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Analysis of neurotrophins in human serum by immunoaffinity capillary electrophoresis (ICE) following traumatic head injury☆

Heather Kalish*, Terry M. Phillips

Ultramicro Immunodiagnostics Section, Laboratory of Bioengineering & Physical Science, National Institute of Biomedical Imaging and Bioengineering, National Institutes of Health, Bethesda, MD USA

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ABSTRACT

Neurotrophins, including brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), and β-nerve growth factor (β-NGF), play an active role in the development, maintenance and survival of cells of the central nervous system (CNS). Previous research has indicated that a decrease in concentrations of these neurotrophins is often associated with cell death and ultimately patient demise. However, much of the research conducted analyses of samples taken directly from the CNS, i.e., of samples that are not readily available in clinical trauma centers. In an attempt to obtain a method for evaluating neurotrophins in a more readily accessible matrix, i.e., serum, a precise and accurate immunoaffinity capillary electrophoresis (ICE) method was developed and applied to measure neurotrophins in serum from patients with various degrees of head injury. The five neurotrophins of interest were extracted and concentrated by specific immunochemically immobilized antibodies, bound directly to the capillary wall, and eluted and separated in approximately 10 min. NT-3, BDNF, CNTF and β-NGF showed a marked decrease in concentration as the severity of the head injury increased: mild versus severe: 91% decrease for NT-3; 93 % decrease for BDNF; 93 % decrease for CNTF; and a 87 % decrease for β-NGF. This decrease in concentration is consistent with the neuro-protective roles that neurotrophins play in the maintenance and survival of neuronal cells. The results obtained by the ICE method were closely comparable with those generated by a commercially available ELISA method.

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1. Introduction

Head injury, known technically as traumatic brain injury (TBI), can be classified as mild, moderate or severe depending on the extent of the damage to the brain. The clinical outcome of TBI patients is determined not only by the primary brain lesions, but also by the extent of secondary brain damage [1]. Secondary brain damage after TBI involves neuro-inflammatory mechanisms, mainly dependent on the intracerebral production of specific biomarkers, such as neurotrophic factors or neurotrophins [2]. However, the roles that neurotrophins play in the outcome following TBI remain unclear and have been the subject of numerous research studies [2–4].

Neurotrophins are a class of proteins involved in the development, maintenance and ultimate survival of neurons. These proteins include brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), and β-nerve growth factor (β-NGF). CNTF, a survival factor for neuronal cell types including dorsal root ganglion sensory neurons, sympathetic ganglion neurons hippocampal neurons and embryonic motor neurons, has been shown to promote axon growth [5], to be upregulated in reactive astrocytes [6], and to be neuro-protective by activating transcription 3 (STAT3), a neuronal survival factor in dendrites, nuclei and cytoplasm of the motor neurons [7]. BDNF and NT-3 are required for the differentiation and survival of specific neuronal subpopulations in both the central and peripheral nervous system. The role of BDNF and NT-3 after TBI remains controversial as some research cites them as being neuro-protective [8–12], while other studies implicate them as either being neuro-degenerative [13], or having no effect on recovery [2,8,14]. NT-4 is secreted at sites of inflammation, is thought to contribute to tissue regeneration and shows potential as a therapeutic strategy after TBI [15,16]. β-NGF plays a crucial role in the development and preservation of the sensory

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* Corresponding author at: Ultramicro Immunodiagnostics Section, Laboratory of Bioengineering & Physical Science, National Institute of Biomedical Imaging and Bioengineering, National Institutes of Health, Building 13, Room 3E42, 13 South Drive, Bethesda, MD, USA. Tel.: +1 301 435 5498; fax: +1 301 496 6608.
E-mail address: KalishH@mail.nih.gov (H. Kalish).

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and sympathetic nervous systems and may play an important role in the regulation of the immune system. The concentration of β-NGF following TBI has been shown to correlate strongly with the severity of TBI [2,3,17], and increased concentrations may play an important role in the TBI treatment [14].

Most of the research focused on correlating TBI outcome with the increase or decrease of neurotrophin concentration is based on experimental models where samples are taken directly from the brain tissue. However since most human head trauma cases are treated in emergency rooms or trauma centers, where brain tissue and cerebral spinal fluid (CSF) samples are not readily available. Therefore, a less invasive sampling technique, such as serum collection, that correlates neurotrophin concentration with TBI outcome would be a valuable tool.

The standard approach to assessing neurotrophins in biological fluids is enzyme-linked immunosorbent assays (ELISA) using specific antibodies for the isolation and detection of each specific analyte. ELISA along with tissue immunohistochemistry localization was employed by Pan et al. [18] to detect and measure BDNF, CNTF, NGF and NT-3 in post-injury regeneration of rat sciatic nerves following a crush injury in a cell culture system. ELISA was also used to study the secretion of the same neurotrophins from monocytes derived from allergic patients during clinical episodes [19]. Chiaretti et al. [20] employed ELISA to study the expression of NGF and BDNF in the CSF and plasma of children at 2 h and 24 h following severe head injury. They demonstrated that high concentrations of BDNF could be found in CSF following injury and represented a potential early marker for head injury [20]. However, in all these studies, multiple assays had to run in parallel for the measurement of the analytes within the same sample.

Capillary electrophoresis (CE) is a fairly rapid analytical technique capable of measuring cytokines, neurotransmitters, hormones and other biomarkers from urine, blood, saliva and cerebral spinal fluids [21–24]. Samples ranging from human albumin solutions [25], plasma and serum for proteome analysis [26] to urine with metabolized MRI contrast agents [27] have all been analyzed by CE. The high sensitivity coupled with the small amount of sample required for analysis and the robustness of CE make it particularly applicable to clinical testing. Additionally, the small amount of reagent and solvent needed for analysis coupled with a relatively short analysis time give CE an advantage over traditional means of clinical analysis such as immunoassay. CE can generate results in a relatively short period of time, requires little pretreatment of biofluids prior to injection, requires minimal reagent volumes for analysis and can detect many analytes in the nano- to pico-molar range[21]. Immunoaffinity capillary electrophoresis (ICE) increases the sensitivity of traditional CE through the use of antibody capture, performs both a cleanup and pre-analysis concentration, which is an advantage when working with biological samples. Additionally, the ability to immobilize multiple different antibodies within the same capillary gives ICE the advantage of being a multiple analyte measuring system. ICE applications to clinical samples include the analysis of tissue samples obtained by microdialysis [24], phosphorylated and non-phosphorylated forms of STAT proteins [23], total IgE quantification in serum [28], and erythropoietin glycoforms in biological fluids [29]. Although ELISA is still the traditional method of analysis and was used to compare the results found in the present study, ICE offers several advantages including less reagent and sample use, greater resolving power and less false positive results [30]. In this paper, a newly developed and validated ICE method was used to analyze and quantify five neurotrophins found in the serum of emergency room patients of different TBI degrees.

2. Experimental

2.1. Chemicals and reagents

The ImmunoPure $F(Ab)'_2$ preparation kit, dithiothreitol (DTT), sulphosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SSMCC) and 0.5 M sodium borate buffer, pH 8.5, were purchased from Pierce (Rockford, IL, USA). 3-Aminopropyltriethoxysilane was purchased from Polysciences (Warrington, PA, USA). The recombinant human neurotrophins NT-3, NT-4, BDNF, CNTF and β-NGF and their corresponding antibodies were obtained from R&D Systems (Minneapolis, MN, USA). AlexaFluor 633 was obtained from Molecular Probes, Invitrogen (Carlsbad, California, USA). ELISA kits for BDNF, CNTF, β-NGF and NT-3 were purchased from Ever Systems Biology Laboratory Inc. (Sacramento, CA), and the ELISA kit for NT-4 was purchased from Antigenix America Inc. (Huntington Station, NY). Formamide, Brij 35, 1 M HCl solution, 1 M NaOH solution, sodium bicarbonate, sodium borate and HPLC grade distilled water were purchased from Sigma–Aldrich Chemicals (St. Louis, MO, USA). Sodium phosphate buffer, 0.1 M, pH 7.0, for CE was purchased from Microsolv Technologies (Eatontown, NJ, USA). Prior to use, all solutions were passed through a 0.2 μm nitrocellulose filter (Millipore, Bedford, MA, USA).

2.2. Preparation of stock solutions

A stock solution of 0.1 M sodium bicarbonate was prepared by dissolving 8.4 g of sodium bicarbonate into 1000 mL of HPLC grade distilled water and titrating to pH 9.0 with 1 M NaOH. The neurotrophins were reconstituted to a final concentration of 1 μg/mL by dissolving the lyophilized stock in 0.1 M phosphate buffer, pH 7.0. These neurotrophin solutions were used to spike normal human serum to make final solutions containing 10, 50, 100 and 500 pg/mL for each neurotrophin. A 5 ng/mL solution of AlexaFluor 633 was prepared by dissolving 5 mg of dye solid in 50 % formamide in 0.1 M sodium bicarbonate, pH 9.0.

2.3. Patient samples

Serum samples were obtained from patients with TBI sustained by either an automobile accident or by falling. Consent to use the samples were obtained from relatives and no name indicators were assigned to the samples, apart from the clinical status of the subject as assessed by standard clinical procedures. Thirty patients were classified into three groups each consisting of ten subjects: (a) mild head injury with an average hospital stay of 1–2 days; (b) moderate head injury with an average hospital stay of 3–4 weeks; and (c) severe head injury with hospital stay of greater than 2 months or death. Five milliliter of whole blood was collected in sterile tubes containing a mixture of protease inhibitors from a cohort of 30 patients seen at the emergency center at the George Washington University Medical Center Washington, DC, USA. The cohort was composed of 15 female and 15 male subjects aged between 18 and 35 years. The whole blood was allowed to clot at room temp for 20 min, and 2 mL of sterile serum was collected from each subject. All samples were stored at −80 °C until analyzed.

2.4. Instrumentation

ICE analyses were performed on a Crystal 660 capillary electrophoresis system (Prince Technologies, Amsterdam, Netherlands) equipped with a on-capillary laser induced fluorescence (LIF) detector (Picometrics, Cambridge, MA, USA) incorporating a 12 mW red light emitting, 633 nm helium–neon laser. Signals from the detector were filtered through a 650 nm interference filter before

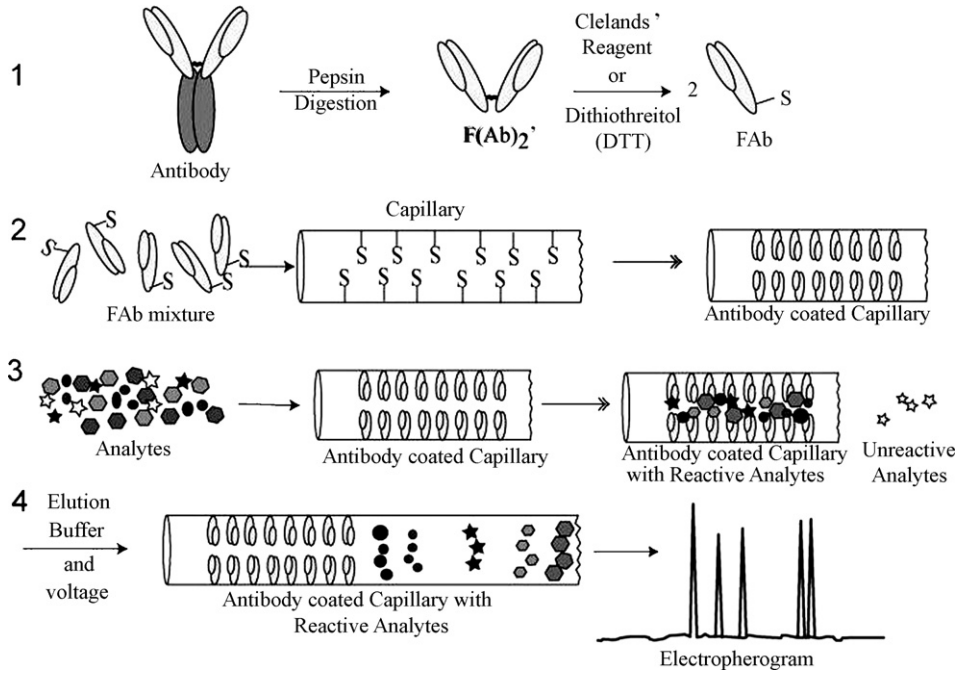

Fig. 1. Diagrammatic representation of the steps used to prepare an ICE capillary. Step 1: The antibody is digested by pepsin digestion, yielding the $F(Ab)'_2$ fragment, which is further reduced by Clelands' reagent to 2 FAb fragments. Step 2: The antibody fragments are loaded by capillary action into the derivatized capillary. Step 3: The AlexaFluor labeled analytes are introduced into the capillary and bind to the immobilized antibody. Non-reactive materials are removed. Step 4: An elution buffer is introduced into the capillary, breaking the antibody/analyte bond and releasing the analytes, which separate and are detected as an electropherogram.

being transferred to a computer running Windows XP Professional and analyzed using DAx data acquisition software (Prince Technologies). The samples and the capillary were maintained at 5 °C using the sample tray and capillary cooling chambers built into the CE system and controlled by the instrument operating system. This system also controlled the injection mode of the CE. A hydrodynamic pressure injection of 6 s at 10 mbar was used throughout the study to ensure that all of the analytes were equally introduced into the capillary in a reliable manner. Recovery of the injected volume by exerting a 20 mbar pressure at the outlet for 10 s and measuring the expelled fluid demonstrated that the injection volume was 50 ± 4 nL. All samples were run in 0.1 M phosphate buffer, pH 7.0, at a constant current of 100 μA.

ELISA was performed on all of the neurotrophin-spiked standards and the patient serum samples using peroxidase high sensitivity kits according to the manufacturer's instructions. The entire assay steps were performed at room temperature in the buffers provided in the kit. The results were read in a Bio-Rad Laboratories Multiscan ELISA plate reader (Hercules, CA, USA) at 450 nm with 570 nm background subtraction.

2.5. Preparation of the immunoaffinity capillary

The immunoaffinity capillary was prepared as described previously [31,32]; this procedure is graphically depicted in Fig. 1. Briefly, each anti-neutrophin capture antibodies was checked for specificity by dot blot against a panel containing all of the analytes of interest. Additionally, their binding characteristics for both native and AlexaFluor labeled analytes were checked by performing immunoprecipitation curves [33]. Antibodies with reasonably matched association/dissociation constants and high specificity were used to perform the immunoaffinity isolation of the specific neurotrophins. Pierce Immunopure $F(Ab)'_2$ preparation kits were used, according to the manufacturer's instructions, to enzymatically digest each anti-neurotrophin antibody into divalent $F(Ab)'_2$ fragment. Further reduction of the $F(Ab)'_2$ fragments to two monovalent FAb fragments was carried out by DTT. The $F(Ab)'_2$ were incubated with an equal volume of 0.2 M DTT for 30 min at 37 °C [34]. The inside wall of a single fused silica capillary with an unmodified surface and an inner diameter of 100 μm (PolyMicro Technologies, Pheonix, AZ, USA) was coated using a mixture containing equal amount of each anti-neurotrophin FAb. The capillary used was 100 cm in length with a detection cell placed 65 cm from the inlet. The detection cell was made by removing a 2 mm section of the capillary polyimide coating with a commercially available CE window maker (MicroSolv).

Into the inlet (the end furthest from the detector), 500 nL of an aqueous solution of 10% 3-aminopropyl-triethoxysilane was introduced by capillary action and incubated at 100 °C for 60 min. This process was repeated four times. Then, the capillary was incubated at 100 °C for another 60 min with 0.1 M HCl, which was introduced by capillary action. The capillary was washed once with distilled water before continuing with the preparations. A 500 nL solution of 1 mg/mL SSMCC dissolved in 0.5 M sodium borate buffer, pH 8.5, was introduced into the capillary by capillary action, and the capillary was incubated at 30 °C for 60 min. Following a capillary flush with 0.5 M sodium borate buffer, it was filled by capillary action with the FAb mixture. The capillary was incubated overnight at 4 °C and then it was ready for use. By employing this procedure to modify the internal capillary surface, an approximately 50-fold molar excess of each antibody was immobilized on the capillaries internal surface. Since the amount of neurotrophin in each serum sample is estimated to be in the pico-molar amounts, loss of sample would significantly affect the calculated concentrations of each analyte. Careful consideration of maximizing the amount of analyte captured at 100% versus avoiding significant non-specific binding during the short antibody capture time (5 min) determined the excess amount of antibody used in the capillary surface coating [35]. Finally, the capillary was washed three times in 0.1 M phosphate buffer, pH 7.0, before being mounted onto the CE.

2.6. ICE analysis of neurotrophins in spiked normal and patient samples

All patient samples were analyzed as previously described [35]. Briefly, direct spectrophotometry at 260/280 nm was used to measure the protein content of each serum sample. 20 μL fractions of each sample were measured and the protein concentration was calculated using the following equation: $[(A/\varepsilon_{percent})\ 10 = \text{concentration in mg/mL}]$, where $\varepsilon_{percent}$ is assumed to be $10\,(g/mL)^{-1}\,cm^{-1}$ because although any given protein can vary significantly from $\varepsilon_{percent} = 10\,(g/mL)^{-1}\,cm^{-1}$, the average for a mixture of many different proteins likely will be approximately 10 [36]. Phosphate buffer (0.1 M, pH 7.0) was then added to the sample to dilute the calculated protein content to approximately 1 ng/mL. An aliquot (20 μL) of the standard AlexaFluor 633 prepared solution were added to the adjusted samples, the mixture was placed in black tubes and shaken for 20 min. The samples were analyzed by ICE after the labeling was complete. To determine the concentrations of the neurotrophins in the standards and the serum samples from TBI patients, approximately 50 nL was injected by vacuum injection into the CE. A 5 min incubation allowed the immobilized FAb's to capture their respective analytes. Then, the unbound material was purged from the capillary by applying a 200 μL wash (0.1 M phosphate/ 0.01% Brij 35, pH 7.0). Recovery of the bound analytes was achieved by changing the buffer to an elution buffer (0.1 M phosphate buffer, adjusted to pH 1.5 with 1 M HCl), containing 0.01% Brij 35. A 100 μA constant current was applied across the capillary to separate and advance the analytes past an on-line LIF detector. Comparison of the areas of each resolved peak with a standard curve (constructed from known amounts of pure neurotrophins added to normal human serum and analyzed under identical conditions) allowed calculation of the concentration of each neurotrophin in the patient serum samples.

2.7. Comparison of ICE with standard ELISA

Each serum sample (both spiked serum and patient sample) was split and ran by ICE as described above and by a commercially available ELISA according to the manufacturer's instructions.

3. Results

3.1. Characteristics of the ICE system

Immunochemical characterization of the anti-neurotrophin antibodies demonstrated each antibody was specific for its appropriate antigen and exhibited no cross-reactivity against the other neurotrophins. Affinity binding using the five different anti-neurotrophin antibodies and their appropriate antigen revealed that the affinity constants (K_a) for each anti-neurotrophin antibody were: $1.21 \times 10^6\ M^{-1}$, $1.13 \times 10^6\ M^{-1}$, $1.08 \times 10^6\ M^{-1}$, $1.32 \times 10^6\ M^{-1}$, and $1.41 \times 10^6\ M^{-1}$ for anti-NT-3, anti-NT-4, anti-BDNF, anti-CNTF, and anti-β-NGF, respectively. In similar studies using the same anti-neurotrophin antibodies and AlexaFluor-labeled antigens no significant loss in affinity binding was seen. The calculated K_a values using the dye-labeled antigens were: $1.16 \times 10^6\ M^{-1}$, $1.10 \times 10^6\ M^{-1}$, $1.02 \times 10^6\ M^{-1}$, $1.27 \times 10^6\ M^{-1}$, and $1.37 \times 10^6\ M^{-1}$ for anti-NT-3, anti-NT-4, anti-BDNF, anti-CNTF, and anti-β-NGF, respectively.

The ICE capillary was found to contain approximately 140 ng of the immobilized FAb mixture. When operated at room temperature the ICE capillary was stable for 100–150 runs, but the capillaries life span could be increased to almost 200 runs if the capillary was used at 5 °C and stored at 4 °C when not being used. The lower limit of detection of the method was determined to be each 0.1 fg/mL for BDNF, NT-3 and NT-4, and 0.15 fg/mL for CNTF and β-NGF in normal human serum. The upper limit of detection of the method was determined to be approximately 3.5 ng/mL for all neurotrophins in normal human serum. A typical electropherogram showing the resolution pattern of a 100 pg/mL mixture of each neurotrophin in normal human serum is shown in Fig. 2. All neurotrophins were separated and eluted in the order: BDNF, CNTF, NT-3, NT-4, β-NGF. Baseline-separation was achieved for BDNF, CNTF and β-NGF, whereas NT-3 and NT-4 ran closely together. Normal human serum spiked with individual neurotrophin standards were used to calculate the recovery rates of the ICE system as shown in Table 1.

Intra- and inter-assay precision (relative standard deviation, RSD) was determined by 5-fold injection of a 100 pg/mL mixture of the neurotrophins added to normal human serum into the ICE system. Intra-assay precision was 4.6%, 5.1 %, 3.9 %, 6.0% and 4.8 %, whereas inter-assay precision was determined to be 4.3 %, 4.9

Table 1
Precision (RSD, %) and accuracy (recovery, %) of the ICE method for neurotrophins in normal human serum.

Neurotrophin	Added (pg/mL)	Measured[a] (pg/mL)	Precision (%)	Recovery (%)
NT-3	10	9.8 ± 1.1	11.2	98.0
	50	48.7 ± 1.3	2.7	97.4
	100	97.9 ± 2.1	2.1	97.9
	500	490 ± 5.7	1.2	98.0
NT-4	10	9.7 ± 1.5	15.5	97.0
	50	46.1 ± 2.1	4.6	92.2
	100	96.1 ± 4.3	4.5	96.1
	500	491 ± 8.9	1.8	98.2
BDNF	10	9.9 ± 1	10.1	99.0
	50	48.4 ± 1.3	2.7	96.8
	100	98.9 ± 2.2	2.2	98.9
	500	496 ± 3.6	0.7	99.2
CNTF	10	6.5 ± 1.1	16.9	65.0
	50	48.2 ± 1.5	3.1	96.4
	100	96.3 ± 3.3	3.4	96.3
	500	494 ± 5.8	1.2	98.8
β-NGF	10	9.7 ± 1.2	12.4	97.0
	50	48.8 ± 1.1	2.3	97.6
	100	97.9 ± 1.8	1.8	97.9
	500	496 ± 3.5	0.7	99.2

[a] Values are presented as the mean ± SD, $n = 5$, for each concentration analyzed.

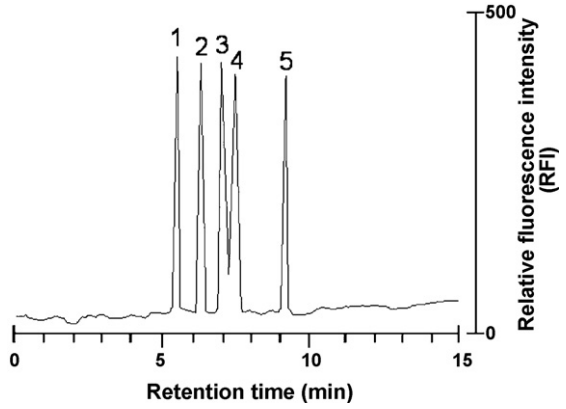

Fig. 2. A typical ICE electropherogram produced by running a mixture containing 100 pg/mL of each neurotrophin standard spiked into normal human serum under the conditions described in Section 2.6. Peak identification: 1. BDNF, 2. CNTF, 3. NT-3, 4. NT-4, 5. β-NGF.

%, 4.1 %, 6.0 % and 5.6 % for NT-3, NT-4, BDNF, β-NGF and CNTF, respectively.

3.2. Comparison of neurotrophin standards by ICE with ELISA

In order to confirm that ICE was comparable to ELISA, we analyzed neurotrophin standards by both assays. The four neurotrophin standard solutions (neurotrophins spiked to normal human serum samples) were analyzed by both ICE and commercially available ELISA kit, and the results were compared by the least squares linear regression analysis using GraphPad 4 (GraphPad Software, San Diego, CA). Analysis of the results obtained by ICE (y) and ELISA (x) for these neurotrophin standards yielded the following regression equations: $y = -5.3 + 2.6x$, $r^2 = 0.9$ for NT-3, $y = -3.6 + 2.0x$, $r^2 = 0.9$ for NT-4, $y = -4.0 + 2.6x$, $r^2 = 0.9$ for BDNF, $y = -6.6 + 4.1x$, $r^2 = 0.9$ for CNTF, and $y = -6.3 + 2.8x$, $r^2 = 0.9$ for β-NGF. The deviation of the slopes from 1 indicates that despite correlation between the ELISA and ICE results, they disagree in their absolute values.

3.3. Neurotrophin measurements from TBI patients

Fig. 3 shows typical electropherograms for each of the three patient groups and Table 2 summarizes the neurotrophin concentrations measured in the patients serum samples. All five neurotrophins were analyzed within approximately 10 min. BDNF showed the overall highest concentration in all three patient groups, while NT-4 was found in the lowest concentration within the groups. NT-3 (by 91 %), BDNF (by 93 %), CNTF (by 93 %) and β-NGF (by 87%) showed a decrease in concentration as the severity of the head injury increased (when comparing mild versus the severe group). The concentration of NT-4, however, actually increased from 19 ± 6 pg/mL in the mild and moderate groups to 34 ± 5 pg/mL in the severe injury group.

Table 2
Serum neutrophin concentrations measured by ICE in the three TBI patient groups.

Neurotrophin	Serum concentration (pg/mL, mean ± SD)		
	Patient group		
	Mild TBI (n = 10)	Moderate TBI (n = 10)	Severe TBI (n = 10)
NT-3	105.6 ± 10.4	37.4 ± 5.0	9.30 ± 1.6
NT-4	19.0 ± 6.2	19.0 ± 6.6	33.6 ± 5.0
BDNF	707 ± 72	223 ± 29	51.1 ± 9.4
CNTF	459 ± 58	201 ± 30	32.7 ± 11.2
β-NGF	91.6 ± 10.9	37.7 ± 7.5	11.6 ± 1.9

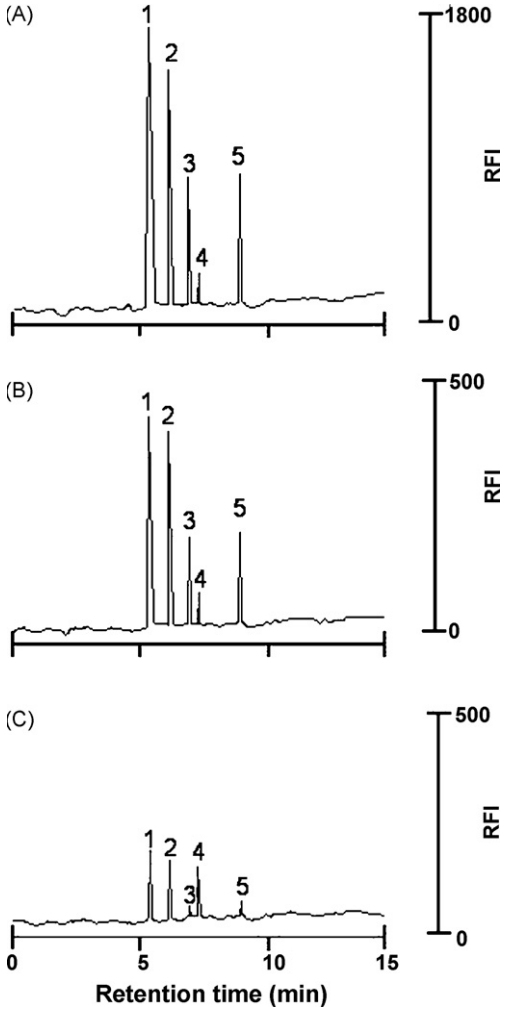

Fig. 3. Electropherograms from ICE analyses of unspiked serum samples of patients suffering from (A) mild, (B) moderate, and (C) severe head trauma. Analyses were performed under the conditions described in Section 2.6. Peak identification: 1. BDNF, 2. CNTF, 3. NT-3, 4. NT-4, 5. β-NGF.

Comparison of the ICE data with ELISA demonstrated high degree of correlation when comparing the patient samples by the two systems (Fig. 4). Least squares linear regression analysis of the different analyte concentrations as measured by ICE and ELISA resulted in r^2 values between 0.96 and 0.99.

4. Discussion

There is a growing need for the development of analytical procedures for use in clinical studies that employ minimally invasive sampling techniques. In the case of TBI, blood sampling is considered minimally invasive when compared to sampling of CSF or brain tissue itself. Analytical techniques such as CE are well suited to analyzing clinical samples as such techniques require minimal sample volume and reagents, thus reducing not only the trauma of invasive sampling but also the costs. Combining immunoaffinity extraction and concentration of specific analytes with CE separation greatly enhances the efficiency of the system, allowing the investigator to study the analytes of interest without further complex extraction or cleanup steps [37]. In our study, ICE effectively isolated and measured all five neurotrophins from both normal neurotrophin-spiked serum and from patient native serum samples with minimal processing. The results obtained by ICE correlated closely with the results obtained by commercially available ELISA

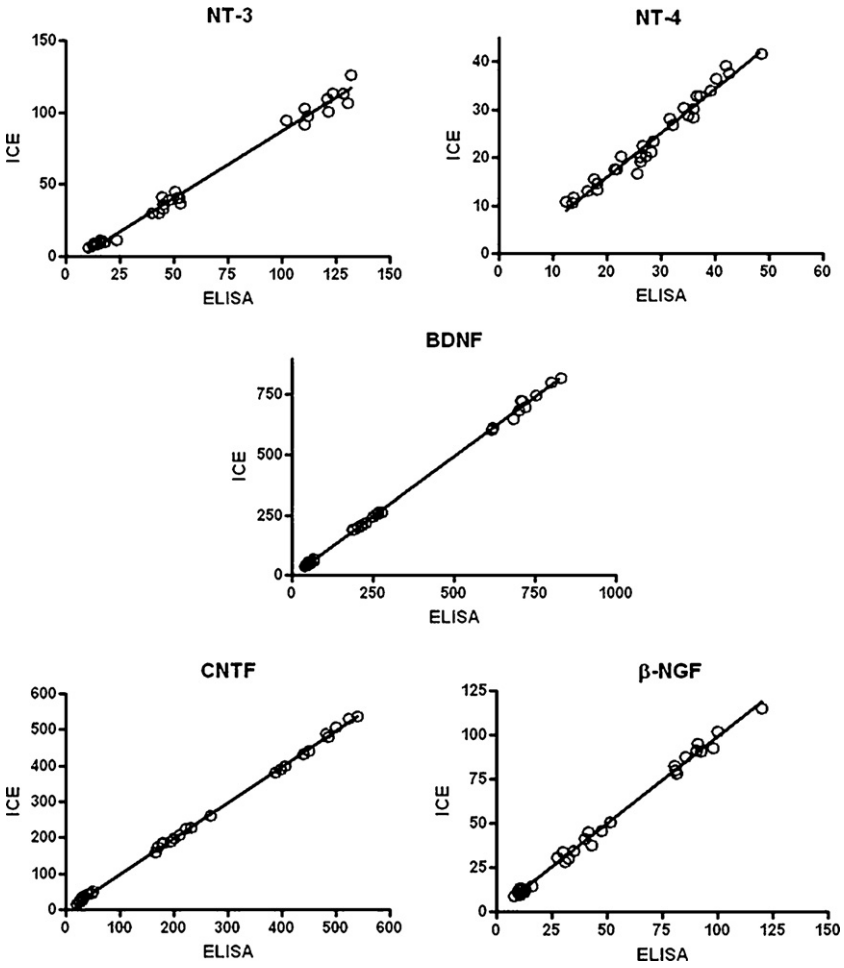

Fig. 4. Linear regression analysis of the results obtained by analyzing the patient samples from each TBI group ($n = 10$ each) by ICE (y) and ELISA (x). The patterns for all of the neurotrophins, except NT-4, clearly indicate the three patient groups. Regression equations: $y = -5.62 + 1.32x$, $r^2 = 0.99$ for NT-3; $y = -2.41 + 0.97x$, $r^2 = 0.96$ for NT-4; $y = -2.38 + 2.6x$, $r^2 = 0.99$ for BDNF; $y = -0.15 + 1.4x$, $r^2 = 0.99$ for CNTF; and $y = 0.55 + 0.82x$, $r^2 = 0.99$ for β-NGF. Here, the slopes are closer to 1.0 which means that ICE and ELISA agree much better than in the normal serum samples.

kits for the neurotrophins analyzed. The time required to run a sample by ICE was 40 min including sample loading, extraction, elution and detection. Although, this is not a rapid assay, the advantage of ICE is that multiple analytes can be measured simultaneously in the same sample. In the present study, five different analytes were measured in a 50 nL sample aliquot, which provides certain advantages over conventional immunoassays. For example, ELISA usually requires 50–200 μL per sample and can analyze one analyte per run. Further, due to the hyphenated nature of ICE, electrophoretic separation in the second phase allows visualization of non-specific materials, thus reducing the incidence of false positive reactions [30,37].

The head trauma patients were grouped as mild, moderate or severe cases according to their ultimate recovery, which is a reflection on the degree of tissue injury. Since neurotrophins play a role in the protection of neuronal cells, it is expected that as nervous system damage is greater, the amount of neurotrophin present should decrease. This correlates with the data from the present study that found such a decrease in four neurotrophins but not in NT-4. We found that there was a small increase in the detectable concentration of this neurotrophin with increasing severity of head trauma, i.e., 19 pg/mL in the mild case versus 34 pg/mL in the severe case. Since NT-4 is expressed ubiquitously throughout the CNS, in contrast to the other neurotrophins [38,39], the observed increase in NT-4 among patient groups may be unrelated to head trauma-induced NT-4 formation.

The ability to correlate patient prognosis for recovery from head trauma with concentration of prevalent neurotrophins in a patient's blood would be an invaluable tool in the clinical setting. Most head trauma cases are seen in emergency rooms or trauma units where blood samples can be easily obtained, but samples directly from the brain cannot. However most research has concentrated on correlating a patient's prognosis for recovery with samples obtained directly from the site of injury, which is not practical for clinical settings. Additionally, research shows that concentration of neurotrophins can vary depending on the site of extraction [40,41]. We feel that ICE is sensitive enough to be able to extract and measure neurotrophins from more easily accessed biological fluids such as serum and that the results from this study indicate a correlation between ICE measurements and the patient's clinical outcome.

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