	c_4	0.125	0.124 ± 0.007	99	5.6

Table 5.2 Accuracy and precision of four concentrations (c) of amino acid standards with rhodamine 6G as an internal standard. Experimental c : mean ± SD (n = 5 for each concentration), accuracy: mean/real value x 100%.

taurine, which was in good agreement with the precision data based on peak-height ratios (Chapter 3, Table 3.3).

The linearity, sensitivity, accuracy and precision data all suggest that this CE-LIF technique might be very useful for the accurate and precise determination of the five amino acid neurotransmitters in brain dialysates.

5.3.3 *In vivo* studies of the brain dialysates

This system was evaluated for monitoring amino acid neurotransmitters in brain microdialysates. The electropherograms of a baseline dialysate sample prior to PLZ injection and a dialysate sample at 50 min after PLZ injection are shown in Figure 5.1. Five peaks of amino acids, including GABA, glutamine, glutamic acid, alanine and taurine, were identified by spiking the sample with standards. In addition, several other unidentified peaks were seen. These peaks corresponded to other substances present in the dialysate and taken up from the brain.

The analysis took around 10 min, which was much faster than HPLC (30 min) [21]. The measurement of the five amino acids in brain dialysates required very small volumes. In the present experiments, we need a sample volume of 4 μL for the measurements. The final volume of the derivatized sample was 200 μL, although 350 pL was actually injected for analysis. This ensures that the analysis of each sample can

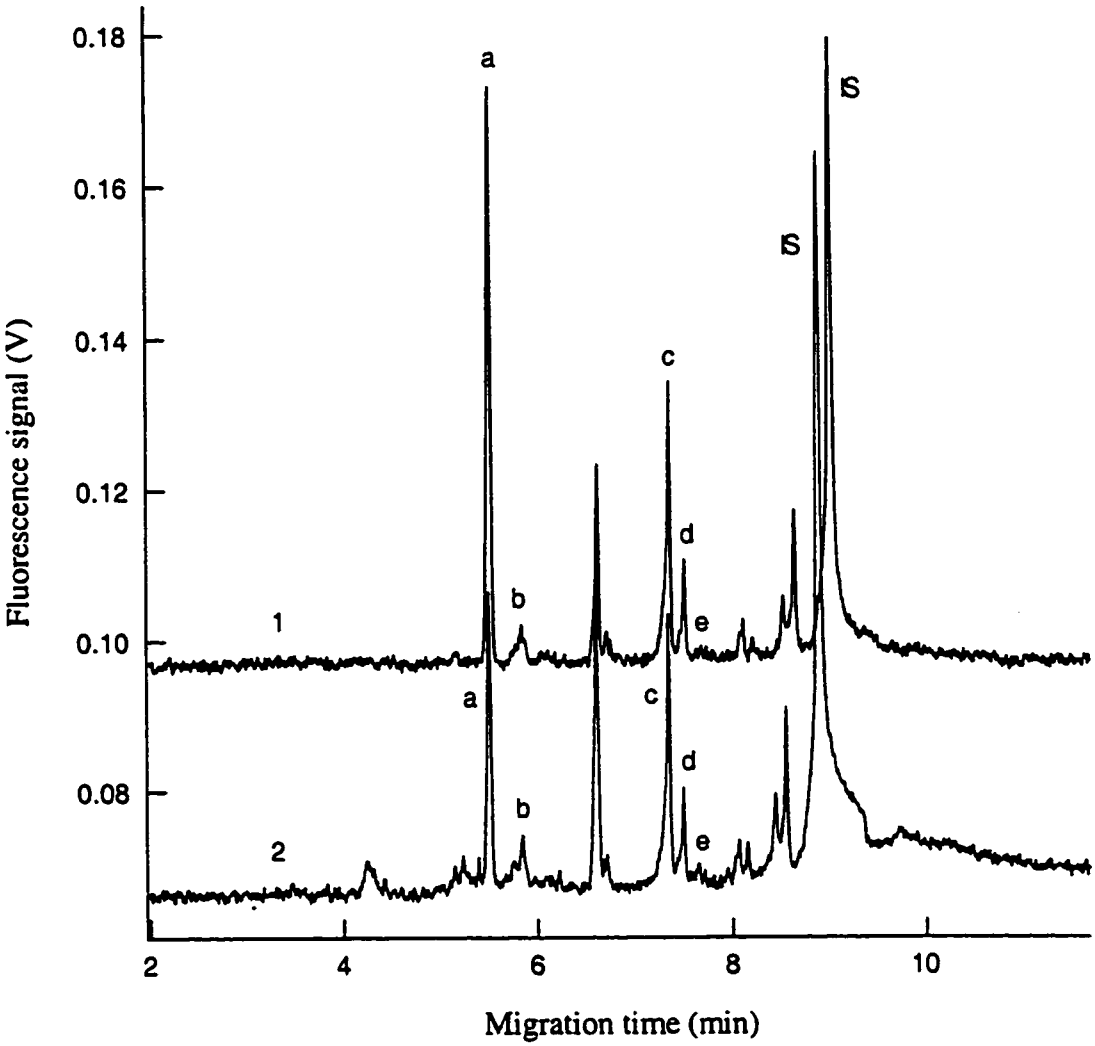


Figure 5.1 Electropherograms of FQ-labeled brain dialysates. (1) A baseline sample before phenelzine (PLZ) injection, (2) a dialysate sample at 50 min after i.p. injection of 30 mg/kg PLZ. Peaks: a. Gln, b. Glu, c. Ala, d. Taur, e. GABA, IS: rhodamine 6G. Capillary: 47 cm x 15 μm i.d. x 145 μm o.d.; injection: 9.4 kV x 3 s; running voltage: 18.8 kV; separation buffer: 45 mM SDS, 15 mM borate and 5 mM β-CD (pH 8.5).

be repeated many times as long as the sample does not decompose or there are no other side effects. In a similar study using HPLC with fluorescence detection, 5μL sample was derivatized and injected for each analysis [21].

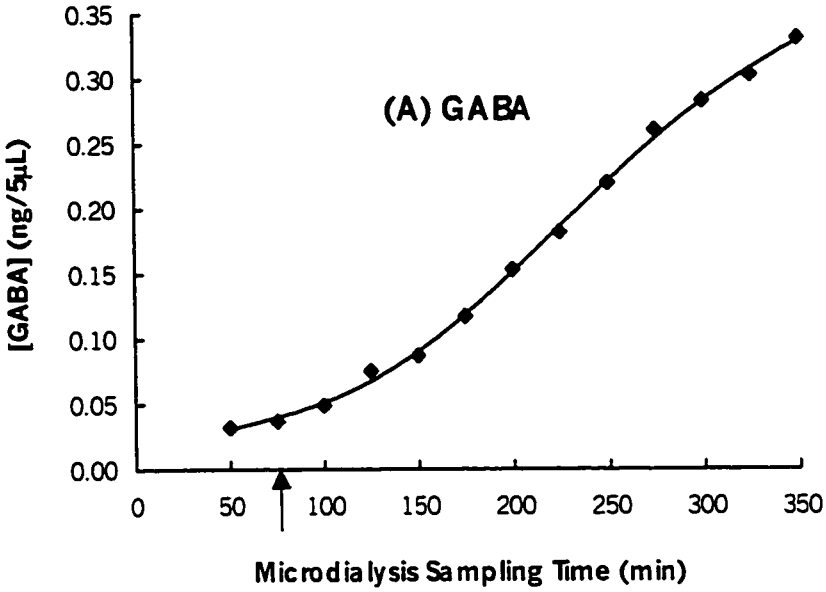
The concentration levels of the five amino acids were continuously monitored prior to and after PLZ (30 mg/kg, i.p.) administration over a period of 350 min, and the results are presented in Table 5.3. Figure 5.2 shows the concentration-time curves obtained for these five amino acids. After 75 min of collecting dialysate samples of the basal amino acid concentration levels, PLZ administration was applied. A significant increase in extracellular levels of GABA and alanine, plus a considerable decrease in levels of glutamine, and minimal changes in glutamic acid and taurine in brain dialysates were observed.

5.3.3.1 Analysis of GABA

The administration of PLZ (30 mg/kg, i.p.) resulted in a marked increase in levels of GABA in microdialysates from rat septum, about a 10-fold increase from 0.03 ng/5μL to 0.33 ng/5μL (Table 5.3). The smooth curve is to guide the eye (Figure 5.2 A). These values are in good agreement with a previous report using HPLC with fluorometric detection after formation of *o*-phthaldialdehyde (OPA) derivatives of the amino acids [21]. Since PLZ is a hydrazine compound, it is likely that inhibition of GABA-transaminase (GABA-T), the catabolic enzyme for GABA contributes to the GABA elevations observed [14]. However, it has been reported that inhibition of

Sampling time (min)	GABA (ng/5μL)	Glutamine (ng/5μL)	Glutamic acid (ng/5μL)	Alanine (ng/5μL)	Taurine (ng/5μL)
50	0.032	27.67	0.24	1.81	0.67
→ 75	0.037	29.01	0.23	2.61	0.92
100	0.049	25.89	0.29	2.23	0.97
125	0.075	19.85	0.29	2.97	0.99
150	0.087	18.61	0.32	3.44	0.98
175	0.117	13.73	0.30	3.88	0.58
200	0.153	10.59	0.34	4.30	0.63
225	0.182	10.24	0.35	4.28	0.76
250	0.220	10.17	0.27	4.78	0.90
275	0.261	10.25	0.27	5.07	0.90
300	0.283	10.05	0.38	5.66	1.25
325	0.303	9.54	0.34	5.77	1.35
350	0.331	9.42	0.37	7.39	1.54

Table 5.3 Concentrations of five amino acids prior to and after PLZ administration (30 mg/kg, i.p.) in microdialysates measured by CE-LIF. Values for the first two sampling times are baseline samples. The arrow marks the time when PLZ was administered.


(A) GABA
[GABA] (ng/5μL)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
50
100
150
200
250
300
350
Microdialysis Sampling Time (min)

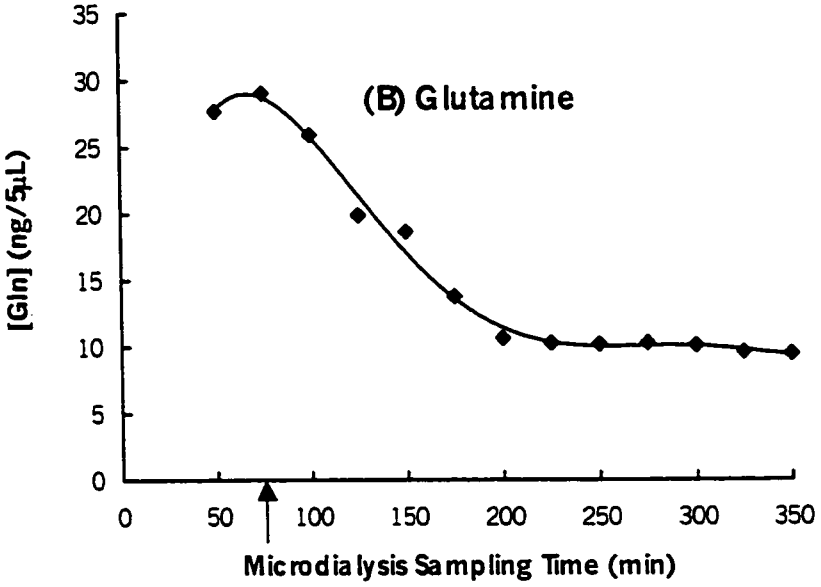
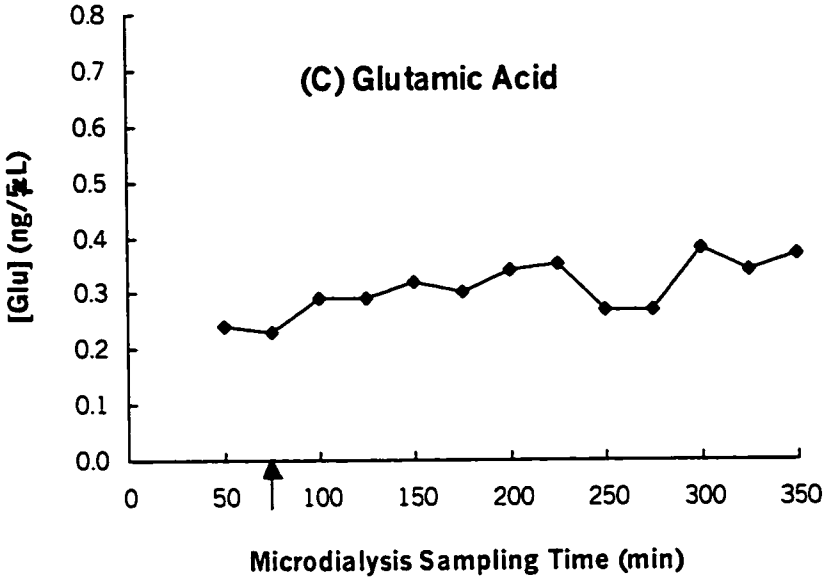

(B) Glutamine
[Gln] (ng/5μL)
35
30
25
20
15
10
5
0
0
50
100
150
200
250
300
350
Microdialysis Sampling Time (min)

Figure 5.2



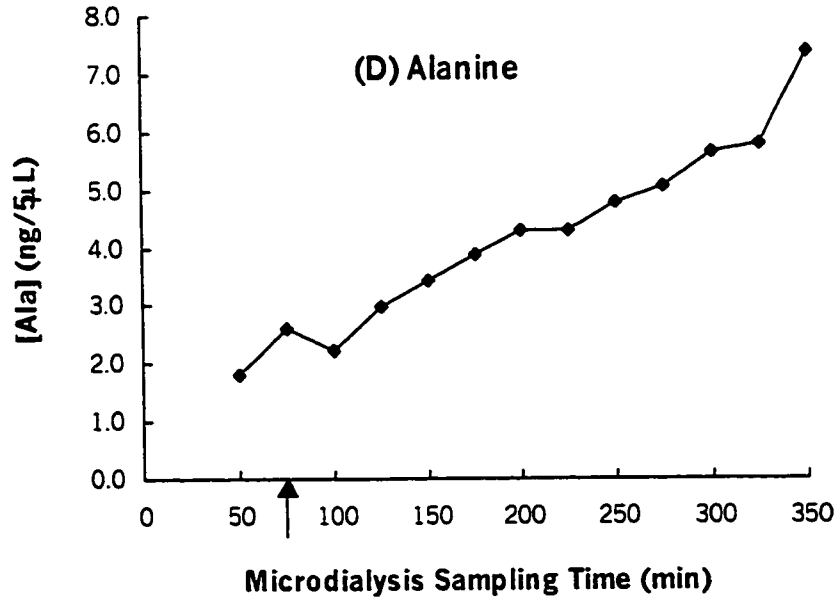


Figure 5.2 (cont'd)

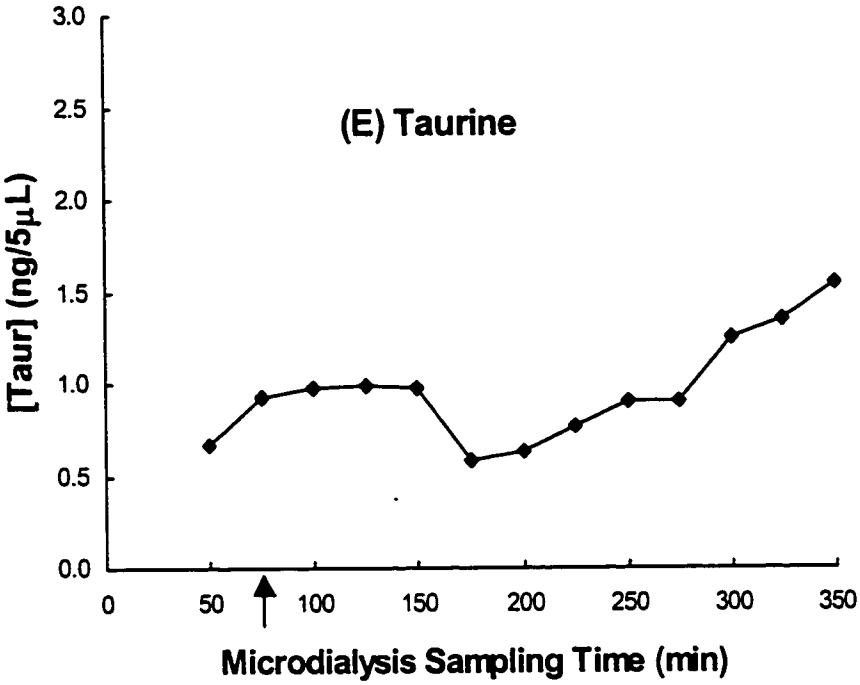


Figure 5.2 (cont'd)

GABA-T in whole brain was considerably less than that of MAO at the same drug doses [14, 23]. In fact, the mechanisms involved in the elevation of GABA are not yet clearly understood, although inhibition of GABA-T may play a role [23]. There is increasing evidence for the possible role of GABA in the actions of antidepressant and antipanic drugs [24], and it is feasible that the increase in GABA observed with PLZ may contribute to its action in both depression and panic disorder [16].

5.3.3.2 Analysis of glutamine

During the experiments, it was found that the concentrations of each analyte in the microdialysis samples varied significantly, which produced more difficulty in the simultaneous quantitation of multiple analytes. Glutamine existed in much higher concentration than the other four amino acids (Table 5.3); it was so far off-scale in another study using HPLC-fluorescence detection that other researchers had to do a second experiment just to measure glutamine (personal communications with Dr. Glen Baker). However, the determination of glutamine could be achieved together with other amino acids in a single run using this CE-LIF system. This is an advantage of this CE method.

The concentration of glutamine was found to be out of the calibration range initially done, therefore a second calibration of glutamine covering the concentration range of 5 - 30 ng/5μL was performed, using the standard solution of glutamine. The

plot of normalized peak height *vs* concentration was fitted by a straight line with the equation $y = 0.016x - 0.127$ ($r^2 = 0.991$).

The levels of glutamine in brain dialysates decreased 3-fold from 30 ng/5μL to about 10 ng/5μL 125 minutes after injection of PLZ and had a minimal decrease during the subsequent sampling period (Figure 5.2 B). It is possible that PLZ and/or its metabolite(s) inhibit glutamine synthetase or stimulate glutaminase [18].

5.3.3.3 Analysis of glutamic acid

The levels of glutamic acid in dialysates varied little even after PLZ administration (Figure 5.2 C), which suggests that PLZ has no effect on glutamic acid.

5.3.3.4 Analysis of alanine

PLZ also caused long-lasting elevation of alanine in brain dialysates, from 1.8 ng/5μL to 7.4 ng/5μL (Figure 5.2 D). A similar pattern was observed using HPLC with fluorescence detection of OPA derivatives [25]. In fact, this is, to our knowledge, the first study utilizing CE to reveal an alanine increase in microdialysates in rat brain after administering PLZ. PLZ may increase the levels of alanine by the binding of the hydrazine of PLZ to pyridoxal 5'-phosphate, an effect produced by several hydrazine drugs [26], but this relationship has not yet been adequately demonstrated. Pyridoxal 5'-phosphate is a cofactor required by alanine transaminase.

5.3.3.5 Analysis of taurine

PLZ does not have much effect on taurine. The concentration levels of taurine in brain septum slightly varied from 0.58 to 1.54 ng/5μL (Figure 5.2 E).

Although this system has been demonstrated specifically for the determination of GABA, glutamine, glutamic acid, alanine and taurine, it can potentially be applied to any primary amine-containing analyte that occurs at concentrations above 0.01 ng/5μL in dialysate samples. By changing the separation conditions, other amino acids as well as biogenic amines could be detected along with these five amino acids.

5.4 CONCLUSIONS

One of the main advantages of CE as an analytical tool for microdialysis sample analysis over the more commonly used HPLC is that it is more amenable to small sample volumes. We developed a rapid CE-LIF method for simultaneous determination of multiple amino acids in brain microdialysates. The method was validated by linearity, sensitivity, accuracy and precision, and microdialysis sampling combined with CE-LIF proved to be a very powerful tool for future *in vivo* neurochemical studies. Although this system did not take the advantage of CE to achieve better temporal resolution, shortening sampling time to faster detect neuronal responses by CE would be very useful for other systems.

The antidepressant/antipanic drug phenelzine (PLZ) increased the extracellular levels of GABA and alanine and decreased the glutamine levels in rat brain. No effects were observed on glutamic acid or taurine.

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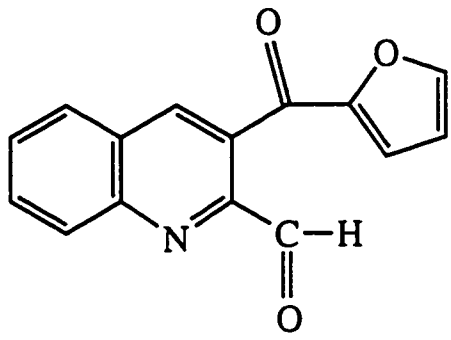
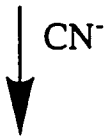
APPENDIX. FQ labeling reaction with an amino acid.

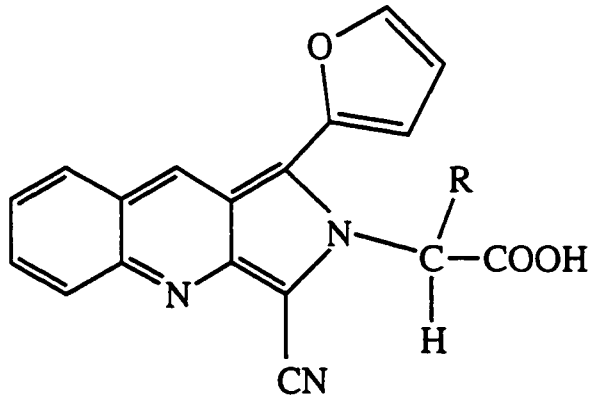


+ H_2N-CHR-COOH

FQ

Amino acid





Fluorescent product