Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Chromatography A, 1111 (2006) 106–111
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Measurement of neuropeptides in clinical samples using chip-based immunoaffinity capillary electrophoresis☆

Terry M. Phillips*, Edward Wellner

Ultramicro Analytical Immunochemistry Resource, Division of Bioengineering and Physical Science, Building 13, Room 3N15, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892, USA

Received 21 June 2005; received in revised form 23 January 2006; accepted 24 January 2006
Available online 10 February 2006

Abstract

The current interest in micro-fabrication has extended to the clinical arena where there is a growing lobby for promoting these for point-of-care purposes. The advantages of such devices are their relative speed of analysis, lower reagent costs, and their application to clinical screening and diagnosis. Two chip-based capillary electrophoresis systems have been designed and their performance evaluated for rapidly measuring the concentrations of inflammatory neuropeptides in tissue fluids of patients with neuropeptide-associated muscle pain. Both chips were manufactured to fit a commercially available chip electrophoresis system. One chip was designed to perform electrokinetic flow immunoassays while the other utilized an immunoaffinity port, containing an array of immobilized antibodies, to capture the analytes of interest. Comparison of the results to commercially available high-sensitivity immunoassays demonstrated that both chip-based systems could provide a relatively fast, accurate procedure for studying inflammatory biomarkers in complex biological fluids. However, the immunoaffinity capture system proved the superior of the two chips. Using this system, twelve different inflammation-associated mediators could be determined in approximately 2 min as compared to 30 min when using the flow immunoassay chip. With the ever-expanding array of antibodies that are commercially available, this chip-based system can be applied to a wide variety of different analyses.

Keywords: Chip-based CE; Immunoassay; Neuropeptides; Inflammatory cytokines; Muscle pain

1. Introduction

Tissue injury induces the secretion of a number of mediators resulting in localized inflammation and pain. Immune mediators such as interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor-alpha (TNF-α) result in localized tissue edema leading to pressure on nerve endings in the immediate vicinity and release of neural mediators. Further, it has been documented by a number of investigators that neuropeptides such as substance P (SP), calcitonin gene-related peptide (CGRP) and vasoactive intestinal peptide (VIP) are responsible for the induction of localized pain and can regulate the local inflammatory response [1–6]. These events can lead to sustainable injury causing, in the most severe cases, loss of function. Muscle injury is a prime example of localized tissue injury involving neuropeptides [7–9] that can lead to a spectrum of conditions ranging from mild discomfort to restricted use of a limb. Measurement of these inflammatory markers could become a useful diagnostic tool for determining not only the extent of the tissue injury but also the efficacy of treatment. However, a major disadvantage of measuring these mediators is the need for sampling techniques capable of obtaining samples within the site of tissue injury. Peripheral concentrations of most cytokines and neuropeptides bear little resemblance to the events taking place at the actual injury site. In order to overcome this problem, investigators have devised a number of different techniques ranging from in-situ microdialysis [10,11] to tissue biopsy. No matter, which approach, is used, the sample available for analysis is usually extremely small requiring specialized procedures to analyze multiple analytes.

Capillary electrophoresis (CE) is a suitable technique for applying to small sample analysis because of its ability to analyze multiple analytes within a single sample. When coupled to the selective power of immunoaffinity extraction this technique becomes even more useful. Immunoaffinity CE has been

☆ Presented at the 28th International Symposium on Capillary Chromatography and Electrophoresis, Las Vegas, NV, 22–25 May 2005.

* Corresponding author. Tel.: +1 301 435 2737; fax: +1 301 496 6608.
E-mail address: phillipt@mail.nih.gov (T.M. Phillips).

0021-9673/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2006.01.102

successfully applied to measurement of cytokines and other analytes in a variety of human biofluids [12–14]. Immunoaffinity CE holds several advantages over other analytical techniques in that it is able to analyze samples of 1 μL or less, when direct capillary injection is employed. Additionally, detection of the selected analytes can be performed with high sensitivity, when laser-induced fluorescence (LIF) detection is used. A drawback to standard CE with immunoaffinity extraction is the time frame required to perform analysis of cytokines and neuropeptides; analyses taking up to 1 h to complete.

The development of micro-fluidic devices enabling chip-based CE to be performed has greatly improved separation time [15–18]. In the present study, two chip-based CE systems, one with a pre-separation immunoextraction port and the other a electrokinetic immunoassay system, were evaluated for their ability to measure multiple inflammation-associated neuropeptides and cytokines in muscle fluid samples from patients with myofascial pain.

2. Materials and methods

2.1. Reagents

Recombinant human neuropeptides (substance P (SP), calcitonin gene-related peptide (CGRP), brain derived neurotrophic factor (BDNF), vasoactive intestinal peptide (VIP), neuropeptide Y (NY), neurotrophic factor-4 (NT-4), β-endorphin, adrenocorticotropic hormone (ACTH), corticotropin releasing hormone (CRH)) and their reactive antibodies were obtained from Bachem (King of Prussia, PA, USA). Recombinant human cytokines (interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumour necrosis factor-alpha (TNF-α)) and their corresponding anti-cytokine antibodies were obtained from R&D Systems (Minneapolis, MN, USA). All of the neuropeptides and cytokines were reconstituted to stock solutions of 1-μg/ml in 0.1M phosphate buffer, pH 7.4. Sulfosuccinimidyl 4-(*N*-maleimido-methyl) cyclohexane-1-carboxylate (SSMCC), ImmunoPure $F(Ab)'_2$ preparation kit, and Cleland's reagent were purchased from Pierce Biotechnology (Rockford, IL, USA). All other chemicals were purchased from Acros Chemicals, Inc. (Fisher Scientific, Pittsburgh, PA, USA). Immediately prior to use, all solutions were passed through 0.2-μm nitrocellulose filters (Millipore, Bedford, MA, USA).

2.2. Instrumentation

The CE studies were performed on a Micralyne μTK microfluidic electrophoresis system (Micralyne, Edmonton, Canada) equipped with eight platinum electrodes and a 635-nm, 8-mW red diode laser. Detection was achieved using a epiluminescence confocal microscope coupled with a Hamamatsu H5773-03 photomultiplier tube and 16-bit data acquisition. The system was equipped with a chip stage form-fitted to accept Microlyne standard 16 mm × 95 mm × 2.2 mm deep CE chips. The entire system was run on a personal computer running Microsoft Windows 2000 with a compiled LabView interface.

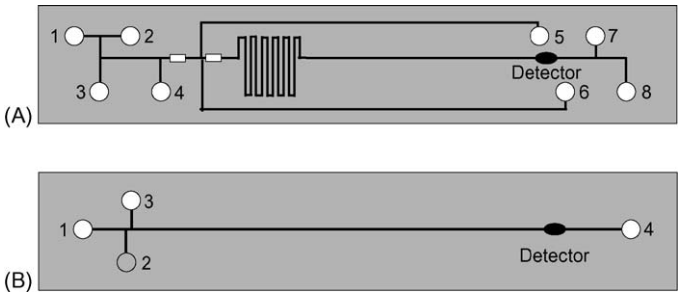

Fig. 1. Diagrammatic representation of the two chip formats. (A) Electrokinetic lab-on-a-chip format and (B) immunoaffinity format. The different ports in (A) are labeled as described in Section 2.6 and the ports in (B) are labeled as described in Section 2.7.

2.3. Analytical chip formats

Low fluorescence "Borofloat" glass CE chips were purchased from Micralyne as either custom-designed laboratory-on-a-chip (lab-on-a-chip) format (Fig. 1A) or as standard chip format with two 20 μm deep × 50 μm wide semi-circular channels arranged in a cross formation (Fig. 1B). In both formats, the channels ended in a 2.0 mm diameter × 0.1 mm deep port, which was used for loading samples or buffers into the chip. The lab-on-a-chip format had a serpentine separation channel of either 81 mm (75 mm to the detector) to mimic the separation channel length of the standard chip or 120 mm (110 mm to the detector). The standard chip possessed a total separation channel of 80.9 mm (75.0 mm to the detector) from the intersection of the sample loading channel to port 4 and a sample-loading channel of 9.64 mm. To aid loading and manipulation of reagents, Upchurch NanoPort assemblies (Upchurch Scientific, Oak Harbor, WA, USA) were attached to each port. In the present studies, the lab-on-a-chip format was used for electrokinetic flow immunoassays while the standard cruciform chip was used for immunoaffinity CE.

2.4. Preparation of the immunoaffinity chip

The immunoaffinity chip was prepared in a similar manner to that previously described [18]. Briefly, ports 1, 3, and 4 were plugged to minimize leakage of reagents from port 2. The inner surface of the port was modified by introducing 0.1 μL of a 10% aqueous solution of 3-aminopropyltriethoxysilane, sealing with adhesive tape, and incubating the chip at 100 °C for 60 min. Following this incubation the fluid was removed and the port filled with fresh silane solution. This treatment was repeated four times before filling the port with 10 mM hydrochloric acid and incubating at 100 °C for a further 60 min. The port was washed twice in distilled water and filled with 0.1 μL of a solution containing 1 mg/mL SSMCC dissolved in 50 mM sodium borate, pH 7.6, and incubated at 30 °C for 60 min. Finally, the port was flushed with 50 mM borate, pH 9.5. $F(Ab)'_2$ fragments were prepared from each of the reactive antibodies using the Pierce ImmunoPure $F(Ab)'_2$ preparation kit according to the manufacturer's instructions. The fragments were further reduced to monovalent FAb fragments by incubation with equal volumes

of 200 mM Cleland's reagent and equal volumes of each FAb were mixed together prior to being immobilized in the immunoextraction port (port 2) of the chip via their free sulfhydral groups. Following the FAb coating, the port was flushed three times with 100 mM phosphate buffer, pH 7.4, ports 1, 3 and 4 unplugged and the chip mounted onto the CE stage.

2.5. Standards and patient samples

Standards of 10, 50, 100, and 500 pg/mL were prepared for each analyte by dissolving a stock solution containing 1 mg/mL of each recombinant analyte in 0.1 M phosphate buffer, pH 7.4. These standard solutions were used to construct calibration curves from which analyte concentration in the patient samples were calculated.

Tissue fluid samples were obtained via needle aspiration from healthy volunteers and patients with mild or intense muscle pain. Samples were obtained pre and post treatment with intramuscular injections of the anti-inflammatory agent, cortisol. The subjects were divided into three groups, each of 12 subjects. Group 1: 12 normal volunteers; Group 2: 12 subjects with mild complaints of muscle pain; and Group 3: 12 subjects with severe muscle pain and/or diminished muscle function. In the third group, samples were collected every 5 min, from the affected muscle, to study the kinetics of mediator release. Consent to use the samples were obtained from the subjects and no name indicators were assigned to the samples. Approximately 10-μL samples were collected in sterile tubes containing a cocktail of protease inhibitors from a cohort of 16 males and 20 females (aged 21–45 years) seen at a muscle pain clinic at the George Washington University Medical Centre, Washington, DC, USA. The samples were passed through a 50 kDa cut-off ultramicro Spin Con dialyzer (The Nest Group, Southborough, MA, USA) to remove extraneous macromolecules prior to analysis. Each sample was analyzed by direct spectrophotometry at 280/260-nm using a NanoDrop ND-1000 micro spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) for total protein content and adjusted to a protein concentration of 1 μg/mL in 100 mM phosphate buffer, pH 7.4 prior to analysis.

2.6. Electrokinetic flow immunoassay

The sample was introduced into port 1 and AlexaFluor633 laser dye (Molecular Probes) labeled antibody was placed in port 3. Port 2 was filled with 100 mM phosphate buffer, pH 7.0, as were all of the chip channels by introducing the buffer through port 8. In the present assay format, ports 5 and 6 were permanently plugged and remained unused. Port 4 was used as a mixing chamber and port 7 as a waste chamber when stacked samples were run. Approximately 100 nL of labelled antibody was moved to port 4 by maintaining 1 kV between ports 3 and 4 for 1 min. Once this had been achieved, approximately 100 nL of sample was moved to port 4 by placing 1 kV between ports 1 and 4 for 1.2 min. These parameters were developed from experiments using either labelled antibody or normal human serum to measure the amounts of materials migrating from one port to another under the influence of a defined electrical current. The port receiving the sample was filled with 500 nL of buffer prior to the electrokinetic transfer and increases in volume measured at 10 s intervals using a calibrated 1 μL syringe pipette attached to a Picoliter Microinjection System (Harvard Scientific Apparatus, Holliston, MA, USA). It was calculated that a pulse of 1 kV for a duration of 1 min transferred 96.4 ± 0.6 nL of labelled antibody and that a pulse of 1 kV for 1.2 min transferred 94.9 ± 1.3 nL of normal human serum. These values were calculated from 10 repetitive injections for both solutions. The mixture in port 4 was allowed to react for 2 min before separation and on-line detection. Separation was achieved by applying 6 kV between ports 4 and 8; the immune complex formed by interaction between the labeled antibody and its antigen being detected as the primary peak while free antibody was detected as a secondary peak. By alternating between port 4 and ports 7 and 8 it was found that up to four stacked samples could be run before the chip needed cleaning.

2.7. Immunoaffinity immunoassay

Polyether ether ketone (PEEK) tubing (50 μm I.D., 360 μm O.D., Upchurch Scientific) was attached to all 4 ports and the system channels filled with 100 mM phosphate buffer, pH 7.0 by pumping at a flow-rate of 1 μL/min through initially port 1 and then port 3. This was achieved using a Harvard syringe pump (Pump "11" – Harvard Scientific Apparatus). Once the system channels were filled, the PEEK lines were removed and ports 1, 3, and 4 filled with the same buffer. Five hundred nanoliters of sample was introduced into port 2 and allowed to remain in contact with the immobilized FAb fragments for 5 min. During this time, the immobilized FAb's interacted with and bound their respective analytes. In the present studies, the amounts of each analyte present in the patient's samples was unknown, and to ensure that the maximum amount of analyte was captured the immunoextraction port was built with a potential 50-fold excess of immobilized FAb. This was based on previous experience (data not shown) that demonstrated a minimal ratio of antibody to analyte of 50:1 was required for efficient analyte capture in clinical samples. Following the incubation, the sample was recovered and 500 nL of a 1 μg/mL solution of AlexaFluor 633 laser dye dissolved in 100 mM phosphate buffer was introduced into the port and incubated for a further 5 min. The dye solution was removed and the port washed five times by introducing 500 nL of phosphate buffer. Finally, the port was filled with 100 mM phosphate buffer, pH 1.5 to elute the captured analytes and the CE electrodes placed into all of the four ports. Using the LabView interface, the CE run was programmed to electrokinetically introduce 100 nL of sample into the intersection of the sample arm with the main separation channel. This was achieved by applying a 1 kV potential between ports 2 and 3 for 8 s while keeping ports 1 and 4 at ground. Applying a 6 kV potential between ports 1 and 4 then separated the injected sample, the components of which was detected on-line by the LIF detector. To aid in comparison of the two systems, both the electrokinetic and immunoaffinity assays were run in 100 mM phosphate buffer, pH 7.0 at 6 kV during the separation phase of the assay. All assays were run at room temperature.

In both assay formats, the individual concentrations of each analyte was calculated by comparison of the area under the curve to that similar values generated by constructing a calibration curve from known standards run under identical conditions.

3. Results

Assay parameters such as lower limit of detection (LOD), recovery and precision as well as intra- and inter-assay variance were examined using known concentrations of each analyte. The lower limit of detection was calculated by running dilutions of each analyte in both systems until they could no further be detected. The electrokinetic system exhibited LODs of 1.6, 1.8, 2.2, 1.7, 2.3, 2.4, 1.6, 2.2, 3.4, 1.1, 1.3, 1.1 pg/mL for SP, CGRP, BDNF, VIP, NY, NT-4, β-endorphin, ACTH, CRH, IL-1β, IL-6, and TNF-α, respectively. The immunoaffinity system exhibited LODs of 0.7, 0.9, 1.4, 2.0, 1.5, 0.7, 1.6, 1.9, 2.0, 0.6, 1.2, 0.8 pg/mL for the same order of analytes. Saturation studies demonstrated that both assay formats had a greater than 1.6 ng/mL binding capacity. The recovery and precision of the two chip-based assays were reasonable and well within limits required for clinical diagnosis. All analytes could be recovered by both systems at 95.4% or above (Table 1) with intra-assay and inter-assay RSDs between 4.21 and 6.68% for both systems.

Chip design was found to be an important issue especially in the electrokinetic assay system. The length of the separation channel greatly dictated the efficiency of the system in its ability to separate complexed antibody-analyte complexes from non-complexed labelled antibody. Chips with short separation channels (equivalent to the immunoaffinity chip) failed to adequately separate these two components thus making measurement of the analyte of interest impossible. Lengthening the separation channel from 81–120 mm resolved this problem but greatly lengthened the analysis time, making the electrokinetic assay format relatively slow when compared to the immunoaffinity capture system. As shown in Fig. 2, the immune complex containing anti-β-endorphin bound to its specific analyte took approximately 200 s to resolve and the excess free-labeled antibody took a further 80 s to resolve. In comparison, as shown in Fig. 3, the immunoaffinity capture technique resolved β-endorphin at ∼100 s and all 12 analytes in 160 s. Even with stacked loading of the lab-on-a-chip system, it was found that no more than four analytes could be fully processed in a 10 min time-frame, whereas the other system could resolve all of the analytes within 5 min including incubation and elution time. This ability to monitor multiple analytes simultaneously was found to be an added advantage. Further, the lab-on-a-chip system required the entire sample to complete all twelve assays whereas the immunoaffinity chip system could measure all of the analytes in a 100-pL sample, thus saving the remainder of the precious samples for further studies.

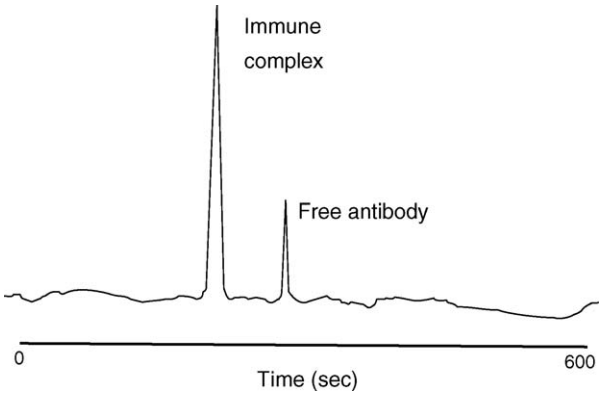

Fig. 2. Electropherogram of an anti-β-endorphin immunoassay performed in the electrokinetic immunoassay lab-on-a-chip format according to the procedure described in Section 2.6.

The Micralyne system is reasonably portable especially when the detector is connected to a laptop computer. The whole apparatus easily fits on a standard laboratory cart and can be used at both point-of-care and in operating room annexes. Using the immunoaffinity chips, we found that we could process between 10–12 samples per hour when using the chips once. Cleaning the chips was tedious and presented a disadvantage to the system. Likewise, labeling the captured analytes in-situ considerably

Table 1
Analyte recovery in the two chip-based systems

Analyte	Amount added (pg/mL)	Electrokinetic assay Amount recovered (pg/mL)	Immunoaffinity assay Amount recovered (pg/mL)
SP	100	97.5 ± 0.9	98.2 ± 0.8
CGRP	100	97.1 ± 1.3	97.5 ± 1.1
BDNF	100	98.3 ± 0.9	98.1 ± 0.6
VIP	100	97.6 ± 1.5	97.3 ± 1.3
NY	100	97.2 ± 1.3	97.7 ± 0.9
NT-4	100	98.5 ± 1.7	98.4 ± 1.4
β-Endorphin	100	95.9 ± 1.2	96.8 ± 0.8
ACTH	100	96.2 ± 0.8	97.7 ± 1.2
CRH	100	98.2 ± 1.1	97.8 ± 1.4
IL-1β	100	96.4 ± 0.8	98.3 ± 1.0
IL-6	100	96.9 ± 1.3	98.2 ± 1.5
TNF-α	100	97.8 ± 1.6	98.5 ± 0.9

All values ± S.E.M. Represents 10 replicates.

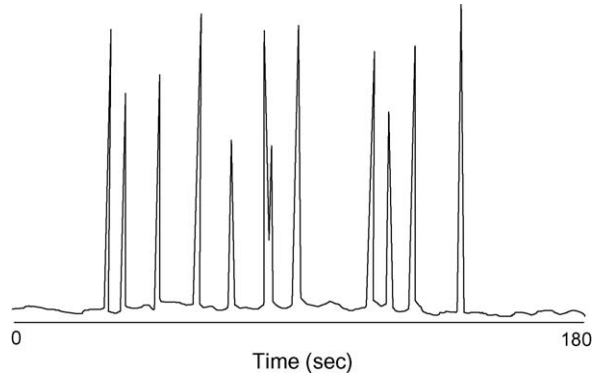

Fig. 3. Multi-analyte electropherogram produced by the immunoaffinity chip as described in Section 2.7. Peaks in order of appearance from left to right: SP, CGRP, BDNF, IL-1β, VIP, NY, NT-4, β-endorphin, ACTH, CRH, IL-6, and TNF-α.

Table 2
Concentrations of the 12 inflammation-associated markers in the three study groups as measured by the two chip-based CE systems

Analyte	Group 1	Group 2	Group 3
Electrokinetic system			
SP	10.9 ± 1.3	68.7 ± 14.5	295.5 ± 49.7
CGRP	12.6 ± 1.9	70.6 ± 12.4	202.1 ± 52.6
IL-1β	11.8 ± 1.7	71.2 ± 13.5	240.6 ± 51.8
IL-6	13.6 ± 1.9	49.8 ± 18.1	271.5 ± 57.5
TNF-α	10.2 ± 1.4	80.3 ± 14.1	329.7 ± 52.7
β-Endorphin	11.6 ± 2.8	65.9 ± 10.2	300.4 ± 48.9
VIP	9.2 ± 1.3	17.5 ± 10.8	81.5 ± 18.6
NY	14.5 ± 1.1	10.6 ± 4.9	110.8 ± 28.3
NT4	12.1 ± 1.8	15.4 ± 6.2	68.8 ± 14.8
ACTH	10.2 ± 1.6	15.9 ± 3.5	120.4 ± 26.1
CRH	16.1 ± 2.1	14.6 ± 5.2	91.7 ± 14.5
BDNF	8.4 ± 0.7	10.4 ± 2.9	73.6 ± 21.3
Immunoaffinity system			
SP	12.4 ± 1.1	65.3 ± 12.1	280.5 ± 52.2
CGRP	10.4 ± 2.3	73.2 ± 10.8	215.8 ± 49.4
IL-1β	14.6 ± 2.1	68.5 ± 15.4	235.4 ± 56.3
IL-6	10.4 ± 1.6	53.9 ± 16.5	280.1 ± 60.1
TNF-α	13.5 ± 1.9	77.4 ± 10.8	315.9 ± 49.7
β-Endorphin	8.6 ± 2.4	68.2 ± 14.4	290.6 ± 51.3
VIP	6.5 ± 1.6	21.4 ± 11.7	90.4 ± 20.6
NY	10.3 ± 0.9	13.1 ± 8.2	106.3 ± 24.4
NT4	8.8 ± 1.3	12.6 ± 5.5	88.1 ± 18.5
ACTH	6.7 ± 0.7	15.5 ± 2.9	116.7 ± 22.7
CRH	11.1 ± 2.6	10.3 ± 4.7	95.3 ± 16.2
BDNF	5.2 ± 0.8	8.1 ± 3.5	68.8 ± 18.9

All of the values are expressed in pg/mL and represent the pre-treatment concentrations of each marker.

shortened the life and usefulness of the immunoaffinity port as the laser dye bound to the antigen binding sites of free antibodies thus preventing them from capturing further analyte. This could be circumvented by labeling the sample prior to immunoaffinity extraction thus preventing loss of reactive antibody activity through steric hindrance of the dye. In other studies (data not shown) we have determined that the immunoaffinity ports can be regenerated, following electroelution, up to 25 times before they lose their binding capacity. This greatly enhances the life of the immunoaffinity chip and greatly reduces the cost of each analysis.

Examination of the three study groups demonstrated that there were clear differences between the normal subjects and those with muscle pain (Table 2). The pre-treatment samples from subjects with mild (group 2) or severe pain (group 3) expressed elevated concentrations of the neuropeptides SP, CGRP, and β-endorphin as well as the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α. The other neuropeptides demonstrated concentrations similar to those of the normal volunteers (Group 1). This was surprising considering that all of these analytes have been implicated as mediators of muscle pain but only six mediators changed during treatment. Following intra-muscular cortisol treatment, these concentrations were considerably lowered 30 min post-treatment (Table 3). Examination of the 5 min samples taken from subjects in Group 3, demonstrated that dramatic falls in the concentration of these inflammation-associated factors took place over 25 min (Fig. 4).

Table 3
Concentrations of the six most important inflammation-associated markers pre- and 30 min post-treatment in subjects from study group 3

Analyte	Pre	Post
Electrokinetic system		
SP	269.3 ± 50.8	31.6 ± 11.8
CGRP	211.7 ± 51.6	27.2 ± 10.1
IL-1β	229.9 ± 59.1	49.3 ± 15.7
β-Endorphin	286.6 ± 49.8	50.4 ± 10.9
IL-6	275.6 ± 66.2	30.5 ± 11.6
TNF-α	309.5 ± 51.7	53.8 ± 17.1
Immunoaffinity system		
SP	280.5 ± 52.2	35.1 ± 10.6
CGRP	215.8 ± 49.4	26.4 ± 11.8
IL-1β	235.4 ± 56.3	44.3 ± 13.5
β-Endorphin	290.6 ± 51.3	53.7 ± 12.6
IL-6	280.1 ± 60.1	33.2 ± 9.9
TNF-α	315.9 ± 49.7	50.5 ± 14.2

All of the concentrations are expressed as pg/mL as measured by the immunoextraction both chip-based CE system.

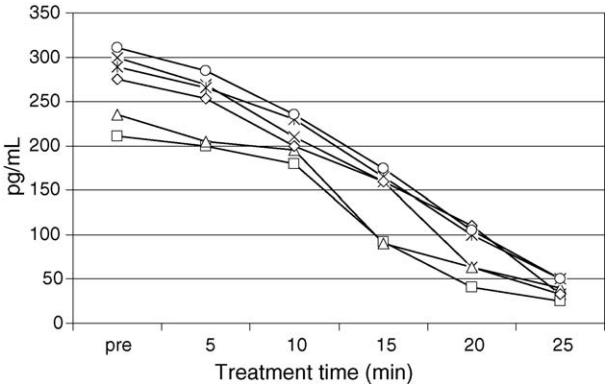

Fig. 4. Analysis of the changes seen in the important inflammatory neuropeptides and cytokines during a treatment of a subject from group 3. Key: (×) = SP; open square = CGRP; open triangle = IL-1β; asterisk = β-endorphin; open diamond = IL-6; open circle = TNF-α. Measurements performed by immunoaffinity chip-based CE as described in Section 2.7.

4. Discussion

The application of CE to clinical testing holds great potential due to the small sample size (ca. 1 μL) required for analysis and high sensitivity when used in conjunction with LIF or electrochemical detectors. Additionally, the use of immunological selectivity in CE adds a further advantage has specific antibodies can be employed either as immunoextraction or immunoaffinity matrices or in electrokinetic immunoassays. The former utilizes either a pre-analysis extraction column [19,20] or immobilization of antibodies directly into the capillary [12,14] to select the analytes of interest. Immunoaffinity CE has been applied to the analysis of multiple different clinically relevant samples where multiple analytes have been measured in the same sample [12,18,21–24].

Electrokinetic immunoassays can be performed as direct binding or competition assays, the amount of analyte being determined from the labeled immune complex. The Kennedy group [25,26] described competitive CE immunoassays cou-

pled to reverse-phase liquid chromatography for the detection and measurement of neuropeptide Y and glucagon in biological materials. Han et al. [27] developed a competitive immunoassay for the measurement of vasopressin in cerebral spinal fluid. This technique used fluorescein-labeled antigen and LIF detection to measure both the immune complex and free-labeled vasopressin within 10 min with a high degree of reproducibility. Wang et al. [28] developed an immunoassay for the detection of bone morphogenic protein-2 based on peroxidase-labelled antigen, which produced a chemiluminescent signal upon reaction with luminol.

The need for analysis of ultramicro samples has led to the development of chip-based systems, especially those designed to perform complete analyses on chip. Such devices have been called "lab-on-a-chip" and are considered the future in protein analysis. The application of chip-based CE to clinical samples is not new. Colyer et al. [29] described a chip-based CE for separation of human serum proteins. The chip was designed for post separation labeling coupled with LIF detection but was not used on true samples due to lack of detection sensitivity. Wang and Chatrathi [30] reported the use of chip-based CE with amperometeric detection for simultaneously determining four markers of renal function. All four markers could be measured within 5 min with creatinine and creatine being measured within 2 min. Guijt et al. [31] reviewed the role of bio-affinity in chip-based analytical systems, with special reference to electrokinetic immunoassays. We have previously described an immunoaffinity chip-based CE system for measuring inflammatory cytokines in cerebral spinal fluid from patients with head injury. This system relied on antibody specificity to isolate the analytes of interest and LIF detection for increased sensitivity [18]. The system was able to isolate six different inflammation biomarkers in circa 5 min and could run up to 12 samples per hour. A major advantage of chip-based systems is that reduction in reagent costs translates to a lowering of the cost of diagnostic assays. There is also the issue of sample size, which becomes important when pediatric and precious archival samples are concerned. Additionally, chip-based CE provides an excellent platform for the development of rapid clinical assays with analysis times almost becoming "real-time". In the present study, the application of chip-based CE to assessment of inflammatory markers in acute and severe muscle pain demonstrated that immunoextraction prior to CE analysis was superior to electrokinetic immunoassay; the former measuring 12 analytes in circa 160 s whereas the latter system measured one analyte per 200 s. Pre-selection of a panel of analytes by immobilized antibodies is a useful approach to isolating analytes of interest from complex biological materials by removing unwanted materials prior to analysis. The addition of antibody extraction to chip-based CE separation greatly improves the efficiency of the system allowing the investigator to study a series of specific analytes without further cleanup. This also helps resolution in chip-based systems, where the removal of unwanted proteins lowers the degree of non-specific adherence to the separation channel wall. The electropherogram was further enhanced by adding a detergent to the running buffer. The immunoextraction chip was capable of running up to 12 samples on an hourly basis, which is compatable with the requirements for clinical use and the speed of analysis (ca. 2 min) makes this a rapid diagnostic screening tool.

5. Conclusions

The coupling of immunoaffinity to CE analysis greatly enhanced the selectivity of the analytical process. Two chip-based systems were tested and the immunoextraction chip was found to be more efficient in the analysis of multiple analytes within the same sample than the electrokinetic immunoassay chip. Both systems had good recovery of all 12 analytes with the immunoextraction chip demonstrating recoveries of greater than 98% for all analytes with intra-assay = 3.51–4.2 and inter-assay = 3.35–4.11. Additionally, 10–12 samples could be run each hour and with pre-labeled samples, the chip was reusable for 50–60 runs. The immunoextraction chip-based CE system is suitable for use bedside or in treatment room studies and is reasonably portable for other types of "field" studies.

References

[1] U. Pedersen-Bjergaard, L.B. Nielsen, K. Jensen, L. Edvinsson, I. Jansen, J. Olesen, Peptides 12 (1991) 333.
[2] G.N. Onuoha, E.K. Alpar, Life Sci. 65 (1999) 1351.
[3] T.M. O'Connor, J. O'Connell, D.I. O'Brien, T. Goode, C.P. Bredin, F. Shanahan, J. Cell. Physiol. 201 (2004) 167.
[4] R. Guillemin, Ann. N. Y. Acad. Sci. 1038 (2004) 131.
[5] H.G. Schaible, A. Del Rosso, M. Matucci-Cerinic, Rheum. Dis. Clin. North Am. 31 (2005) 77.
[6] D. Pozo, M. Delgado, FASEB J. 18 (2004) 1325.
[7] K.A. Sluka, J. Orthop, Sports Phys. Ther. 24 (1996) 240.
[8] S. Kopp, J. Orofac, Pain 15 (2001) 9.
[9] C. Malm, T.L. Sjodin, B. Sjoberg, R. Lenkei, P. Renstrom, I.E. Lundberg, B. Ekblom, J. Physiol. 556 (2004) 983.
[10] L. Rosendal, B. Larsson, J. Kristiansen, M. Peolsson, K. Sogaard, M. Kjaer, J. Sorensen, B. Gerdle, Pain 112 (2004) 324.
[11] N. Desvigne, J.C. Barthelemy, D. Frere, J.P. Gay-Montchamp, F. Costes, Eur. J. Appl. Physiol. 94 (2005) 216.
[12] T.M. Phillips, B.F. Dickens, Electrophoresis 19 (1998) 2991.
[13] S.A. Brown, A.J. Mayberry, J.A. Mathy, T.M. Phillips, B. Klitzman, L.S. Levin, Plast Reconstr. Surg. 105 (2000) 991.
[14] T.M. Phillips, P.D. Smith, Biomed. Chromatogr. 17 (2003) 182.
[15] S. Mouradian, Curr. Opin. Chem. Biol. 6 (2002) 51.
[16] J.W. Cooper, Y. Wang, C.S. Lee, Electrophoresis 25 (2004) 3913.
[17] U. Backofen, F.M. Matysik, C.E. Lunte, Anal. Chem. 74 (2002) 4054.
[18] T.M. Phillips, Electrophoresis 25 (2004) 1652.
[19] N.A. Guzman, Electrophoresis 24 (2003) 3718.
[20] N.A. Guzman, Anal. Bioanal. Chem. 378 (2004) 37.
[21] T.M. Phillips, J.J. Chmielinska, Biomed. Chromatogr. 8 (1994) 242.
[22] N.A. Guzman, J. Chromatogr. B 749 (2000) 197.
[23] N. Delaunay-Bertoncini, M.C. Hennion, J. Pharm. Biomed. Anal. 34 (2004) 717.
[24] P. Su, X.X. Zhang, W.B. Chang, J. Chromatogr. B 816 (2005) 7.
[25] I. German, R.T. Kennedy, Anal. Chem. 72 (2000) 5365.
[26] I. German, M.G. Roper, S.P. Kalra, E. Rhinehart, R.T. Kennedy, Electrophoresis 17 (2001) 3659.
[27] K.Y. Han, E. Ban, Y.S. Yoo, J. Chromatogr. A 1013 (2003) 215.
[28] J. Wang, W. Huang, Y. Liu, J. Cheng, J. Yang, Anal. Chem. 76 (2004) 5393.
[29] C.L. Colyer, S.D. Mangru, D.J. Harrison, J. Chromatogr. A 781 (1997) 271.
[30] J. Wang, M.P. Chatrathi, Anal. Chem. 75 (2003) 525.
[31] R.M. Guijt, E. Baltussen, G.W. van Dedem, Electrophoresis 23 (2002) 823.