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CAPILLARY ZONE ELECTROPHORESIS ANALYSIS AND DETECTION OF MID-SPECTRUM BIOLOGICAL WARFARE AGENTS

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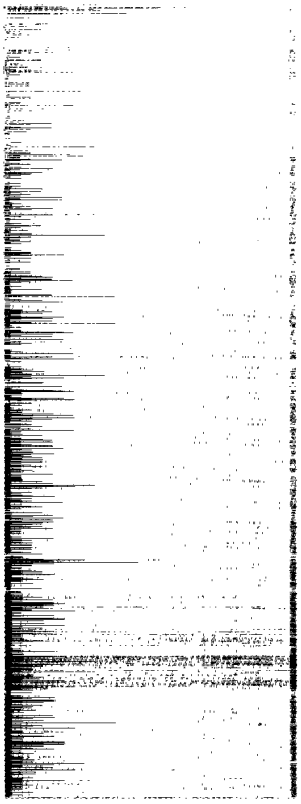
CAPILLARY ZONE ELECTROPHORESIS ANALYSIS AND DETECTION OF MID-SPECTRUM BIOLOGICAL WARFARE AGENTS

BY

CAMILLE A. BOULET and CAROL TOWNSLEY*



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CAPILLARY ZONE ELECTROPHORESIS ANALYSIS AND DETECTION OF MID-SPECTRUM BIOLOGICAL WARFARE AGENTS

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Camille A. Boulet and Carol Townsley*

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Camille A. Boulet and Carol Townsley, *Capillary Zone Electrophoresis Analysis and Detection of Mid-Spectrum Biological Warfare Agents*, Suffield Memorandum 1463, April 1995

EXECUTIVE SUMMARY

DRE Suffield has initiated a research program to develop methods and equipment for field detection and laboratory identification of mid-spectrum biological warfare agents, molecules of biological origin such as proteins, peptides and toxins. These mid-spectrum agents are often difficult to analyze and often require individually developed bio- or immunoassay methods for detection and identification. A chromatographic-based analytical system which could separate and detect all of the target compounds would represent an important advance in detection and identification capability. Additional or new mid-spectrum agents can be more easily accommodated in a chromatographic analytical system as these systems use a generic principle for detection rather than custom prepared immunoassays.

Capillary electrophoresis (CE) is an important, emerging technique for the separation and quantitation of peptides and proteins providing separation efficiencies up to two orders of magnitude greater than high performance liquid chromatography (HPLC). It can analyze a broad range of compounds, has a simple instrument design which can be fully automated, and has very low volume requirements for both sample and run buffers which would substantially reduce the maintenance and logistics burden for fielding a CE based instrument for biological detection . CE also uses a novel principle for liquid handling, electroosmotic flow, which requires no moving parts to transfer reagents that could be adapted to other analytical devices.

In this study, a highly efficient and reproducible capillary zone electrophoresis method was developed to separate and identify a series of nine peptides of defence interest: bradykinin, bradykinin fragment 1-5, substance P,[arg^8]-vasopressin, luteinizing hormone releasing hormone, bombesin, leucine enkephalin, methionine enkephalin, and oxytocin. Several of these peptides have been identified as potential mid-spectrum agents.

Three strategies, which could be used in a fully automated field detection and identification system, were demonstrated for the identification of unknown peptides: comparison of migration times, comparison of electrophoretic mobilities, and co-injection of multiple reference standards. These experiments demonstrate that a separation based analytical method such as capillary electrophoresis could form the basis of a generic detection system for mid-spectrum protein and peptide toxins.

TABLE OF CONTENTS

ABSTRACT

DRE Suffield has initiated a research program to develop methods and equipment for field detection and laboratory identification of mid-spectrum agents, molecules of biological origin such as proteins, peptides and toxins. In this study, a highly efficient and reproducible capillary zone electrophoresis method was developed to separate and identify a series of nine peptides of defence interest: bradykinin, bradykinin fragment 1-5, substance P,[arg^8]-vasopressin, luteinizing hormone releasing hormone, bombesin, leucine enkephalin, methionine enkephalin, and oxytocin. Using a 50 µm x 47 cm capillary column, 22.5 kV separation voltage and a 100 mM pH 2.5 phosphate buffer, all nine peptide could separated in under 10 minutes. Three strategies, which could be used in a fully automated field detection and identification system, were demonstrated for the identification of unknown peptides: comparison of migration times, comparison of electrophoretic mobilities, and co-injection of multiple reference standards. These experiments demonstrate that a separation based analytical method such as capillary electrophoresis could form the basis of a generic detection system for mid-spectrum protein and peptide toxins.

INTRODUCTION

DRE Suffield has initiated a research program to develop methods and equipment for field detection and laboratory identification of mid-spectrum agents, molecules of biological origin such as proteins, peptides and toxins. These mid-spectrum agents are often difficult to analyze and often require individually developed bio- or immunoassay methods for detection and identification. A chromatographic-based analytical system which could separate and detect all of the target compounds would represent an important advance in detection and identification capability. Capillary electrophoresis , which is highly sensitive, has high resolving power, and is capable of analyzing a broad spectrum of biomolecules, could allow for the detection of all mid-spectrum agents within a single analytical technology.

Mid-spectrum agents are typically bacterial, fungal, or animal toxins as well as human bioregulatory peptides which have very potent physiological effects. Some representative agents are listed in Table 1.

Table 1: Target proteins and peptides for detection as mid-spectrum agents.

Protein	Description	Molecular Weight
Botulinum Toxins (A-F)	protein	~150,000
Conotoxins	peptide	1438-2750
Ricin	protein	62,000
Shiga toxin	protein	60,000
Staphylococcal enterotoxins	protein	27800
Microcystin	peptide	994

Capillary electrophoresis (CE) is an important, emerging technique for the separation and quantitation of peptides and proteins(*1*)(*2*) (*3*)(*4*) providing separation efficiencies up to two orders of magnitude greater than high performance liquid chromatography (HPLC). It can analyze a broad range of compounds, has a simple instrument design which can be fully automated, and has very low volume requirements for both sample and run buffers (nanoliter injection volumes are typical). The analytical efficiency, with exceptional resolving power and peak efficiencies greater than 1,000,000 theoretical plates, is superior to many other chromatographic methods.(*5*) This high separation efficiency coupled with the small sample requirements, ease of automation, and the possibility of direct quantitation(*6*)(*7*)(*8*) are ideal for the development of a highly specific and sensitive detector for mid-spectrum agents.

CE employs narrow bore (10-200 µm i.d., 370 µm o.d.) polyimide coated capillaries to perform separations of molecules based on their mobilities in an electric field. The instrument is fairly simple (Figure 1) consisting of two buffer reservoirs connected via a hollow capillary column, a high voltage power supply, and a detection system.(*9*)

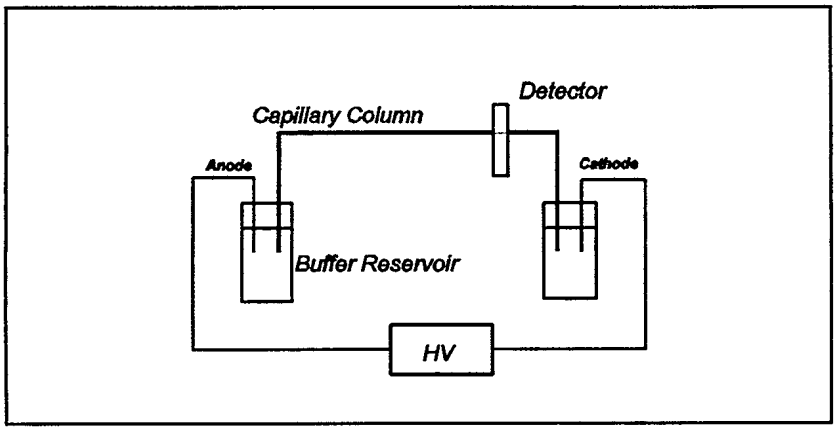

Figure 1: Generalized Capillary Electrophoresis Instrument

In a CE experiment a mixture of different analytes is introduced onto the column either electrokinetically, using an applied potential, or hydrodynamically, using pressure on the sample reservoir to force the sample into the capillary. A potential is then applied across the capillary and the analytes are induced to move (migrate) in this applied field. Because of differences in the effective mobilities, resulting in different migration velocities of different substances in an electric field, the mixture separates into spatially discrete zones of individual substances. The analytes are detected on-column through a small window on the capillary where the polyimide coating has been removed.(*10*)(*11*)

Capillary zone electrophoresis (CZE)(*12*) separation is based on differences in solute size and charge at a given pH which lead to differences in migration rates. CZE uses bare fused silica capillaries containing surface silanol groups which are partly ionized in the presence of the electrophoretic buffer medium or "run buffer" (pH range 2.5-10). This leads to the formation of a double layer of immobile cations tightly bound to the negative charges on the silica resulting in a potential difference (zeta potential) at the wall of the capillary. Adjacent to this layer is a layer of weakly bound cations and their sphere of hydration. When a potential is applied across the capillary, these solvated cations migrate towards the cathode resulting in a net flow of liquid known as electroosmotic flow (EOF). Because the EOF can be significantly greater than the electrophoretic mobilities of the individual ions in the sample, both anions and cations can be separated in the same run. Because the magnitude of the EOF can vary greatly depending on the pH of the run buffer, a stable and reproducible EOF is an important experimental consideration in method development.(*13*)(*14*)(*15*)

Any factors contributing to band broadening will decrease the efficiency and resolution of the separation. A major source of band broadening in CE is the heat generated by the passage of electrical current through a resistor which is known as Joule heat. The increase in temperature within the CE column depends on the power generated (Power = Voltage x Current) and is determined by the capillary dimensions, conductivity of the buffer and the applied voltage. Excessive heat causes non-uniform temperature gradients and changes in viscosity which results in band broadening. Thus an inherent advantage of performing CE in narrow-bore capillaries as compared to traditional electrophoresis is the rapid heat dissipation and reduction in the effects of heating. (*16*) It is evident that the efficiency can be increased by increasing the electric field but this will also result in increased Joule heating. Experimental parameters which can be optimized to limit Joule heating include decreasing the electric field (reducing applied voltage), reducing the capillary internal diameter, decreasing the buffer ionic strength or concentration, and active temperature control.

Identification[1] of components in CE can be made on the basis of absolute migration time, relative migration times or by measurement of an analyte's electrophoretic mobility. Measurement of the electrophoretic mobility can compensate for variability in the migration time due to unstable electric field, changes in buffer capacity or composition, temperature fluctuations, and changes to the capillary surface conditions.(*17*)(*18*)(*19*) The apparent electrophoretic mobility (μ_{app}) is characteristic for a given species at a certain pH and results from the sum of the mobility due to attraction to the cathode and the mobility due to the EOF. Apparent mobility can be calculated using

[1]In this report, the term identification refers to identification on the basis of reference electrophoretic data and comparison to authentic standards. Unambiguous identification requires spectrometric or spectroscopic data.

Equation 1. The apparent mobility is a result of the both attraction to the cathode and the EOF ($\mu_{app} = \mu + \mu_{EOF}$).

Equation 1

$$\mu_{app} = \frac{\nu}{E} = \frac{L_d}{t}\frac{L_t}{V}$$

ν = velocity of ion
L_d = length of capillary to detector
L_t = total length of capillary
t = migration time
V = applied voltage

The purpose of this study was to develop an efficient and reproducible capillary zone electrophoresis method for the analysis of bioactive peptides of defence interest. In addition, strategies for the detection and identification of these peptides based on migration time and electrophoretic mobilities were also examined.

EXPERIMENTAL

Sample preparation

Samples were dissolved in triple distilled, deionized water to a concentration of 1 mg/mL and serially diluted with 10% run buffer in water. Individual peptide standards, the nine peptide standard mixture, and Dynorphin A fragment 1-13, were obtained from Sigma Chemical Co. (St. Louis, MO). A 0.1 M phosphate buffer at pH 2.5 was used for all peptide separations.

Instrumentation

Samples were run on a Beckman P/ACE System 5010 CE unit (Beckman Instruments, Palo. Alto. Calif.). 57 cm x 75 µm i.d. (Beckman Instruments, Palo. Alto. Calif.); 57 cm x 50 µm i.d. capillary, 47 cm x 50 µm capillary (Polymicro Technologies) were used. Data acquisition, analysis, and instrument control were done with Beckman System Gold software.

Standard Conditions

Unless otherwise noted, all samples were run at 23 ^{0}C and were injected hydrodynamically for 5 seconds with 0.5 psi pressure (30 nL injection/ 57 cm x 75 µm i.d. capillary; 8.0 nL/ 47 cm x 50 µm capillary; 6.0 nL for the 57 cm x 50 µm i.d. capillary). UV detection was performed on-column (deuterium lamp, 200 nm).

Capillary Equilibration and Maintenance

Pre-run conditioning:

- 10 min with regenerator solution (0.1 M NaOH or 0.1 M HCl)
- 10 min with doubly distilled, deionized water
- 10 min with run buffer

Post sample rinses:

- 1 min with regenerator solution (0.1 M NaOH or 0.1 M HCl)
- 1 min with doubly distilled, deionized water
- 1 min with run buffer

Pre-storage rinses:

- 10 min with water
- 5 min with nitrogen gas

Peptide Separation Conditions

Capillary	47 cm x 50 μm i.d.
Voltage	22.5 kV
Injection	1.6 nl/sec x 5 sec = 8 nl
Run time	10 min

RESULTS AND DISCUSSION

A series of model peptides were chosen for development of the CZE method (Table 2). Although these peptides are commercially available they are also low-molecular weight peptides of defence interest. The effect of capillary dimensions and applied voltages were examined to develop a CZE method for the separation of all nine bioactive peptides in as rapid a run time possible while maintaining baseline separation for each peptide.

A number of bioactive peptides have been previously analyzed by CZE . Chen et al (*20*) examined the electrophoretic separation of a series of dipeptides and found that the reproducibility of the migration time was better in acidic buffer than in alkaline buffer due to fluctuations in the EOF at the higher pH. The CE analysis of bradykinin, LHRH, oxytocin, leucine enkephalin, methionine enkephalin and bombesin was examined by Johansson et al(*21*) using a 57 cm x 50 μm i.d. capillary and 20 mM ammonium formate pH 2.5 buffer. The use of low pH high ionic strength buffers, typically pH 2.5 and 50-100 mM concentrations with other constituents to adjust the ionic strength, have been shown to give good, reproducible separations. (*22*) For these experiments, a 100 mM, pH 2.5 phosphate buffer was selected. Samples were prepared in 10% buffer/water to maintain conductivity. This also minimizes sample dispersion and peak broadening through "stacking" conditions. In zones of lower conductivity, ie the sample plug, a higher electric field causes higher migration velocities. This causes the ions to stack at the buffer boundary giving higher peak

efficiencies. (*23*)(*24*)

Table 2: Bioactive peptide standards for CE analysis.

PEPTIDE	**SEQUENCE**	**FW** (free base)
Bradykinin	Arg-Pro-Pro Gly-Phe-Ser-Pro-Phe-Arg	1060.2
Bradykinin Fragment 1-5	Arg-Pro-Pro-Gly-Phe	527.7
Substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2	1347.6
[Arg^8]-Vasopressin	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2 (1-6 disulphide)	1084.2
Luteinizing Hormone Releasing Hormone	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2	1182.3
Bombesin	pGlu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2	1619.9
Leu Enkephalin	Tyr-Gly-Gly-Phe-Leu	555.6
Met Enkephalin	Tyr-Gly-Gly-Phe-Met	573.7
Oxytocin	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly (1-6 disulphide)	1007.2

The optimum applied voltage for a given capillary and buffer were determined using Ohm's law plots, a plot of current versus applied voltage. Because of the linear relationship between voltage and current, $V = I \times R$, a CE experiment obeying Ohm's Law should give a linear plot of voltage versus current. To determine the run voltage, the voltage was increased in a step-wise manner and the current monitored. The point where the plot begins to deviate from linearity was an indication that Joule heating is becoming a significant effect. Under practical conditions, slightly higher values were used without any perceptible change in separation efficiency.

Initially the analysis was run using a 57 cm x 75 μm capillary, 0.1 M phosphate buffer at pH 2.5 and 20 kV applied voltage (Figure 2). In this sample mixture, oxytocin is the last peptide to migrate at 16.2 minutes and baseline separation was obtained for each of the peptides. Hydrodynamic injection performance was tested by performing a series of nine 5 second injections of the individual peptides. Table 3 gives the migration time and peak area data for two representative peptides methionine enkephalin and leucine enkephalin.

Table 3: Reproducibility of migration time and peak area for 2 representative peptides .

Inj. No.	Methionine Enkephalin		Leucine Enkephalin	
	Mig. Time (min)	**Peak Area**	**Mig. Time** (min)	**Peak Area**
1	8.27	3.43	8.02	6.43
2	8.31	3.53	8.04	6.43
3	8.34	3.57	8.04	6.36
4	8.30	3.30	8.01	6.47
5	8.21	3.53	8.01	6.36
6	8.24	3.56	8.03	6.43
7	8.28	3.62	8.04	6.59
8	8.27	3.48	8.33	6.71
9	8.26	3.41	8.04	6.58
$\bar{x}$	8.28	3.49	8.06	6.48
$\sigma n-1$	0.040	0.097	0.10	0.12

The reproducibilities of the migration times and peak areas for electrokinetic and hydrodynamic injections were compared using substance P. The electrokinetic injections were performed for 5 sec at 10 kV while the hydrodynamic injections were performed for 5 sec using 0.5 psi of pressure. For each mode, the injections were repeated nine times and the migration time and peak area data are given in Table 4.

As is evident from this data, the reproducibilities of the migration times and peak areas are very good; hydrodynamic injections gave RSD for met enkephalin and leu enkephalin of 0.48% and 1.3% respectively. The electrokinetic injection of substance P gave better reproducibility for migration time, 0.09 % RSD, whereas the hydrodynamic injection gave much better quantitative data, 2.7 % RSD as compared to 11.3 % RSD for the electrokinetic injection. This is a result of a systematic sampling bias with electrokinetic injections where differences in the electrophoretic mobility of the solutes and the sample medium can result in differences in the amount of analytes loaded on the column.(*25*)(*26*)

Table 4: Electrokinetic and hydrodynamic injection for substance P

Inj. No.	Pressure Injection		Voltage Injection	
	Mig. Time (min)	**Peak Area**	**Mig. Time** (min)	**Peak Area**
1	5.99	3.30	5.86	15.01
2	6.00	3.31	5.86	14.90
3	5.97	3.51	5.86	13.74
4	5.97	3.43	5.85	14.06
5	5.97	3.30	5.85	12.65
6	6.00	3.35	5.85	12.48
7	5.97	3.48	5.85	12.09
8	5.97	3.52	5.85	11.26
9	5.97	3.46	5.86	11.01
x̄	5.98	3.41	5.85	13.02
σn-1	0.0136	0.093	0.00527	1.48

For this study, a detection limit was estimated for a representative peptide to give a reasonable injection volume and sample concentration for the method development. A serial dilution of bradykinin in water/buffer was prepared and injected hydrodynamically. Figure 3 shows the electropherogram from an injection of 40 pg (5 µg/mL) of bradykinin which gave a S/N of approximately 10 suggesting practical detection limit of approximately 10 pg (10^{-14} mol).

To further reduce the analysis time, a shorter, smaller diameter 50 µm x 47 cm (for more efficient heat dissipation) capillary as used. Because this capillary also provided less resistance, the voltage, as determined by an Ohm's Law Plot, was set to 22.5 kV. Figure 4 is the electropherogram of the separation of the nine peptide with oxytocin now migrating in under 10 minutes while still maintaining baseline separation for all of the peptides. This was considered acceptable for the purposes of this study and further improvements were not attempted. To ensure that there was no interaction between the peptides, the average migration times used for the identification experiments and calculation of the mobility data were determined using the mixed standard (Table 5).

To test the CZE method for detection and identification, a series of unknown peptide mixtures were prepared from the bioactive peptide standards. Three methods for identification were tested: identification by comparison of migration times, comparison of electrophoretic mobilities, and

co-injection of a standard mixture. Four unknown samples each containing 1-5 of the individual bioactive peptides were prepared by an independent analyst and analyzed by CZE using the optimized conditions (47 cm x 50 μm, 22.5 kV, 0.1 M phosphate buffer, pH 2.4) in order to identify the individual peptides.

Table 5: Average migration times (n=6) and electrophoretic mobilities of the bioactive peptides.

Peptide	Ave Mig Time (min)	σn-1 ($x 10^{-2}$)	%RSD
Bradykinin	5.55	5.55	1.79
Bradykinin Frag. 1-5	5.91	5.91	1.97
Substance P	6.15	6.15	2.26
Vasopressin	6.62	6.62	2.26
LHRH	7.06	7.06	3.39
Bombesin	8.02	8.02	3.87
Leu Enkephalin	8.76	8.76	3.87
Met Enkephalin	9.03	9.03	4.23
Oxytocin	9.76	9.76	4.62

Initial comparison of the migration of the unknown samples and the migration times of the standard peptides was sufficient for identification within this test sample (Table 6). Because of the excellent reproducibility of the migration times, automatic identification of the components was possible using the system software (Figure 5a). For this mixture, the commercial software correctly identified all the peptides by comparison to the reference migration times.

The unknowns were also identified by co-injection of a multiple internal standard. The Beckman P/ACE 5010 autosampler allows for multiple injections from different reservoirs thus allowing for the injection of a standard without premixing which preserves the original sample for further analyses. The applied voltage then causes the individual analytes to focus ("stack") into individual bands and co-migrate without affecting the electrophoretic separation. In this experiment, the unknown sample was hydrodynamically injected onto the CE column followed the nine peptide mixture as a multiple internal standard. A large concentration difference between the sample and the

known peptide mixture was used to clearly differentiate the unknown from the standards. This technique readily identified the unknown peptides as can be seen in Figure 5 b.

Table 6: Migration times and identity of peptides in unknown samples.

STANDARD PEPTIDE (Average Migration Time, min)		**Unknown Sample** (min)			
		1	**2**	**3**	**4**
Bradykinin	5.55		5.59	5.56	
Bradykinin Frag. 1-5	5.91				
Substance P	6.15	6.14			6.19
Vasopressin	6.62		6.67		
LHRH	7.06	7.05	7.13		
Bombesin	8.02				8.11
Leu Enkephalin	8.76	8.77		8.83	
Met Enkephalin	9.03	9.06		9.12	
Oxytocin	9.76	9.79		9.86	

The electrophoretic mobility of the bioactive peptides at a given pH can also be used to identify the unknowns. Because at pH 2.5, the EOF is virtually suppressed and difficult to determine experimentally,(27) the experimental electrophoretic mobilities of the nine peptides were determined using an internal standard, Dynorphin A fragment 1-13. The apparent mobility (μ_{app}) of the reference peptide Dynorphin was found to be 3.05×10^{-4} ($\mu_{Dyn} = L_d \times L_t / t \times V$; L_d=40 cm , L_t=47 cm, t=274.26 sec, V= 22500 Volts). For each peptide, the apparent electrophoretic mobility was calculated and an electrophoretic mobility relative to the reference peptide determined ($\mu_{rel} = \mu_{app} - \mu_{Dyn}$). The electrophoretic mobilities for the nine bioactive peptides are given in Table 7.

The use of Dynorphin A_{1-13} as an internal standard for identification of the peptides was tested with Unknown 1 and Unknowns 2 and 4 which were combined prior to analysis. Table 8 gives the migration times, apparent electrophoretic and relative electrophoretic mobilities for these analyses . The unknowns could also be identified by comparing the electrophoretic mobilities to reference data (Table 7). All three methods described were able to identify the components of the unknown mixtures. Instead of a multiple internal standard experiment, Dynorphin can be co-injected as the internal standard in an automated analysis for calculation of the relative electrophoretic mobilities.

Table 7: Electrophoretic mobilities of nine bioactive peptides.

Peptide	**Mig Time** (min)	μ_{app} (10^{-4} cm^2/ V s)	μ_{rel} (10^{-4} cm^2/ V s)
Bradykinin	5.55	2.52	-0.53
Bradykinin Frag. 1-5	5.91	2.36	-0.69
Substance P	6.15	2.26	-0.79
Vasopressin	6.62	2.10	-0.95
LHRH	7.06	1.97	-1.08
Bombesin	8.02	1.74	-1.31
Leu Enkephalin	8.76	1.59	-1.46
Met Enkephalin	9.03	1.54	-1.51
Oxytocin	9.76	1.43	-1.62

Table 8: Electrophoretic mobilities (internal standard) for identification of unknown peptides.

Peptide	**Unknown 1**			**Unknown 2/4**		
	t (min)	μ_{app}	μ_{rel}	**t** (min)	μ_{app}	μ_{rel}
Dynorphin	4.59	3.03		4.6	3.03	0
Bradykinin				5.51	2.53	-0.5
Brady. Fr						
Sub P	6.11	2.28	-0.75	5.88	2.37	-0.66
Vasopressin				6.6	2.11	-0.92
LHRH	7.03	1.98	-1.05	7.05	1.98	-1.05
Bombesin				8.04	1.73	-1.3
Leu Enk	8.75	1.59	-1.44			
Met Enk	9.03	1.54	-1.49			
Oxytocin	9.79	1.42	-1.61			

CONCLUSIONS

Capillary electrophoresis is highly sensitive, has high resolving power, and is capable of analyzing a broad spectrum of biomolecules. In this study, a highly efficient and reproducible capillary zone electrophoresis method was developed to separate and identify a series of peptides of defence interest. Three strategies, which could be used in a fully automated field detection and identification system, were demonstrated for the identification of unknown peptides: comparison of migration times, comparison of electrophoretic mobilities, and co-injection of multiple reference standards. These experiments demonstrate that a separation based analytical method such as capillary electrophoresis could form the basis of a generic detection system for mid-spectrum protein and peptide toxins.

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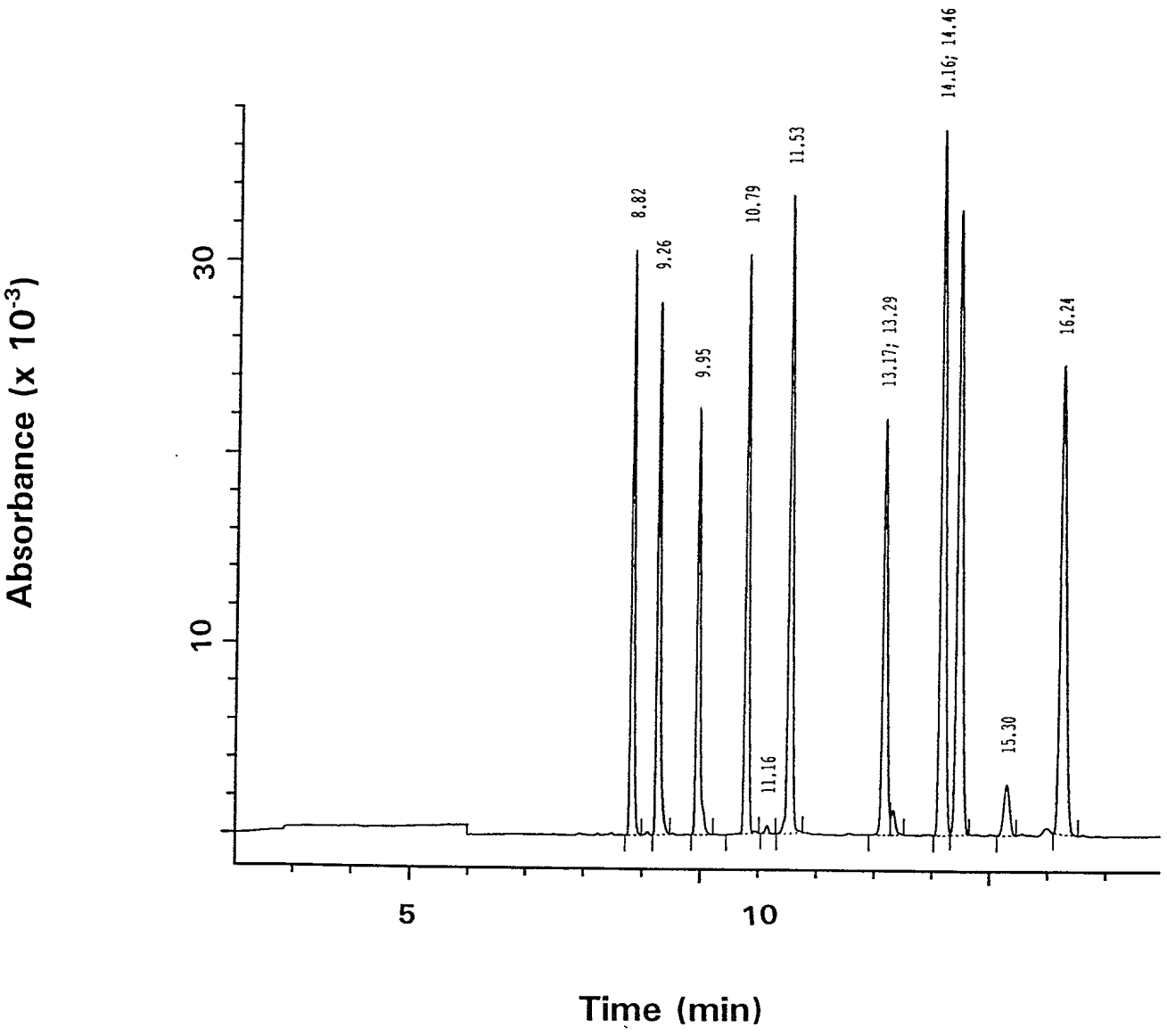

Figure 2: Electropherogram of nine bioactive peptides as analyzed using a 75 µm x 57 cm capillary column (20 kV, peptide concentration 0.5 mg/mL, 30 nL injection volume).

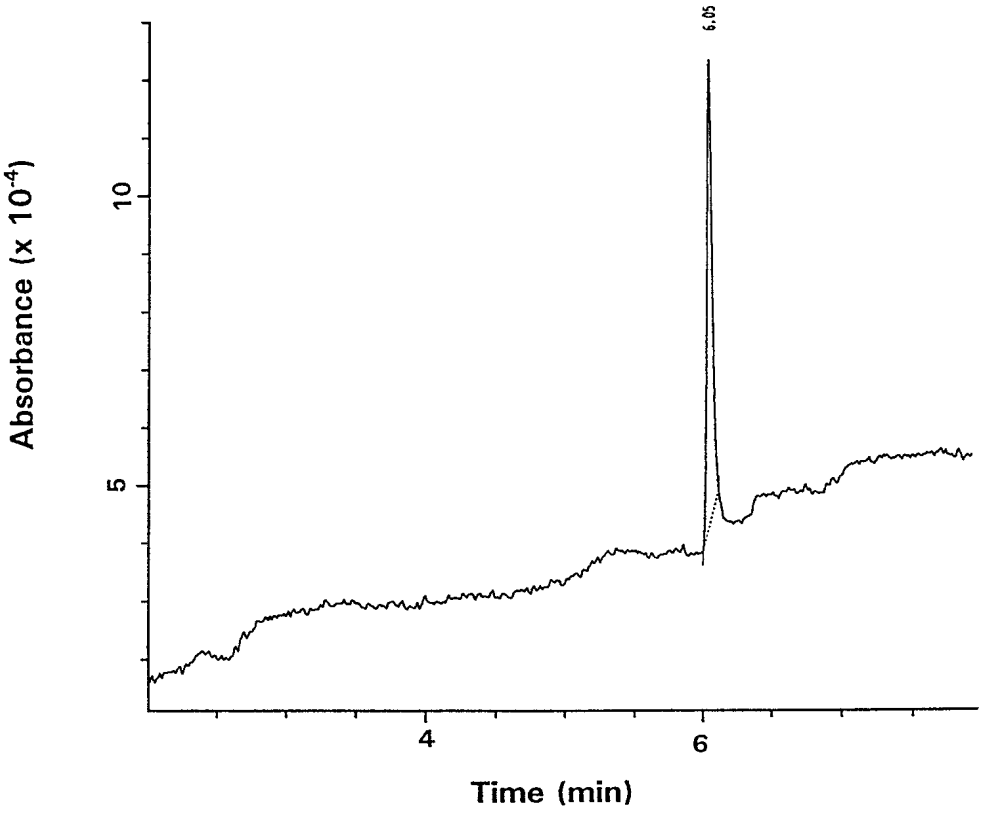

Figure 3: Determination of a practical detection limit for bradykinin using a 50 µm x 47 cm capillary, 22.5 kV, 100 mM phosphate buffer pH 2.5. Electropherogram for a 5 µg/mL (40 pg injected) sample.

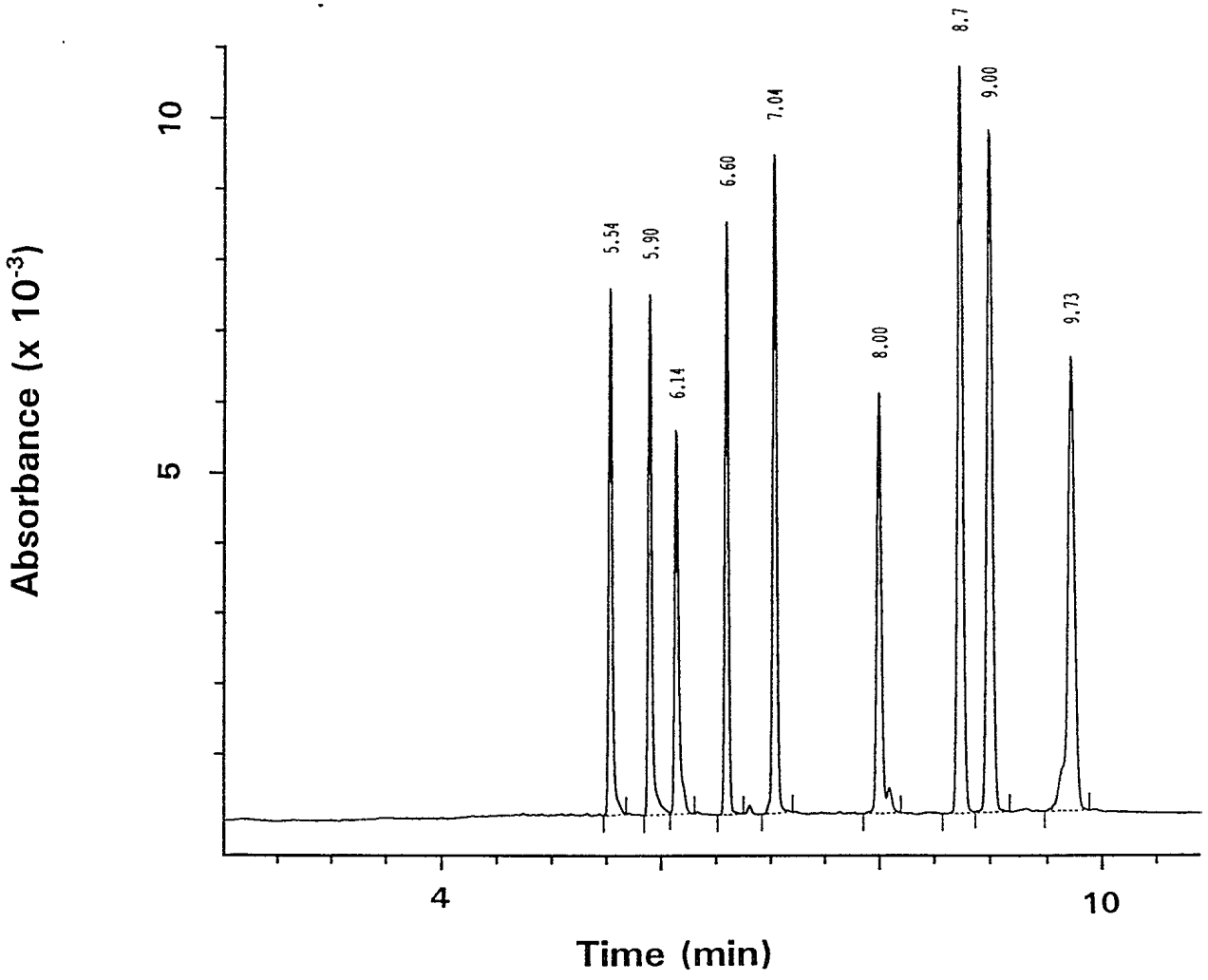

Figure 4: Electropherogram for the optimized analysis of the nine bioactive peptides using a 50 µm x 47 cm capillary column (22.5 kV, peptide concentration 0.5 mg/mL, 8 nL injection volume).

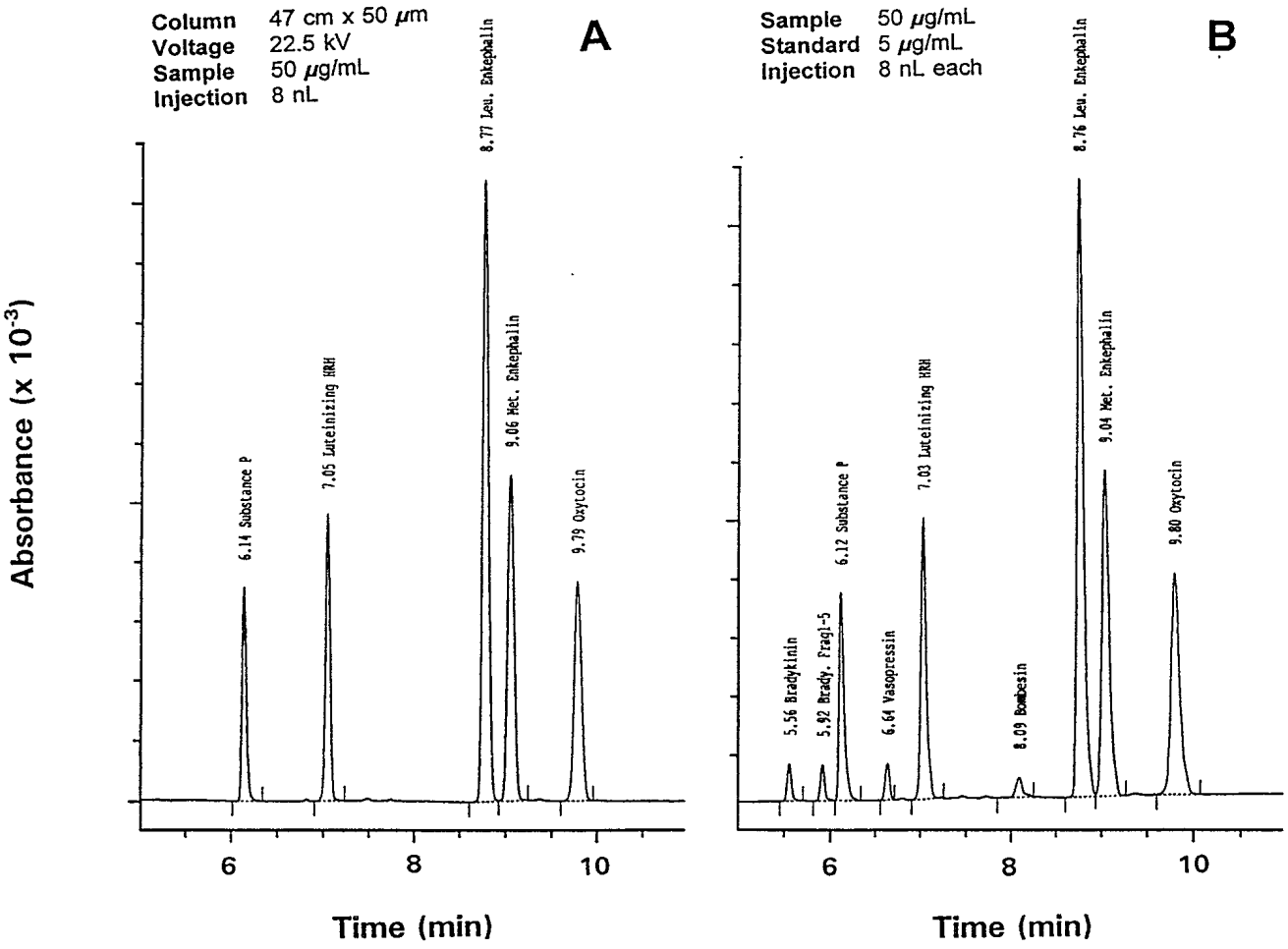

Figure 5: CZE analysis of unknown peptide mixtures: A) electropherogram of Unknown 1 and automatic identification of the components on the basis of migration time (sample concentration 50 µg/mL; and B) co-injection of the peptide standards (standard concentration 5 µg/mL) using standard peptide analysis conditions.

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