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Complete capillary electrophoretic separation of substance P and its metabolites at neutral pH using ionic run buffer additives

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Abstract

Substance P (SP), a bioactive peptide that is present in the extracellular fluid of the brain at picomolar concentrations, has been proposed to be a neurotransmitter and a neuromodulator in the central and peripheral nervous systems. This paper describes the development of analytical methodology for the separation of SP and its metabolites by capillary electrophoresis at neutral pH. Since SP is a cationic peptide, it tends to adsorb to silica surfaces. In this work, phytic acid was employed as a run buffer additive to eliminate the interaction of SP and its cationic N-terminus metabolites with ionized silanol groups. The separation of the C-terminus metabolites was then accomplished through the use of sulfobutyl ether β-cyclodextrin, which imparted a negative charge to these otherwise neutral metabolites through complexation of the phenylalanine residue. The final separation buffer consisted of 150 m*M* boric acid, 15 m*M* phytic acid and 5 m*M* SBE(IV)β-cyclodextrin at pH 7. Under these conditions, it was possible to separate SP from all eight of its reported metabolites.

Keywords: Buffer composition; Substance P; Peptides

1. Introduction

Substance P (SP) is a bioactive peptide in the tachykinin family with the following amino acid sequence: Arg–Pro–Lys–Pro–Gln–Gln–Phe–Phe–Gly–Leu–Met–NH_2. It was first isolated from brain and intestine and was purified in a powdered form, thus giving it the name substance "P" for powder. SP possesses a naturally occurring amidated C-terminus and is postulated to play a role as a transmitter, co-transmitter and neuromodulator in the central and peripheral nervous systems [1]. It is also believed to be involved in vasodilation [2], stimulation of smooth muscle [2,3], transmission of pain sensation [4], cardiovascular regulation [5] and regulation of blood pressure [6]. In addition, abnormal levels of SP are associated with many disease states such as Huntington's [1,7], Parkinson's [1,8], schizophrenia [1,9] and Alzheimer's [10,11]. SP is present at low nanomolar concentrations in the extracellular fluid of the brain.

Currently, there are several different methods by which SP can be detected and quantitated, the most sensitive of which is radioimmunoassay (RIA). Using RIA, it is possible to detect SP at physiologically relevant levels. However, this technique has some disadvantages. First, it is impossible to determine the concentrations of both SP and its metabolites using a single immunoassay. In addition, in some cases, the antibodies directed toward SP have been shown to cross-react with other mammalian tachykinin peptides as well with several SP metabolites, leading to erroneous results [12]. The in vivo concentrations of SP metabolites can be of particular

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importance in pharmacological studies, since some of these compounds exhibit a similar or the same biological response as the parent compound [13]. Another disadvantage of RIAs is that, due to the low binding constants of the SP antibodies, these assays often take 24–48 h.

Fig. 1 shows the structure of SP and its metabolites. A highly efficient separation method is required to determine all nine compounds. Liquid chromatographic (LC) methods have been reported for the separation of SP and some of its metabolites [14–18]. However, to the best of our knowledge, no LC method has been reported in which all of the major metabolites are separated. The most comprehensive separation of SP metabolites was performed by Igwe et al. [15]. In this case, SP and its fragments 1–4, 1–7, 1–9, 2–11 and 5–11 were resolved by reversed-phase liquid chromatography (RPLC) with UV detection. However, several of the C-terminus metabolites (3–11, 4–11 and 7–11) were excluded from this assay. Both anionic and hydrophobic stationary phases were evaluated in this work; however, it was found that the charged N-terminus fragments adsorbed strongly to both of these stationary phases, especially in the case of anionic exchangers. As a result, the separation efficiency of the C-terminus metabolites was generally higher than that of the charged N-terminus metabolites with these columns.

Capillary electrophoresis (CE) is an analytical technique that has been shown to be extremely useful for the separation of peptide mixtures such as in tryptic mapping [16]. In CE, the separation is dependent on the mass-to-charge ratio; thus, it is possible to separate complex mixtures of peptides that differ only slightly in p*I* [17]. Although CE has tremendous resolving power, it is not quite as widely accepted today as RPLC. This is primarily due to problems associated with separation of basic molecules, in particular, peptides and proteins, which are known to adsorb silanols on the capillary surface [18]. There have been numerous attempts to solve this problem, including direct control of the charge density at the capillary wall by manipulation of the pH of the background electrolyte (BGE) [19], modification of the run buffer through addition of carrier electrolytes [20,21] or pseudophase additives [22] and permanent modification of the capillary wall [23,24].

Recently, the separation of SP from related peptides by CE has been reported. Lee and Desiderio [25] separated SP from other synthetic opioid and tachykinin peptides using a low pH run buffer, and Cifuentes and Poppe [26] reported separation of SP from six of its metabolites at pH 2.6. However, at low pH, it was shown that adequate peak efficiency was obtained only when analyzing low concentrations of SP [27]. At high concentrations (mid-to-high micromolar range), build-up of SP on the capillary led to peak tailing, loss of efficiency and irreproducible migration times. Even at low concentrations, build-up of SP on the capillary occurred, although at a slower rate.

Phytic acid has been shown previously to prevent

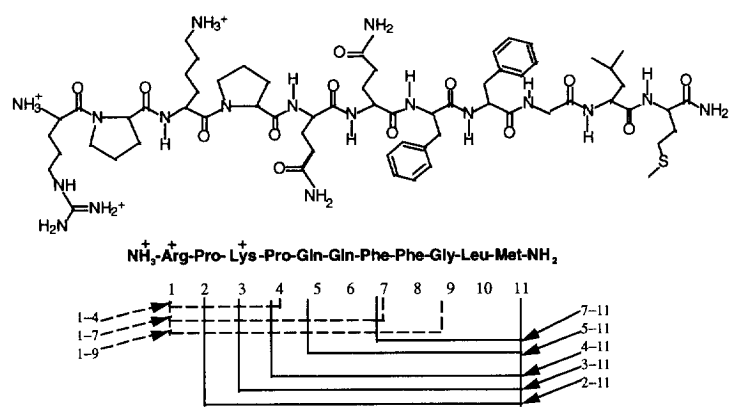

Fig. 1. Structure of substance P (SP) and its C- and N-terminus metabolites.

adsorption of positively charged peptides to capillary walls [28,29]. Another run buffer additive, sulfobutyl ether β-cyclodextrin [SBE(IV)β-CD], has been shown to complex with phenylalanine residues on peptides, increasing their negative electrophoretic mobility [30]. In this paper, a complete separation of SP and all of its metabolites by CE at neutral pH is described. Phytic acid and SBE(IV)β-CD are used as buffer additives to separate the N-terminus and C-terminus peptides, respectively.

2. Experimental

2.1. Reagents

Substance P, 2–11, 1–4, 1–7 and 1–9 were obtained from Bachem Biosciences (King of Prussia, PA, USA). Substance P 4–11 and 5–11 were obtained from Bachem California (Torrance, CA, USA). Substance P 7–11, phosphoric acid, Tris, TAPS and phytic acid were obtained from Sigma (St. Louis, MO, USA). Substance P 3–11 was obtained from Peninsula (Belmont, CA, USA). Sodium cyanide was obtained from Fluka (New York, NY, USA). Boric acid, sodium tetraborate and sodium hydroxide were obtained from Fisher (Fair Lawn, NJ, USA). Mesityl oxide was obtained from Aldrich (Milwaukee, WI, USA). All sulfobutyl ether β-cyclodextrins: SBE(III)β-CD, SBE(IV)β-CD and SBE(V)β-CD were obtained either from Cydex L.C. (Overland Park, KS, USA) or the Center for Drug Delivery Research (CDDR) (Lawrence, KS, USA).

2.2. Stock solutions

Stock solutions of substance P and its metabolites were 2.0 m*M* in concentration and were prepared in Nanopure water (Sybron–Barnstead, Boston, MA, USA). The samples were prepared fresh biweekly in polypropylene microvials and stored at 4°C. Boric acid, phytic acid and sulfobutyl ether β-cyclodextrin were dissolved together in Nanopure water and titrated to pH 7.0 with 1.0 *M* HCl, followed by filtration with a 0.2 μm Acrodisc filter obtained from Gelman Sciences (Ann Arbor, MI, USA). The buffers were then sonicated for approximately 5 min prior to the electrophoretic runs. The other buffer systems —sodium phosphate (pH 6–9), Tris (pH 7–9), TAPS (pH 8–9) and sodium borate— were also prepared using Nanopure water. Exact titrations were made using either 0.1 *M* HCl or 0.1 *M* NaOH. The substance P mixture was prepared by combining appropriate volumes of 2.0 m*M* stock solutions of SP and all of its metabolites to yield a final concentration of approximately 100 μ*M* for SP and each of the metabolites.

2.3. Capillary electrophoresis

All separations were carried out using an ISCO 3140 Electropherograph employing hydrodynamic injection. Fused-silica capillaries (50 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 of the fused-silica capillary was burned off to allow optical detection. The length to the detector was 46 cm and the total length was 73 cm. The temperature of the instrument was maintained at 25°C using a refrigerated water bath. Ultraviolet detection was accomplished at 210 nm. After each run, the capillary was flushed with 1.0 *M* NaOH for 2 min, followed by a 5-min flush with the BGE. The applied voltage was +20 kV for all separations.

Mesityl oxide (1.5 m*M*) was employed as a neutral marker in these studies. Relative migration times for SP and its metabolites are reported with respect to this neutral marker.

2.4. Optimization of the N-terminus metabolites

2.4.1. Effect of pH

To determine the effect of pH and buffer type on the separation efficiency of the N-terminus metabolites, several buffer systems were investigated, including 20, 50 and 100 m*M* phosphate (pH 6–9); 50, 100, 125 and 150 m*M* Tris (pH 7–9); 50, 100, 125 and 150 m*M* TAPS (pH 8–9); 20, 50 and 100 m*M* sodium borate (pH 6–8); and 50, 100, 125, 150 and 175 m*M* boric acid (pH 6.5–10). CE separation conditions were the same as given in Section 2.3.

2.4.2. Effect of phytic acid concentration

The effect of phytic acid concentration on resolution was investigated using a BGE of 150 m*M*

boric acid, pH 7. The separation conditions were the same as given in Section 2.3. The four specific concentrations of phytic acid investigated were 3, 10, 15 and 17 m*M*. After 15 m*M* was determined to be the optimal concentration of phytic acid, an additional study was performed to determine the optimal pH (pH 6.5–10). The effects of separation voltage were also investigated over the range of 10–30 kV.

2.5. Optimization of the C-terminus metabolites

For separation of the C-terminus metabolites, the optimal run conditions previously established for the N-terminus metabolites (150 m*M* boric acid and 15 m*M* phytic acid at pH 7.0) were initially employed. Sulfonated cyclodextrins (SBE(III)β-CD, SBE(IV)β-CD and SBE(V)CD) were dissolved in the BGE at a concentration of 3 m*M*. Several concentrations of SBE(IV)β-CD (0, 1, 2, 3, 4, 5 and 6 m*M*) in conjunction with phytic acid and boric acid were investigated. The optimal resolution of the C-terminus metabolites was obtained by using a BGE containing 150 m*M* boric acid, 15 m*M* phytic acid and 5 m*M* SBE(IV)β-CD at pH 7.0.

2.6. Final separation conditions

The final separation of all C- and N-terminus metabolites was accomplished using 150 m*M* boric acid, 15 m*M* phytic acid and 5 m*M* SBE(IV)β-CD, pH 7.0, with a separation voltage of +20 kV. All other conditions were the same as described in Section 2.3.

3. Results and discussion

In addition to its pharmacological importance, SP is interesting from an analytical standpoint. The metabolites described in this paper can be divided into two categories that are known to have very different physicochemical characteristics as well as physiological properties [1,21]. The N-terminus metabolites are cationic at both neutral and low pH, with three positive charges at the first three residues. In contrast, the C-terminus metabolites are more lipophilic in nature and neutral (with the exception of 2–11 and 3–11) due to the naturally occurring amidated C-terminus. Due to these large differences in physical properties, the electrophoretic separations of these two groups of metabolites were developed independently. The two methods were then combined to obtain a complete resolution of SP and its metabolites.

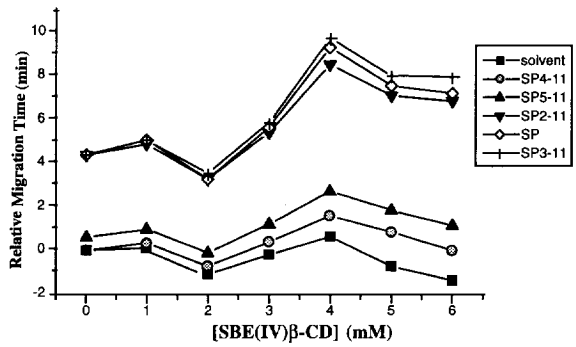

Fig. 2. Effect of phytic acid concentration on SP separation efficiency. The BGE was 150 m*M* boric acid, pH 7. The data for 0 m*M* phytic acid was not included in the plot since the separation efficiency was essentially 0 and no apparent peak was visible. The total column length was 73 cm with a length to the detector of 46 cm, the applied voltage was +20 kV. The concentration of SP injected was 150 μM. Note that each point represents an averaged data point where $n=3$. UV detection was accomplished at 210 nm.

3.1. N-terminus metabolites

The first goal of this work was to improve the separation efficiency of the N-terminus metabolites. These metabolites are the most problematic due to electrostatic interactions of the cationic peptides with the negatively charged surface of the capillary wall, leading to decreased separation efficiency and loss of analyte due to build-up on the capillary [21]. Analyte adsorption also results in a continuously changing zeta potential, which can lead to irreproducible migration times, variable EOF and, eventually, reversal of the EOF.

To minimize peptide–wall interactions, phytic acid, a polyanion containing six phosphate groups with pK_a values ranging from 1.9–9.5, was investigated as an ion-pairing additive. It has been shown previously to be useful for the separation of basic proteins and peptides by CE because it masks all positive charges on the peptide by forming an ion-pair complex [28,29]. The resulting peptide complex

is anionic and is therefore repelled by the negatively charged capillary wall, thereby reducing or eliminating adsorption. One advantage of phytic acid over other ion-paired reagents is that phytic acid results in a lower overall current. This is thought to be due to the increased ionic radius of the hydrated form, in comparison to mono-ions [28,29]. Phytic acid is also relatively inexpensive, especially when compared to the cost of permanently modified capillaries often employed to prevent adsorption of peptides and proteins.

The effect of phytic acid concentration on the separation of the N-terminus metabolites was investigated. A plot of concentration of phytic acid vs. SP separation efficiency is shown in Fig. 2. As it was desirable to maintain a current below 100 μA, concentrations of phytic acid above 17 m*M* were not evaluated. It was found that separation efficiency increased with increasing concentrations of phytic acid. The optimal concentration of phytic acid was determined to be 15 m*M*. Under these conditions, excellent efficiency is still obtained while maintaining a lower overall current. The resulting field strength was 274 V/m. Voltages above +20 kV were not employed because there was some loss of separation efficiency due to Joule heating.

Since the N-terminus metabolites are cationic and vary in their charge-to-size ratio, it was anticipated that these metabolites could be separated by simple manipulation of buffer pH. Fig. 3 shows the effect of BGE pH on the relative migration times of the N-terminus metabolites of SP with respect to the neutral marker mesityl oxide. Although the best resolution was obtained at pH 6.5, as shown in Fig. 3, the analysis time was relatively long. Better peak efficiency and shorter migration times were obtained at pH 7.0, and at this pH the compounds were still adequately resolved. Therefore, pH 7.0 was chosen as optimum for the separation of the N-terminus metabolites. A representative electropherogram using the optimal BGE consisting of 150 m*M* boric acid and 15 m*M* phytic acid at pH 7.0 is shown in Fig. 4.

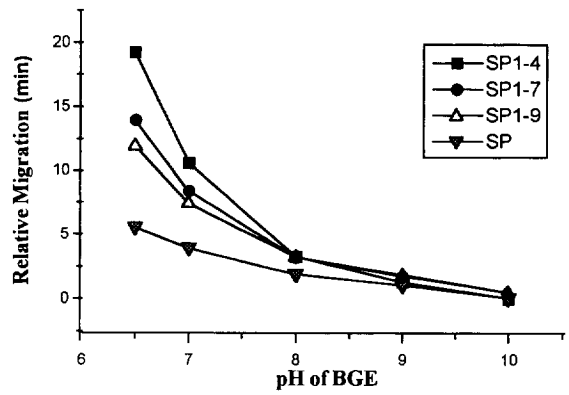

Fig. 3. Effect of BGE pH on relative migration times of SP and its N-terminus metabolites. The relative migration times were calculated with respect to the neutral marker, mesityl oxide. All conditions were the same as in Fig. 2, except the BGE contained 15 m*M* phytic acid and the pH was varied from 6.5–10.0. Approximately 150 μM of each of the N-terminus peptides were injected. Each point represents an averaged data point where $n=3$.

3.2. C-terminus metabolites

After the successful separation of the N-terminus metabolites, the separation of the C-terminus metabolites was investigated. These metabolites exhibit little or no charge difference and, therefore, are difficult to separate by conventional CE. However, all of the C-terminus metabolites possess two phenylalanine residues —one at position 7 and the other at position 8 of the SP peptide. This feature can be exploited in their separation. Cyclodextrins have been shown to form inclusion complexes with aromatic residues of peptides and other compounds [30]. If a negatively charged cyclodextrin is employed, the separation window is greatly expanded, and it should be possible to resolve neutral com-

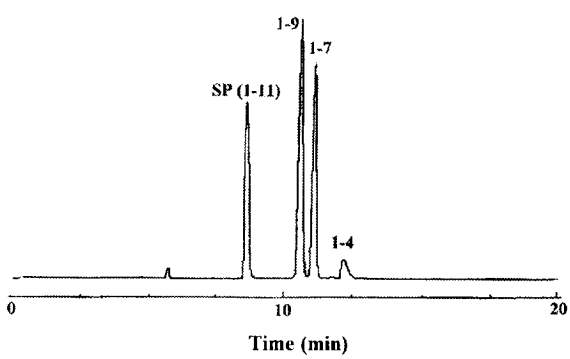

Fig. 4. Electropherogram of the N-terminus metabolites under optimal conditions. BGE consisted of 150 m*M* boric acid and 15 m*M* phytic acid, pH 7. Other separation conditions were identical to those in Fig. 2.

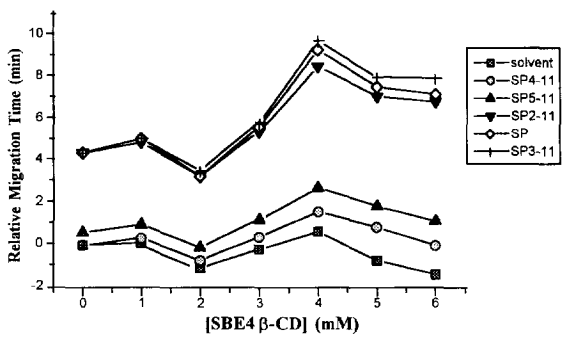

Fig. 5. Effect of the concentration of SBE(IV)β-CD on the relative migration times of SP and its various C-terminus fragments. BGE consisted of 150 mM boric acid and 15 mM phytic acid, pH 7, where various concentrations of SBE(IV)β-CD were included in the BGE. Other separation conditions were identical to those in Fig. 2. The peak labeled solvent was consistently present in all samples as well as the blank runs. Each point represents an averaged data point where $n=3$.

pounds that differ in size [31]. Therefore, a polyanionic derivative of β-cyclodextrin, sulfobutyl ether β-cyclodextrin (SBE β-CD), was evaluated for this purpose [32].

The fact that SBE β-CD is anionic is also beneficial since it will not interact electrostatically with the negatively charged phytic acid additive. SBE β-CD should aid phytic acid in the prevention of analyte adsorption to the capillary wall. SBE β-CD can effect the separation of the phenylalanine-containing peptides by providing a mechanism for differential partitioning as well as by incorporating a negative charge onto the otherwise neutral fragments. Three different types of SBE β-CDs were investigated with different degrees of substitution. These included SBE(III)β-CD, which is substituted at position 3, SBE(IV)β-CD, substituted at position 4 and SBE(V)β-CD, substituted at position 5. It was

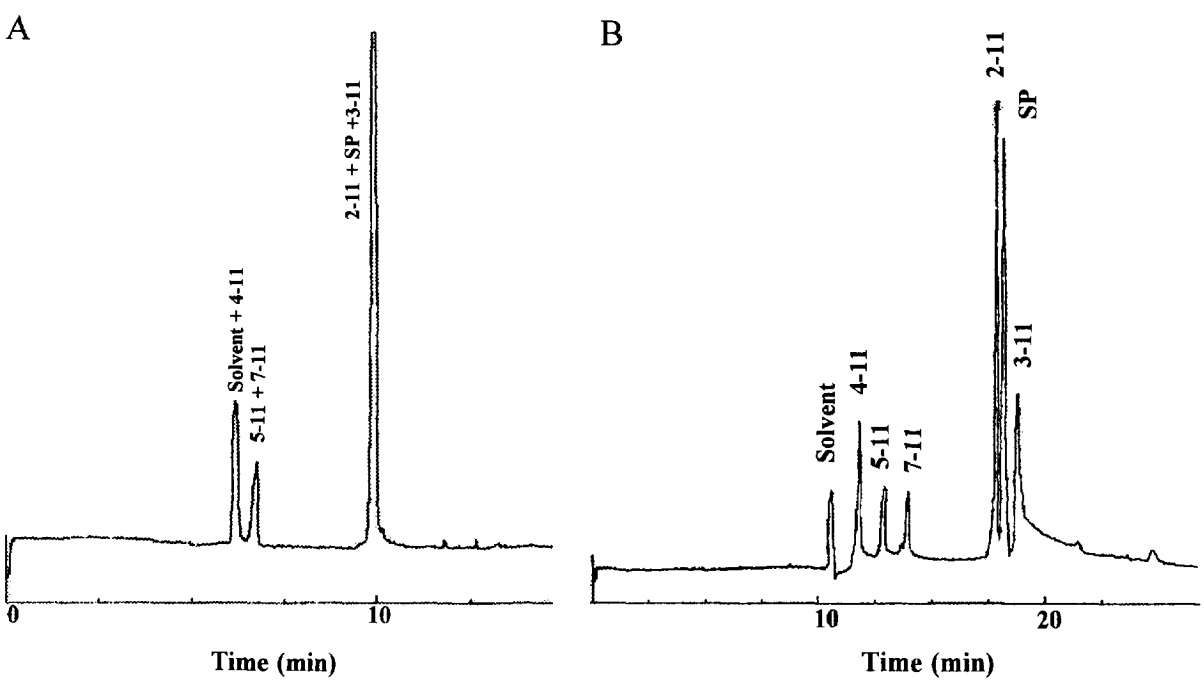

Fig. 6. (A) Electropherogram of the C-terminus metabolites without the addition of SBE(IV)β-CD. The BGE and separation conditions are identical to those in Fig. 5. (B) Separation of the C-terminus metabolites following the addition of 5 mM SBE(IV)β-CD. The BGE was consistent with that in Fig. 4, except for the addition of 5 mM SBE(IV)β-CD.

found that the SBE(IV)-substituted derivative of the β-CD provided the best overall separation efficiency and peak shape for all separations. Therefore, the SBE(IV)β-CD derivative was used for all subsequent studies. The effect of SBE(IV)β-CD concentration on the relative migration times of the C-terminus fragments of SP is shown in Fig. 5. Complete resolution of the C-terminus metabolites was obtained at concentrations above 5 m*M*. Due to the increased current above 5 m*M*, this was also determined to be the optimal concentration of additive.

An electropherogram of the C-terminus metabolites under optimal N-terminus conditions is shown in Fig. 6A. It can be seen that when no cyclodextrin is present in the BGE, there is a large amount of co-migration of peptides. For example, there is co-migration of SP 4–11 with the solvent peak, 5–11 with 7–11 and 2–11 and 3–11 with the parent SP peak. However, with the addition of 5 m*M* SBE(IV)β-CD, there is complete resolution of all five of the C-terminus metabolites as well as SP, as shown in Fig. 6B.

3.3. Combined separation of SP and all metabolites

The final step in this project was to combine the two individual separation methods and obtain a complete separation of SP and its metabolites. In order to evaluate the final BGE composition, the N-terminus and C-terminus metabolites were combined and injected into the CE under the optimized C-terminus separation conditions. It was found that all of the metabolites could be separated under these conditions, so no further optimization was necessary. Fig. 7 shows the final separation of SP and all of the metabolites under the final optimized run conditions of 150 m*M* boric acid, 15 m*M* phytic acid and 5 m*M* SBE(IV)β-CD at pH 7.

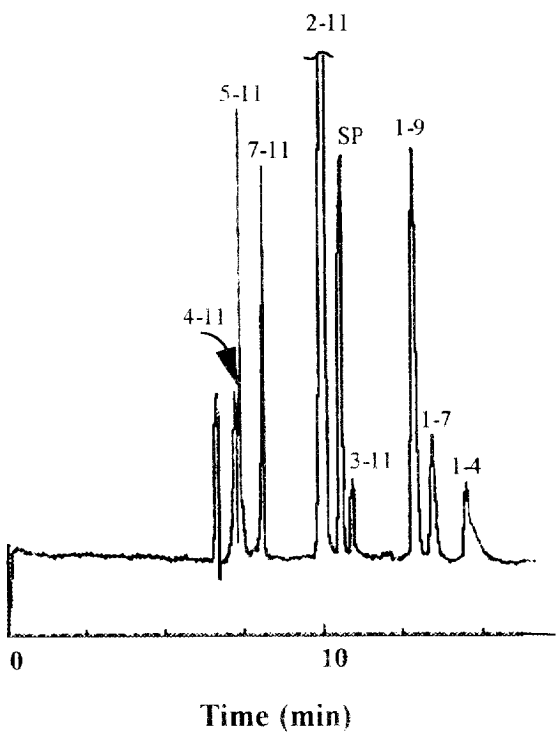

Fig. 7. Combined separation of SP and all of its N- and C-terminus metabolites. The final BGE consisted of 150 m*M* boric acid, 15 m*M* phytic acid and 5 m*M* SBE(IV)β-CD, pH 7.0. All other conditions were consistent with Fig. 5.

4. Conclusions

A complete CE-based separation of substance P and its metabolites was developed using a neutral pH and anionic additives. Future work will focus on the development of a post-column derivatization system to provide better sensitivity than is currently available with UV detection. The ultimate goal is the determination of SP and its metabolites in microdialysis samples.

Acknowledgments

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