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Evaluation of capillary electrophoresis with post-column derivatization and laser-induced fluorescence detection for the determination of substance P and its metabolites

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Abstract

A method for the detection of substance P and its metabolites using capillary electrophoresis with post-capillary derivatization and laser-induced fluorescence detection is described. The post-capillary chemical derivatization system employs naphthalene-2,3-dicarboxaldehyde and β-mercaptoethanol. Two reactor designs were evaluated for the determination of substance P and its metabolites. The fluorescent spectroscopic properties of the derivatives under optimal separation conditions were also examined. The final system was evaluated for the investigation of substance P metabolism in brain following perfusion of the striatum with substance P using microdialysis sampling.

Keywords: Substance P; Naphthalene-2,3-dicarboxaldehyde; β-Mercaptoethanol

1. Introduction

Substance P (SP) is a basic undecapeptide with a hydrophobic C-terminus (Fig. 1). It is present in the extracellular fluid of brain at picomolar concentrations and has been postulated to act as a transmitter, cotransmitter and/or neuromodulator in the central and peripheral nervous systems [1]. SP is also believed to be involved in vasodilation [2], stimulation of smooth muscle [2,3], transmission of pain [4], cardiovascular regulation [5] and blood pressure regulation [6]. Abnormal levels of SP are believed to be associated with such disorders as schizophrenia [7] and Huntington's [8], Parkinson's [9] and Alzheimer's diseases [10,11].

Microdialysis sampling is an in vivo sampling technique that can be employed for continuous monitoring of neurotransmitters in the brain [12,13]. One advantage of this approach is that the dialysates are essentially protein-free and can be injected directly into the analytical system. Enzymes that could continue to degrade the peptide after sampling are excluded as well. In microdialysis, recovery of analyte through the probe increases with decreasing flow-rate; therefore, rates of 1 μl/min or less are typically employed. To obtain the best temporal resolution, highly sensitive techniques capable of analyzing small sample volumes are required.

Because capillary electrophoresis (CE) is capable of analyzing small volume samples, it is useful in cases where one is sample limited, such as the analysis of single cells [14,15], microdialysis samples [16–18] and recombinant proteins [19–24]. CE has been found to be especially advantageous for separating peptides, because the separation is based on charge-to-size ratios. Analysis times are typically shorter than in liquid chromatography (LC), and

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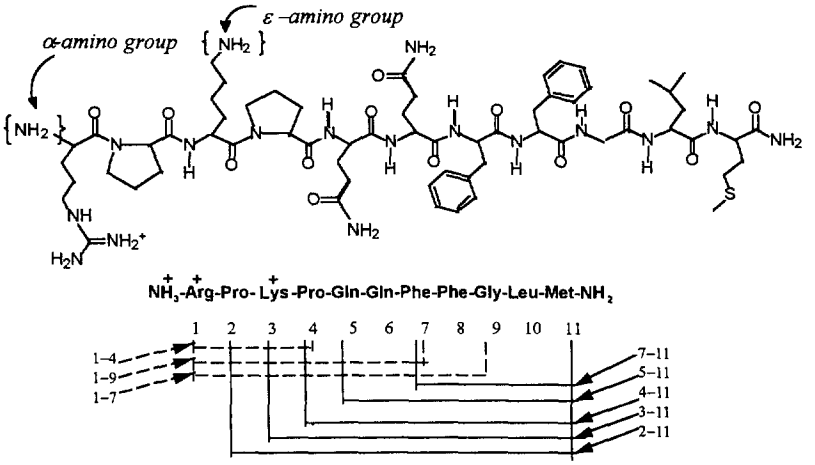

Fig. 1. Structures of substance P and its potential metabolites.

better resolution and higher separation efficiencies are often obtained.

Recently, we reported the separation of SP and related peptides using CE–UV. However, detection limits were typically in the micromolar range, which is not sensitive enough for measurement of SP in biological samples. Laser-induced fluorescence (LIF) is much more sensitive, but chemical derivatization is usually necessary because most peptides, including SP, are not naturally fluorescent.

Derivatization of peptides for CE can be accomplished on-column, pre-column or post-column. In on-column derivatization, some or all of the reagents are present in the background electrolyte (BGE) [25], and derivatization occurs while the separation is taking place. The major difficulty with this approach is that the derivatization and separation conditions must be optimized simultaneously. Pre-column derivatization is most commonly employed with CE. However, because the tag generally reacts with one of the ionizable moieties (NH_2), the overall charge of the analyte may be reduced, making the separation more difficult. In addition, side products from the reaction can hinder quantitation of trace components.

Post-column derivatization used with CE has several advantages. Analytes can be separated prior to detection, based on their native electrophoretic mobilities, facilitating the separation of families of peptides that may be very similar in structure. Additionally, in contrast to pre-column derivatization, no side-products are present to cause interfering peaks. Two reagents that have been employed extensively for post-column derivatization of primary amines are *o*-phthaldialdehyde (OPA) [26–28] and fluorescamine [29,30]. In 1992, Dave et al. [31] demonstrated the use of naphthalene-2,3-dicarboxaldehyde–β-mercaptoethanol (NDA–β-ME) (Fig. 2) as a post-column derivatization agent for the detection of opioid peptides by LC with LIF detection. Later, Gilman and Ewing [32] used these same reagents for post-column derivatization of a mixture of amino acids, neuropeptides and proteins at the high nanomolar level following separation by CE.

One of the major challenges of post-column derivatization for CE is the development of the post-column reactor. The ideal reactor is simple to construct, yields minimal sample loss and permits

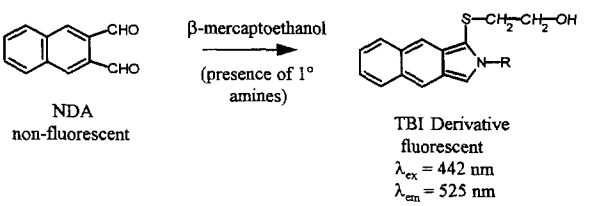

Fig. 2. Reaction of primary amines with NDA–β-ME.

adequate mixing of the reagent with the analyte of interest. The most commonly employed design is the gap reactor (Fig. 3A) [25]. Several variations have been reported, including a gap junction tee or coaxial tee [29,33–35], a pressurized mixing tee [36] and a porous tube [37]. The major drawbacks of the gap-type reactor are that there can be significant sample loss at the junction and sample dilution.

In this paper, we report the evaluation of post-column derivatization with NDA–β-ME in conjunction with CE–LIF for the determination of SP and its metabolites (Fig. 1). An etched reactor and a membrane-based reactor were compared. The membrane-based reactor system was applied in an investigation of SP metabolism by microdialysis sampling following perfusion of rat striatum with SP.

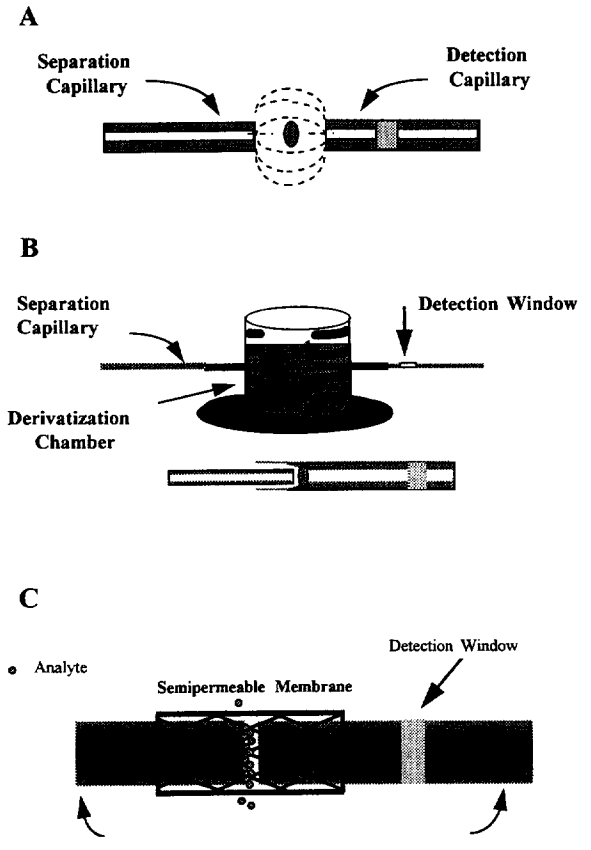

Fig. 3. Post-column reactor designs (A, gap; B, etched; C, membrane).

2. Experimental

2.1. Reagents

SP 1-11, 1-4, 1-7, 1-9 and 2-11 were obtained from Bachem Biosciences (King of Prussia, PA, USA). SP 4-11 and 5-11 were purchased from Bachem California (Torrance, CA, USA). SP 7-11, phytic acid and amino acid standards were obtained from Sigma (St. Louis, MO, USA) and SP 3-11 was purchased from Peninsula (Belmont, CA, USA).

Boric acid, sodium tetraborate and sodium hydroxide were obtained from Fisher (Fair Lawn, NJ, USA). Sulfobutyl ether β-cyclodextrins (IV), SBE(IV)β-CD, were supplied by Cydex (Lenexa, KS, USA) or the Center for Drug Delivery Research (CDDR) (Lawrence, KS, USA). NDA was contributed by Dr. John Stobaugh's laboratory at the University of Kansas (Lawrence, KS, USA). Laser-grade sodium fluorescein was purchased from Kodak (Rochester, NY, USA). Concentrated sulfuric acid and 50% hydrofluoric acid (HF) were obtained from Aldrich (Milwaukee, WI, USA). All other chemicals were of reagent grade and were used as received.

2.2. Apparatus

All separations were carried out with a laboratory-constructed capillary electrophoresis system using a CZE 1000r high voltage power supply (Spellman, Plainview, NY, USA). Fused-silica capillaries (50 μm I.D.×150 μm O.D., 50 μm I.D.×180 μm O.D. and 75 μm I.D.×360 μm O.D.) were obtained from Polymicro Technologies (Phoenix, AZ, USA). A small section (1.0–1.5 cm) of the polyimide coating on the detection capillary, either 50 or 75 μm I.D., was burned off with hot concentrated sulfuric acid to permit optical detection. A pressure injection system and a timer box were built in-house using a Mac valve (225B-111CAAA, 120/60 110/50, 24DVC) from Wolverines (Wixom, MI, USA) and a Potter and Brunfield timer (CNT-36-76) (Princeton, IN, USA). Hydrodynamic injections were made with pressurized nitrogen gas at 13.8 kPa for 5 s, unless otherwise specified. The injection volume was calculated to be 20 nl using the Hogan-Poiseville equation. The high voltage was applied at the injection end of the capillary and the detection end of the

capillary was held at ground. To lock out stray light and isolate the user from the high voltage, all optical components, including the capillary, were enclosed in a black Plexiglas box with slide-in panels on all four sides for easy access. Caution was exercised to avoid touching the post-column reactor or any of the optical components while high voltage was being applied.

Detection was accomplished by LIF using a He–Cd laser (Model 4310N, Liconix, Santa Clara, CA, USA) with 7–10 mW of power at 442 nm as an excitation source. A 442-nm interference filter (Edmund Scientific, Barrington, NJ, USA) was used for rejection of laser plasma. A 1.0-cm focal length biconvex lens (Melles Griot, Irvine, CA, USA) was used to focus the laser beam in the detection window of the capillary. This was accomplished using an $x-y$ positioner, consisting of two precision horizontal translators (no. 16121, Oriel, Stratford, CT, USA). A 20X microscope objective, purchased from Edmund Scientific, was used to collect the fluorescent light. The emitted image was filtered using a glass long-pass filter with a cut-on wavelength of 495 nm (no. 51292, Oriel). Scatter and reflection were reduced using a flexible shield (no. 77356, Oriel). The photomultiplier tube (PMT) (R1527, Hamamatsu, Bridgewater, NJ, USA) was housed in a PMT adapter and operated at between +900 and +1100 V, supplied by a regulated power supply (Model 227, Pacific Photometric, Concord, CA, USA).

Data acquisition was accomplished with a DA-5 system from Bioanalytical Systems (BAS) (West Lafayette, IN, USA) running on a Gateway 2000 4DX2-66 computer. Signal amplification, variable offset and RC filtering (typically 300 ms) were accomplished with a circuit constructed in-house.

2.3. Etched reactor

The etched reactor used in this study was constructed by placing the end of a 30-cm fused-silica capillary (75 μm I.D.×360 μm O.D.) in 40% hydrofluoric acid and sparging helium through the capillary at 6.9 kPa for 45 min. This produced a conical shape at the end of the capillary. Following the etching procedure, the capillary was flushed with water for 15 min to remove residual HF.

Prior to alignment of the reaction capillary with the separation capillary, a reagent reservoir was constructed from a 3-cm section of a 5-ml plastic microsyringe barrel. Two holes were made on each side of the syringe reservoir, using a heated piece of metal tubing approximately 1 μm in diameter. A 1-cm piece of 0.02 in. I.D. PEEK tubing (no. 1532, Upchurch, Seattle, WA, USA) was placed on each side of the plastic syringe reservoir. The two pieces of tubing were aligned by threading a small piece of fused-silica capillary through them, and the tubing was glued to the reservoir using 5-min epoxy. After 1 h, the capillary was removed and the reservoir was ready for use. The separation capillary (50 μm I.D.× 150 μm O.D.) and the detection capillary were inserted into each side of the PEEK tubing and aligned using a microscope (Fig. 3B). Once the alignment was made, the capillaries were secured with 5-min epoxy. A portion of a glass microscope slide was attached to the bottom of the reagent reservoir with epoxy to secure the post-column reactor. Finally, a small section of the polyimide coating on the detection capillary was burned off, at approximately 1 or 20 cm from the reactor for determination of lysine-containing peptides and non-lysine-containing peptides, respectively.

2.4. Membrane reactor

For this reactor, the separation and detection capillaries were of the same dimensions (50 μm I.D.×180 μm O.D.). To produce the reactor, a capillary, 68 or 78 cm in length, was scored at the 48 cm mark and a small section (approximately 1.0–1.5 cm) of a polyacrylonitrile (PAN) microdialysis membrane (240 μm I.D. and 340 μm O.D., M_r cut-off= 29 000) was placed over the mark. The capillary was pulled manually to make a clean break at the score. UV glue (UVEXS, Sunnyvale, CA, USA) was added to the inside of the membrane until it reached approximately 50 μm from the edge of the break in the two sections of the capillary. The glue was allowed to cure for approximately 20 min. The capillaries were separated by a minimal distance of 20–30 μm and glue was used to secure the detection capillary to the other end of the membrane (Fig. 3C).

The membrane reactor was placed inside the reagent reservoir by careful threading the capillaries through the PEEK tubing. The reservoir was con-

structed as described in Section 2.3. Using 5-min epoxy, both ends of the detection capillary were glued to the reagent reservoir. Finally, a 1.0–2.0 cm window was made at a distance of 0.75 cm from the capillary break inside the membrane.

2.5. Capillary preparation

To align the laser with the capillary window, 0.5 m*M* fluorescein was placed in the derivatization reservoir and flushed through the detection capillary using pressurized nitrogen at 5 p.s.i. In order to obtain the highest signal-to-noise ratio possible, with minimal scatter, micromanipulators were employed to focus the laser beam onto the capillary window. The inlet capillary, post-column reactor and outlet capillaries were all kept at equal distances vertically from the laser optical bench to prevent siphoning.

The reaction mixture was then prepared. The derivatization buffer consisted of 100 m*M* sodium tetraborate (pH 9.5) containing 7.0 m*M* SBE(IV)β-CD. Separate stock solutions of β-ME (30 m*M*) and NDA (50 m*M*) containing 5.0 m*M* SBE(IV)β-CD were prepared in MeOH and kept in the dark until use. NDA stock solutions without SBE(IV)β-CD required 10–15 min of sonication for complete dissolution. The solutions were combined to yield a final derivatization mixture consisting of 70 m*M* sodium tetraborate, 8.0 m*M* β-ME, 1.0 m*M* NDA and 5.0 m*M* SBE(IV)β-CD (30% MeOH) at pH 9.5. After performing the above procedures, the post-column reservoir was filled with ca. 1 ml of the derivatization mixture using a syringe.

The CE separation buffer consisted of 150 m*M* boric acid, 15 m*M* phytic acid and 5 m*M* SBE(IV)β-cyclodextrin [38]; the pH was adjusted to 7.0 using HCl. This buffer was prepared fresh weekly, filtered daily and sonicated for 5 min prior to the first run each day to prevent air bubbles from entering the capillary. Due to the high ionic strength of the BGE, the applied voltage was maintained at either +15 kV (etched reactor) or +17.5 kV (membrane reactor). Following focusing, the fluorescein was removed from the reagent reservoir and the reactor was rinsed twice with either 1.0 *M* NaOH (etched reactor) or 0.1 *M* NaOH (membrane reactor) and flushed thoroughly with NANOpure water (Sybron-Barnstead, Boston, MA, USA). The capillary was also flushed with NaOH (at least 30 min), water (15 min) and BGE. A vacuum was applied at the outlet to ensure that the derivatization capillary was fully rinsed.

2.6. Fluorescence studies

Solutions used for fluorescence experiments included 100 m*M* sodium tetraborate (pH adjusted to 7.0, 8.0, 9.0, 9.5 or 10.0 using 1.0 *M* NaOH or 1.0 *M* HCl), either 1.0 or 50 m*M* NDA (100% MeOH) and 100 m*M* β-ME (20% MeOH). Three peptides were evaluated in these experiments, SP, SP 1-7 and SP 5-11. These peptides were prepared in 100% NANOpure water to yield a final concentration of 1.5 m*M*. All derivatization mixtures (final volume 1.0 ml) were placed in 1.0-ml glass cuvettes.

The reaction profiles of the three peptides were monitored using a Perkin-Elmer Model MPF-66 fluorescence spectrophotometer, a Perkin-Elmer 7500 professional computer and a PR-210 printer. At each pH value, 40 μl of a 1.5-m*M* concentration of the respective peptide, 10 μl of 100 m*M* β-ME dissolved in (20:80, v/v) NANOpure water–MeOH and 600 μl of 1 m*M* NDA dissolved in 100% MeOH were added (in that order) to a 350-μl volume of 50 m*M* sodium tetraborate buffer: This resulted in final concentrations of 60 μ*M* peptide, 1 m*M* β-ME, 17.5 m*M* sodium tetraborate buffer and 0.6 m*M* NDA in 60% MeOH. Fluorescence intensity readings were taken every 30 s. The initial time (t_0), the time required to mix the peptides with the post-column derivatization reagents, was also 30 s. Each run was performed in duplicate, and all scans were run over a 6-min interval.

Because phytic acid was found to be necessary for the separation of the SP metabolites, studies were also performed with the fluorimeter to determine the effect of phytic acid and SBE(IV)β-CD on fluorescence intensity. In these studies, a 30-μ*M* stock solution of SP dissolved in the BGE was employed. The derivatization mixture consisted of 5.0 m*M* SBE(IV)β-CD, 2.0 m*M* β-ME, 0.5 m*M* NDA and 1% MeOH. To initiate the reaction, the SP solution was diluted 1:1 with the derivatization mixture. In one study, 15 m*M* phytic acid was added to the BGE mixture and the fluorescence intensity was measured. A second experiment was performed in which SBE(IV)β-CD was excluded from the derivatization

mixture. A background scan was also made where SBE(IV)β-CD and phytic acid were eliminated from both the BGE and derivatization mixture.

2.7. Microdialysis studies

A stock solution of artificial cerebrospinal fluid (ACSF) that consisted of 120 m*M* NaCl, 20 m*M* $NaHCO_3$, 3.0 m*M* KCl, 1.2 m*M* $CaCl_2$, 1.0 m*M* $MgCl_2$ and 0.25 m*M* Na_2HPO_4 was prepared fresh weekly. A concentric cannula probe with a 4-mm membrane (BR4) was used in these studies. The probe was perfused at 0.3 μl/min with ACSF using a Baby-Bee syringe pump. The probe was calibrated in a stirred solution (20 ml) of 300 μm SP dissolved in ACSF at 37°C. A polyethylene container was used to minimize adsorption of SP to the vial. Samples were collected off-line every 30 min using an automated sample collector (CMA Model 142). All of the microdialysis apparatus and probes employed in these studies were from BAS.

A male Sprague–Dawley rat weighing 315 g was anesthetized by i.m. injection of ketamine and xylenol (90 mg/kg and 20 mg/kg, respectively) and was maintained under anesthesia for the duration of the experiment through additional doses, as needed. The microdialysis probe was implanted in the striatum using a guide cannula (coordinates: A: +2 mm, L −3 mm, V −6 mm, with reference to bregma) and perfused with ACSF at 0.3 μl/min using a syringe pump. PTFE tubing (120 μm I.D.) was used for the line connections. Blank samples (30 min collections) were collected from the animal for at least 1 h prior to the introduction of SP. Following this, the perfusate was changed to 300 μ*M* SP in ACSF and dialysis samples were collected every 30 min (9-μl aliquots) for 3 h following the change in perfusate composition.

3. Results and discussion

3.1. Optimization of reaction conditions

The ideal post-column derivatization reagent is not fluorescent itself, but reacts quickly and selectively to produce a fluorescent product that is stable for the time period required to reach the detector. One of the most popular reagents for the determination of primary amines is NDA–CN. However, this reagent has usually been restricted to pre-column derivatization due to its slow reaction kinetics. The use of NDA with β-ME has recently been employed for post-column derivatization of peptides following separation by LC [31] and CE [32]. The reaction is much faster than that with NDA–CN, and the excitation wavelength is compatible with the He–Cd laser. Using CE and post-column derivatization with NDA–β-Me, Gilman and Ewing were able to achieve limits of detection of 1.4 μ*M* and 50 n*M* for glycine and transferrin, respectively.

NDA–β-ME was also investigated for our studies. The derivatization solutions appeared to be stable, with no noticeable increase in background for over 10 h. The maximum concentration of NDA that could be used in our system was determined to be 1.0 m*M*, due to its low aqueous solubility. The fluorescence intensity of the thiobenzylisoindole (TBI) derivative reached a plateau at 8 m*M* β-ME. Therefore, the optimal concentrations of NDA and β-ME were determined to be 1.0 and 8.0 m*M*, respectively. These values were in good agreement with those reported previously [32].

The effects of pH and reaction time on fluorescence intensity were investigated. Three representative peptides were chosen for these studies: SP (lysine-containing), SP 1-7 (lysine-containing) and SP 5-11 (non-lysine-containing). Fig. 4A–C shows the plot of fluorescence intensity vs. time for the three peptides. For the lysine-containing peptides, the maximum fluorescence intensity was achieved in less than 30 s for all pH values, and the highest yield was obtained at pH 9.5. The products of this reaction were very unstable, and the fluorescence intensity began decreasing immediately following t_o. For non-lysine-containing peptides, the time required to reach maximum fluorescence was much greater, approximately 6.0 min. The optimum pH for the non-lysine peptides was 8.0.

3.2. Effect of buffer additives

In the previous development of the CE separation using UV detection, SBE(IV)β-CD was used to resolve the C-terminus metabolites [38]. The $(CBI)_2$ derivative of SP is known to exhibit quenching

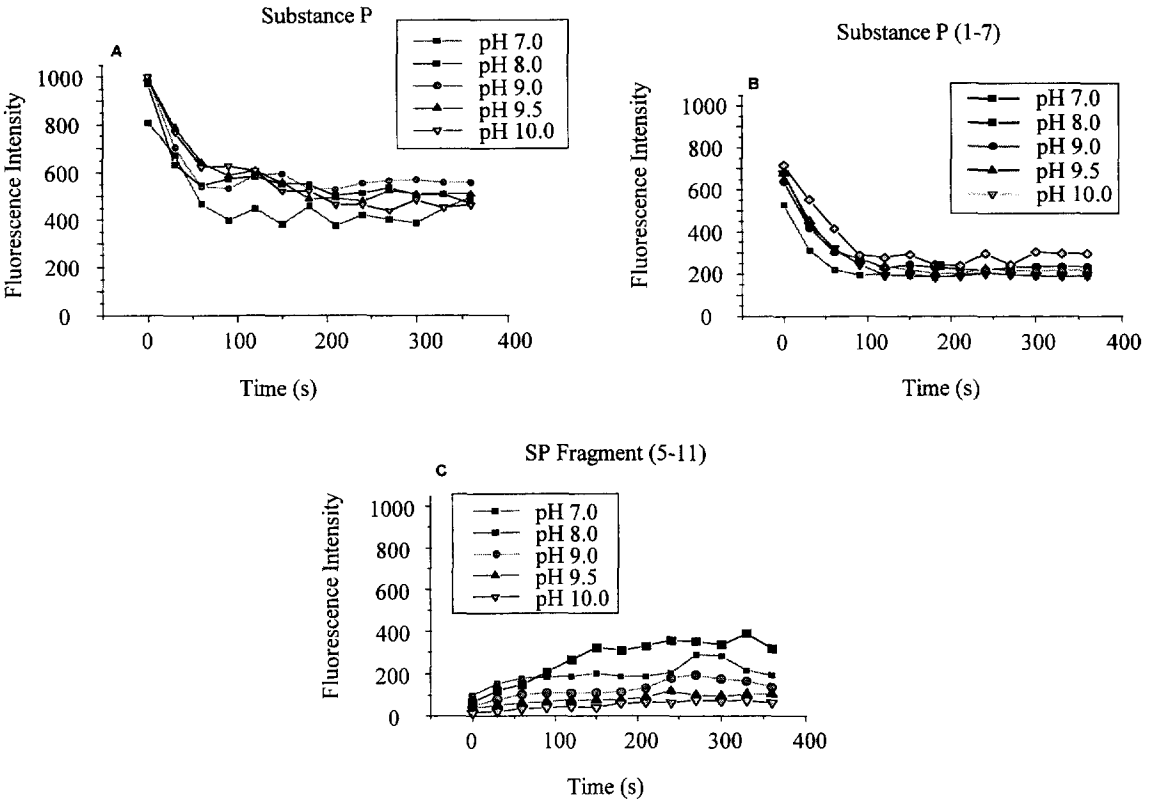

Fig. 4. Reaction profiles of: (A) substance P, (B) SP 1-7 and (C) SP 5-11 with NDA–β-ME. Final concentrations: 60 μ*M* peptide; 1 m*M* β-ME; 17.5 m*M* sodium tetraborate buffer and 0.6 m*M* NDA. Fluorescence intensity readings were taken every 30 s, where t_0=30 s.

behavior [39], and CD has been shown previously to prevent fluorescence quenching [40]. Thus, it was of interest to determine whether SBE(IV)β-CD had an overall effect on the reaction rate or on the fluorescence intensity of the TBI product. The optimal concentration of SBE(IV)β-CD in the BGE needed for the separation was previously determined to be 5 m*M* [38]; therefore, higher concentrations were not investigated.

As shown in Fig. 5, the addition of 5 m*M* SBE(IV)β-CD to the derivatization mixture caused a >100-fold increase in fluorescence intensity at t_0= 30 s, in comparison to the same reaction without CD. Under actual CE separation conditions, it was found that when 5 m*M* SBE(IV)β-CD was added to *both* the BGE and the derivatization mixture, an additional eight-fold increase in fluorescence intensity for the lysine-containing peptides was obtained. This observation is in agreement with the findings of Li and Purdy [41] and Sbai et al. [42], who reported that CDs stabilize the excited state of the molecule within the cyclodextrin cavity in addition to separating the two fluorophores. We believe that the CD also acts to stabilize the reaction product, leading to an increase

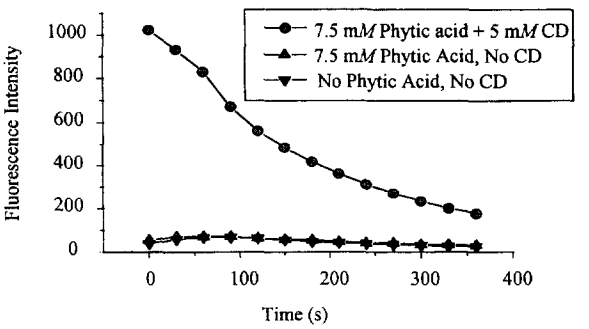

Fig. 5. Effect of phytic acid and SBE(IV)β-CD on fluorescence intensity.

in overall fluorescence intensity under CE separation conditions.

There was also some concern as to whether phytic acid would adversely affect the reactivity or fluorescence intensity of the complex. However, no significant difference in fluorescence intensity was found between derivatization mixtures with or without phytic acid.

3.3. Post-column reactors

Two reactors were evaluated for post-column derivatization with NDA–β-ME. It was found that complete resolution of SP and its metabolites could not be achieved using the etched post-column reactor (Fig. 3B), although this result had been achieved in the earlier CE–UV separation under the same conditions. The separation efficiency for SP with this reactor was 4725, substantially lower than that obtained with the CE–UV separation (18 646). This loss in efficiency is the result of band-broadening, caused by the mismatch in I.D. of the two capillaries. Fig. 6A shows the detection of the lysine-containing metabolites using this reactor at pH 9.5. Fig. 6B shows a separation of the non-lysine-containing peptides at pH 8 with a reaction time of 6 min. In the latter case, the severe loss of resolution is due to the extended reaction time in addition to the mismatch in capillary I.D.

A membrane reactor that was developed previously by Zhou and Lunte [43] for pH adjustment of the BGE prior to CE with electrochemical detection was also evaluated for this application (Fig. 3C). When placed in the derivatization reservoir, the membrane acts as an on-line mixer, allowing the NDA–β-ME to diffuse through the semipermeable membrane and to mix with the analyte of interest. It also acts as a restrictive barrier to limit loss of analyte. This latter factor is especially important for large molecules such as SP. It was found that the sample loss using the membrane reactor was about a factor of five less than with the standard gap reactor design.

In order to determine the optimized detection distance for the lysine-containing metabolites, a study was conducted in which the distance between the gap and the detection window was varied between 0.75 and 1.5 cm. The maximum fluorescence intensity was obtained at 0.75 cm. Unfortunately, with the present reactor design, the minimum distance that could be employed was 0.75 cm. In future studies, alternative configurations that permit detection at shorter distances will be investigated.

Fig. 7 shows an electropherogram of SP and its metabolites using the membrane reactor. The derivatization pH was 9.5 and the reaction time was 10–12 s. The detection limits for SP and its metabolites under these conditions are given in Table 1. The limit of detection (LOD) range was 75–100 n*M* for

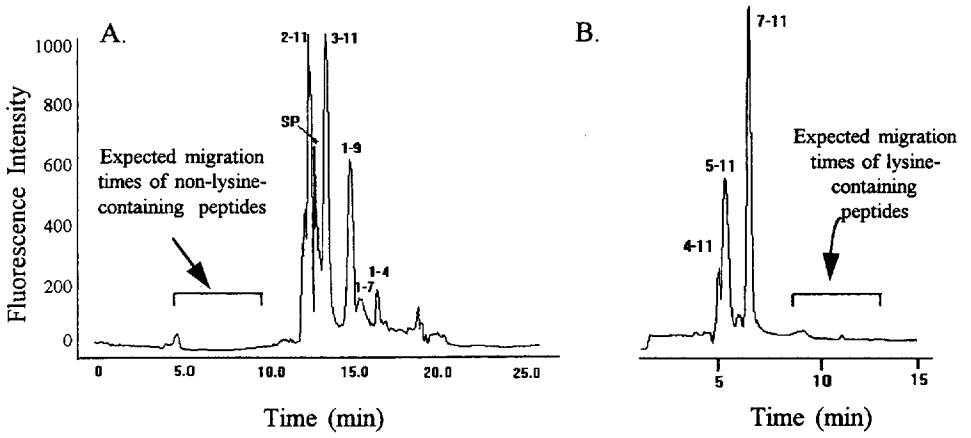

Fig. 6. Electropherogram of SP and its metabolites obtained using the etched reactor design. Separation conditions: BGE, 150 m*M* boric acid, 15 m*M* phytic acid and 5 m*M* SBE(IV)β-CD at pH 7.0; separation voltage, +15 kV; column length, 68 cm; reactor, 48 cm. (A) Conditions optimized for lysine-containing peptides: derivatization, pH 9.5; reaction time, 23 s. (B) Conditions optimized for non-lysine-containing peptides: derivatization, pH 8.0; reaction time, ca. 6 min.

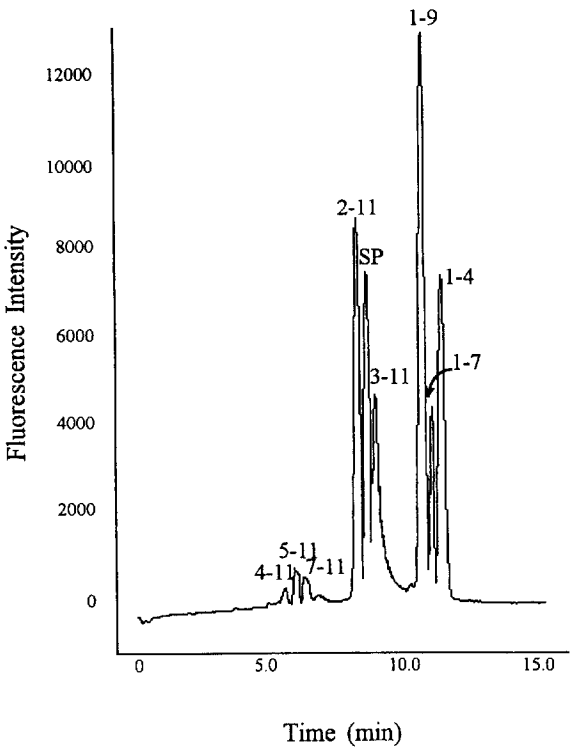

Fig. 7. Electropherogram of SP and its metabolites obtained using the membrane-based reactor design. Separation conditions identical to those described in Fig. 6, except that the separation voltage was +17.5 kV. Derivatization, pH 9.5; reaction time, 23 s.

the lysine-containing peptides and 20–25 μ*M* for the non-lysine-containing peptides at $S/N=3$. The method was linear for SP from 0.50–100 μ*M*. Correlation coefficients for all of the peptides ranged from 0.997 to 0.999. The separation efficiency was much better ($N=11\,577$) than with the etched reactor, but still not as good as obtained with CE–UV. Due to the large disparity in LODs between the lysine-containing- and non-lysine-containing metabolites, only the non-lysine-containing metabolites were determined in the microdialysis study.

3.4. Microdialysis sampling

The post-column reactor was evaluated for the investigation of the metabolism of SP in rat brain using microdialysis sampling. The in vitro recovery for SP using the PAN microdialysis membrane was 33%. Fig. 8A shows an electropherogram of a blank microdialysis sample obtained prior to perfusion with SP and Fig. 8B shows a microdialysis sample spiked with SP. From these electropherograms, it is clear that SP did not co-migrate with any of the endogenous substances in the blank. However, there was a further loss of separation efficiency due to the high salt composition of the microdialysis sample.

Fig. 9 shows five electropherograms of microdialysis samples obtained during continuous

Table 1
Limits of detection for substance P and its metabolites

Metabolite	Lysine-containing[a]	Mass detection limit[b] (fmol)	LOD[c]
1-4	Yes	1.4	75.0 n*M*
1-7	Yes	1.9	100.0 n*M*
1-9	Yes	1.4	75.0 n*M*
Substance P	Yes	1.9	100.0 n*M*
2-11	Yes	1.9	100.0 n*M*
3-11	Yes	1.5	80.0 n*M*
4-11	No	388	20.0 μ*M*
5-11	No	388	20.0 μ*M*
7-11	No	485	25.0 μ*M*

[a] Conditions optimized for lysine-containing peptides; derivatization, pH 9.5 sodium tetraborate; 8 m*M* β-ME; 1 m*M* NDA, 5 m*M* SBE4-β-CD.
[b] Assuming viscosity of 0.801 g/ms (at 30°C); pressure=34.5 kPa for 2 s; total capillary length=68 cm.
[c] Signal-to-noise ratio of 3:1.

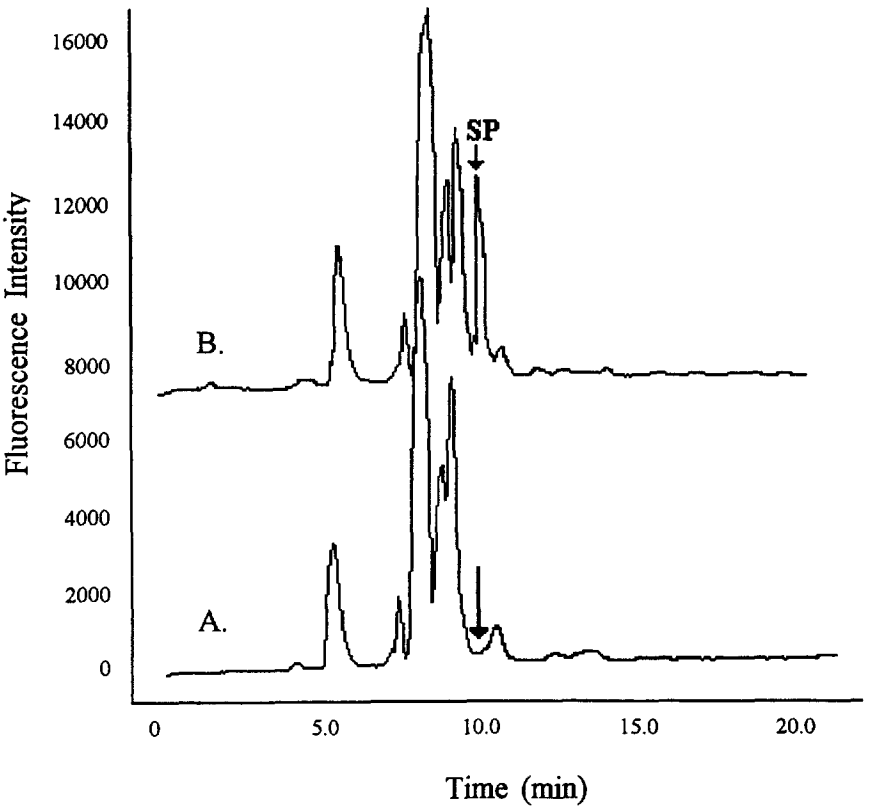

Fig. 8. Electropherogram obtained using the membrane-based reactor design with separation conditions identical to those described in Fig. 7 except that the column length was 78 cm (reactor at 48 cm). (A) Brain microdialysis sample blank. (B) Brain microdialysis sample blank spiked with substance P.

perfusion of the brain with 300 μ*M* SP dissolved in ACSF. Introduction of SP via the probe permits profiling of metabolites formed in the immediate vicinity of the probe. This approach has previously been used by Caprioli's group to investigate SP metabolism by LC–MS [13]. All peptides were tentatively identified based on comparison of their migration times with those of standards. The major metabolite detected in these studies was SP 3-11, which can be produced from SP by a variety of proteolytic enzymes [13,44–48]. Lesser quantities of SP 1-7, 1-9 and 1-4 were seen and could be produced by enkephalinase 24.11 [49], post-proline-converting enzyme [50] or other endopeptidases [45,51,52] known to be located in the striatum. Although we were able to detect only the lysine-containing metabolites, many of the same peptides have been detected under similar conditions by LC–MS [13].

4. Conclusions

Two reactor designs were evaluated for post-column derivatization with CE–LIF using NDA–β-ME. It was determined that the membrane reactor was superior to the etched reactor. Conditions were ultimately optimized for detection of the lysine-containing metabolites of SP. Unfortunately, it was not possible to detect the non-lysine-containing SP

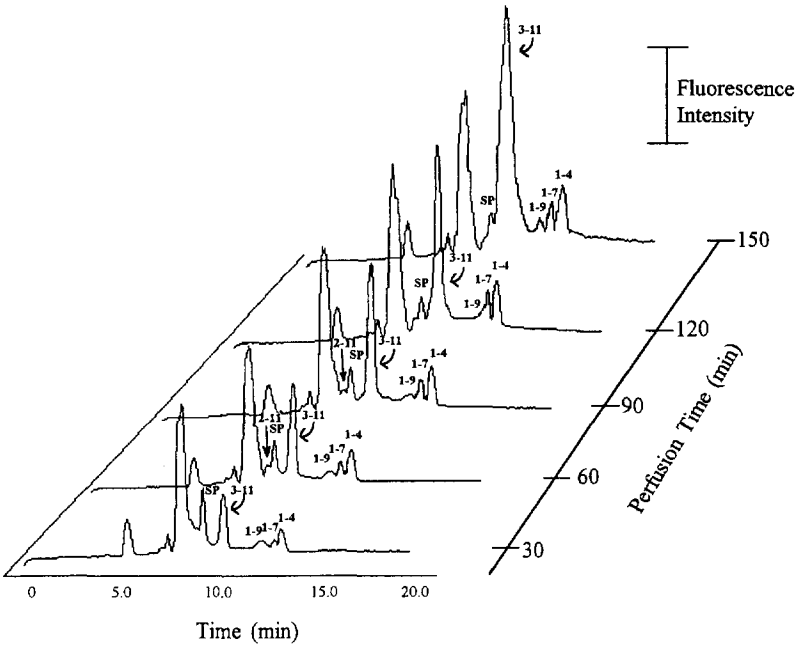

Fig. 9. Electropherograms of microdialysis samples obtained after perfusion of the striatum with 300 μ*M* substance P at 0.3 μl/min. Samples were taken every 30 min and analyzed under the conditions given in Fig. 7.

fragments, 4-11, 5-11 and 7-11, due to the relatively long reaction time of these compounds with NDA–β-Me. An in vivo study of the metabolites of SP in striatum showed 3-11 to be the major detectable metabolite. Future work will concentrate on additional optimization of the reactor and investigation of the transport and metabolism of SP across the blood–brain barrier.

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