JMB 2009; 28 (4) DOI: 10.2478/v10011-009-0026-7

UDK 577.1 : 61 **ISSN 1452-8258**

JMB 28: 274–278, 2009

Original paper
Originalni naučni rad

ADVANCED TECHNIQUES IN CLINICAL PRACTICE: USE OF LAB-ON-A-CHIP ELECTROPHORESIS AND OTHER METHODS IN PROTEIN PROFILING

NAPREDNE TEHNIKE U KLINIČKOJ PRAKSI: ELEKTROFOREZA TIPA LABORATORIJA NA ČIPU I DRUGE METODE ZA PROFILISANJE PROTEINA

Olgica Trenčevska[1], Vasko Aleksovski[2], Kiro Stojanoski[1]

[1]Institute of Chemistry, Faculty of Natural Sciences,
Sts. Cyril and Methodius University, Skopje, Republic of Macedonia
[2]Neurology Clinic, Faculty of Medicine,
Sts. Cyril and Methodius University, Skopje, Republic of Macedonia

Summary: Proteins in clinical practice are analyzed as important parameters in the determination and treatment of different diseases. The scopes of the analyses are mainly concentrated in two levels – analyses of the complete protein profile, or determination of an isolated protein. In this work, despite of the use of conventional methods, mainly electrophoresis, new techniques have been implemented in protein analyses. Lab-on-a-chip is an electrophoretic technique that, when optimized, provides analyses of the total protein profile. When normal samples are compared to samples obtained from patients with different neurological diseases, characteristic patterns can be noted. Also, correlation and comparison can be made between the newly developed microchip electrophoresis method and the results obtained using the conventional techniques. When an analysis of a specific protein is necessary, mass spectrometry has proven to give best results, in both the selectivity and specificity of analyses. It is believed that cystatin C is a potential biomarker in neurological diseases; therefore, the mass spectrometry method has been developed in order to obtain qualitative and quantitative analyses of biological fluids. Using the developed method of mass spectrometry immunoassay (MSIA), cystatin C was easily isolated and analyzed, obtaining complete analysis within minutes. The resulting mass spectra revealed various levels of cystatin C isoforms in serum and CSF samples.

Keywords: cystatin C, lab-on-a-chip electrophoresis, MALDI-TOF-MS, MSIA, neurological diseases, protein profiling

Kratak sadržaj: U kliničkoj praksi, proteini se analiziraju kao parametri važni za određivanje i lečenje različitih bolesti. Postoje dva opsega analiza: analize kompletnog proteinskog profila ili određivanje izolovanog proteina. U ovom radu, pored korišćenja uobičajenih metoda, pre svega elektroforeze, u analizu proteina uključene su nove tehnike. Laboratorija na čipu je elektroforetska tehnika koja, optimizovana, omogućava analize ukupnog proteinskog profila. Kada se normalni uzorci uporede sa uzorcima dobijenim od pacijenata s različitim neurološkim oboljenjima, postaju uočljivi karakteristični obrasci. Takođe, moguće je uspostaviti korelaciju i poređenje između nedavno razvijenih metoda »mikročip« elektroforeze i rezultata dobijenih korišćenjem uobičajenih tehnika. Kada je neophodna analiza specifičnog proteina, masena spektrometrija je dala najbolje rezultate, što se tiče selektivnosti kao i specifičnosti analiza. Postoji uverenje da je cistatin C potencijalni biomarker za neurološka oboljenja, stoga je razvijen metod masene spektrometrije kako bi se postigle kvalitativne i kvantitativne analize bioloških tečnosti. Pomoću metode imunoeseja masenom spektrometrijom (MSIA), cistatin C je lako izolovan i analiziran a kompletna analiza trajala je nekoliko minuta. Maseni spektri koji su nastali kao rezultat otkrili su različite nivoe izoformi cistatina C u serumu i uzorcima cerebrospinalne tečnosti.

Ključne reči: cistatin C, elektroforeza tipa »laboratorija na čipu«, MALDI-TOF-MS, MSIA, neurološka oboljenja, profilisanje proteina

Address for correspondence:

Olgica Trenčevska
Institute of Chemistry
Faculty of Natural Sciences, Sts. Cyril and Methodius University
Arhimedova 5, PO Box 162
1001 Skopje, Republic of Macedonia
phone no: + 389 70 54 24 60
fax no: + 389 2 3226 865
e-mail: olja.trencevska@gmail.com

Introduction

Everyday laboratory analyses are done in order to contribute to precise medical diagnosis in patients with different diseases. The techniques developed include methods which are easy to handle, provide results with satisfying selectivity and sensitivity, and are fast enough to give results in a short time.

Proteins can be analyzed in different biological specimens and in two frames – either in general, obtaining an image of the total protein profile or by analysis of a selected protein, which is considered to have a role as a potential biomarker for a certain disease. Both methods contribute to the precise clinical diagnosis and provide physicians with the required information about the patients' health. Obtaining a complete protein profile is important because information can be provided about the different classes of proteins present in the sample, therefore giving the opportunity to calculate specific indexes that are usually implemented as diagnostic criteria in some diseases (1). On the other hand, there are specific proteins, potential biomarkers, which, when present in the biological fluid, can indicate specific dysfunction (2).

In this work, we have used lab-on-a-chip electrophoresis and mass spectrometry in order to analyze real samples (serum and cerebrospinal fluid) from patients with neurological diseases. We have analyzed proteins in two levels – by obtaining total protein profile through optimized lab-on-a-chip electrophoresis (3), and by analyzing a specific protein, cystatin C, an acute phase protein, which is considered to be a potential biomarker in neurological diseases, by a mass spectrometry immunoassay.

Miniaturized lab-on-a-chip electrophoresis is a novel technique in protein profile analyses (4). The microchip system includes a system of microchannels through which gel is rushed by applying pressure. The gel contains fluorescent dye which serves as a label; therefore protein detection is done by fluorescence analysis. By running the chip, proteins can be separated according to their size, and, as a result, the protein profile can be obtained.

Mass spectrometry is still not widely used in routine clinical practice. However, its importance in analyses of specific proteins as potential biomarkers will lead to its implementation in this field. Matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) is a technique that is used to provide a rapid and sensitive profiling method for molecular mass calculation for proteins, as well as for biomarker discovery, from a variety of biological samples. It is considered that cystatin C, the protein found in biological fluids, is a potential biomarker for several neurological diseases, although its role is still not determined (5). By developing novel MSIA methods, using MALDI-TOF-MS in order to analyze cystatin C levels in the specimen, this technique can provide great results. Using immunoaffinity pipettes it is possible to extract only the targeted protein and to analyze it with MALDI-TOF-MS (6).

Materials and Methods

Materials

Serum samples and cerebrospinal fluid samples (CSF) were obtained from the biochemical laboratory of the Neurology Clinic (Faculty of Medicine, Skopje, Macedonia), and were labeled according to the Declaration of Helsinki Ethical Committee. When analyzed, all samples were compared to protein standard, in order to obtain qualitative analyses. Therefore, high molecular mass (HMW) and low molecular mass (LMW) protein standards, obtained from BioRad Laboratories, were used. Denaturing agents, SDS, β-mercaptoethanol (BME) and urea were obtained from Sigma-Aldrich. Rabbit anti-human polyclonal cystatin C antibodies, DAKO, A0451, 17 g/L, Carpinteria, CA, USA, were used. Affinity pipettes were activated using affinity ligand, labeled as CDI (1,1'-carbonylimidazol), developed according to standard procedure at Intrinsic Bioprobes Inc, Tempe, AZ. MALDI-TOF-MS method was used in order to complete the analyses. Autoflex MALDI-TOF from Bruker Daltonics, Billerica, MA was used to complete the measurements. Zebra software (Intrisic Bioprobes, Tempe, AZ) and PAWS (Proteometrics, NY) programs were used to process the obtained results.

Methods

Serum samples were collected from whole blood by vein punction, and were stored at –20 °C until analyzed. CSF samples were stored at –20 °C for 3 months before being analyzed. Before handling the serum samples, total protein, albumin and immunoglobulin G (IgG) concentrations were measured. The total protein concentration in the serum was determined by a spectrophotometric method, using an Alcyon 200 Bioanalyzer. Turbidimetry method and radioimmunoassay (RIA) were used to analyze albumin and IgG according to the prescript procedure. Conventional DISC PAGE was done at the biochemical laboratory of the Neurology Clinic. SDS-PAGE was done at the Department of Biochemistry of the Faculty of Medicine. Lab-on-a-chip electrophoresis was carried out on the Agilent 2100 Bioanalyzer from Agilent Technologies, Santa Clara, USA. All chips were prepared according to the Agilent protocol provided. The gel and the gel dye mix were prepared as proscribed in the Agilent manual (7, 8).

Samples were prepared using an optimized procedure previously developed (3). Protein standard was included in every run, therefore providing comparison between different runs. The Protein 200 Plus software assay was used to obtain a complete numerical analysis. The reproducibility and statistical analysis were performed using Statsoft Statistica v7.0.61.0.

A novel MSIA method was developed, using MALDI-TOF-MS in order to analyze cystatin C levels in the specimen. Using immunoaffinity pipettes it was possible to extract only the targeted protein and to analyze it with MALDI-TOF-MS. Experimental conditions such as sample dilution and incubation time were optimized in order to provide better results (9).

Results and Discussion

Protein profiling in neurological diseases is usually done by classifying the type of profile into one of the four main groups: normal (N), transudative (T), gammaglobuline (γ) and transudative gammaglobuline (Tγ) (10). This classification has been made by analyzing the five basic protein zones: prealbumine, albumine, alpha-globuline, beta-globuline and gamma-globuline fraction (*Figure 1*). In these areas of protein mobility, different proteins can be identified when compared to protein standard.

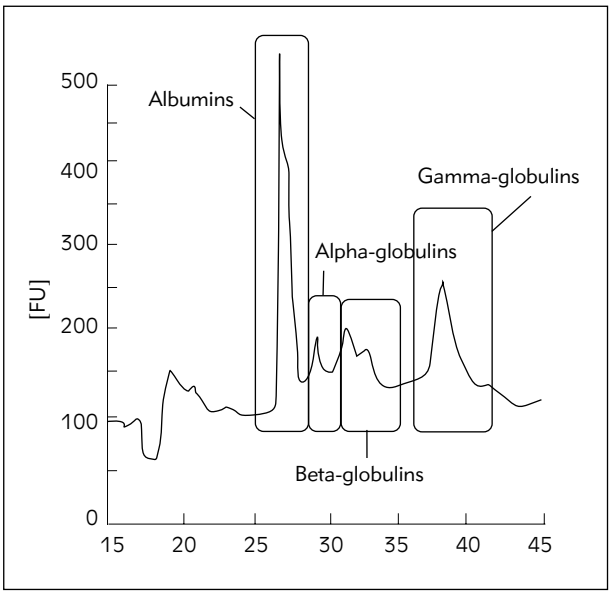

Figure 1 Protein profile obtained from serum sample by lab-on-a-chip electrophoresis. Protein zones are separated and labeled.

The optimized lab-on-a-chip electrophoresis was used to analyze serum and cerebrospinal fluid samples from patients with neurological diseases. It was noted that owing to this advanced technique, protein profiles can be used to obtain satisfactory qualitative analyses, therefore contributing to precise clinical diagnosis. In patients with multiple sclerosis, for example, characteristic electrophoretic patterns were noted, characterized by high IgG concentration (which is evident in 46% of all MS cases, where intrathecal IgG synthesis occurs), and normal total protein levels (11, 12) (*Figure 2* and *Table I*).

For the samples presented, peak area quantities are summarized in *Table I*. Results for albumin and IgG concentration were compared to the ones obtained previously with radioimmunoassay (RIA) analyses, and a satisfactory relation has been established (data not presented). Therefore, we believe that this lab-on-a-chip electrophoresis method can provide reliable results when used for obtaining the protein profile in real samples.

However, when CSF samples were analyzed, significant correlation between the analyses could be made only regarding albumin, but not other protein fraction concentrations. That is a result of the very small protein concentration in CSF when compared to serum samples (*Figure 3*).

The results have shown that by using this optimized technique, the protein profile can be determined in serum samples, but sample preconcentration is necessary in order to obtain quantitative analysis in CSF samples.

Immunoaffinity mass spectrometry is becoming an important analytical approach for analyzing specific proteins from complex biological tissues (13). MSIA combines targeted protein affinity-extraction with rigorous characterization using MALDI-TOF mass spectrometry. Since cystatin C is considered to be a potential biomarker, this selected protein has been analyzed both in serum and CSF samples. The presence of truncated cystatin C isoforms in CSF samples obtained from patients suffering from multiple sclerosis has been the subject of a number of recent investigations (14).

Table I Protein composition in analyzed specimen from *Figure 1*.

Sample	TP[1]	Alb[2]	Alb (%)	α-globulins (%)	β-globulins (%)	γ-globulins (%)
S2/10	61	45	27.9	11.5	16.6	21.9
S4TP/67/5	67	45	20.6	8.44	23.9	33.1
S6TP/70/5	70	25	17.3	6.73	15.6	40.2
S5TP/72/2	72	30	20.2	8.89	25	24.9
S3/10	68	46	30.4	7.26	23.5	20.5

[1]TP – total protein concentration given in g/L
[2]Alb – albumin concentration given in g/L

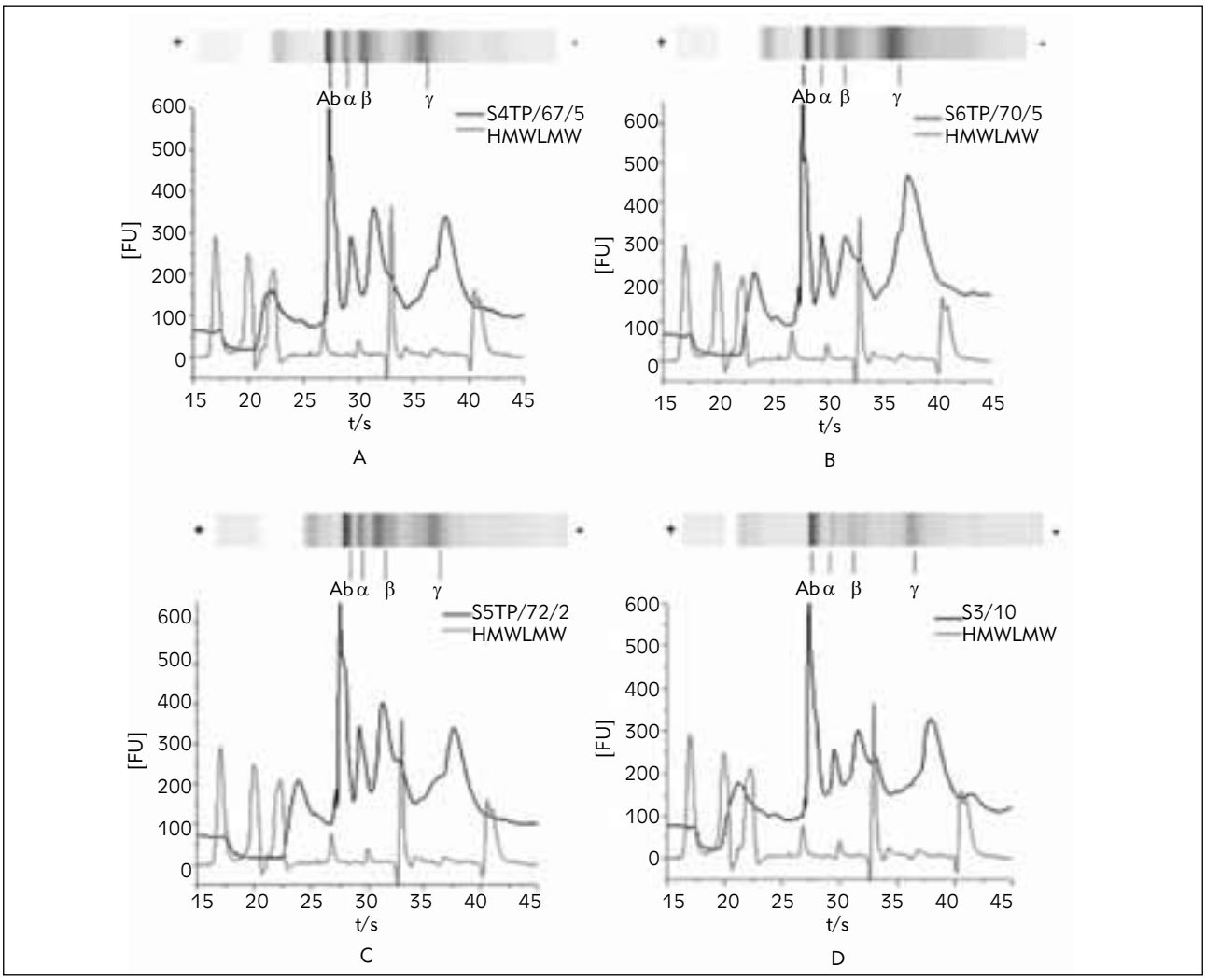

Figure 2 Protein profile in samples from patients with different neurological diseases compared to protein standard.
(A) control sample, (B) sample obtained from patient with MS, (C) sample obtained from patients with dysfunction of blood-brain barrier and intrathecal IgG synthesis, (D) sample obtained from patients with dysfunction of blood-brain barrier, without intrathecal IgG synthesis.

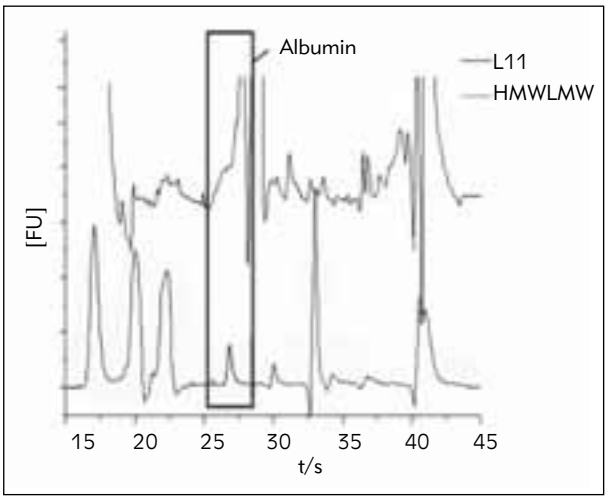

Figure 3 Protein profile in CSF sample from patient with MS. Albumin concentration gives significant fluorescence signal and can be compared to albumin determined with RIA.

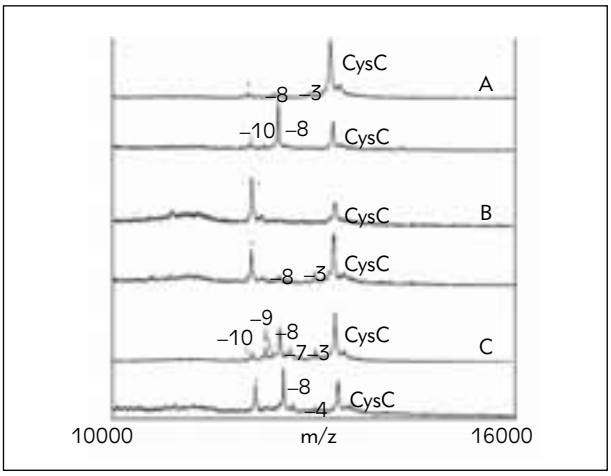

Figure 4 Mass spectra resulting from developed MSIA analyses.

(A) paired control samples, serum (down) and cerebrospinal fluid (up); (B) paired CSF and serum samples from patient with MS; (C) paired CSF and serum sample from patient with MS.

In our analyses, the presence of expected isoforms has been noted, both in the control group and in the samples obtained from patients (*Figure 4*).

Cystatin C appears as control peak in all the samples. Also, specific isoforms lacking 3 and 8 N-terminal amino acids are noted. However, in the samples obtained from patients with multiple sclerosis, additional trunctated isoforms are noted, missing additional 4, 7, 9 and 10 N-terminal amino acid residuals. Additional analyses are in progress in order to establish the relation between the type and state of disease and the trunctated isoforms of cystatin C.

Conclusion

Advanced techniques implemented in clinical practice provide great results in protein profile determination when optimized. Miniaturized electrophoresis, such as lab-on-a-chip GE, can be used in routine analyses of the protein patterns in biological fluids. Protein profiles obtained from serum sample analyses are comparable with the patterns from conventional electrophoresis techniques, therefore indicating potential implementation of this advanced technique into routine practice. In CSF sample analyses, additional optimization should be done, primarily preconcentration, probably due to the small initial total protein concentration.

From the diagnostic point of view, it is a great advantage to analyze and assess biological disease markers from several biological fluids from the same individual. Albeit on a small scale, the results shown here indicate that such studies are possible if the right assays are utilized. The developed MSIA method provides a unique way of delineating protein isoforms and their abundance in serum and CSF. This way, additional population proteomics studies can be done, that will provide further insight into the physiology of biological processes and diseases.

However, additional studies should be done in order to simplify and implement these types of analyses in everyday routine clinical practice.

Acknowledgements: This work was supported in part by Grant No. 094505 from the Ministry of Education and Science of the Republic of Macedonia. Mass spectrometry analyses were done thanks to Prof. Dobrin Nedelkov, PhD, Intrinsic Bioprobes Inc., Tempe, AZ, USA.

Support from the Clinic of Neurology and the Department of Clinical Biochemistry, PhD Angel Mitrevski, as well as CEEPUS program for student exchange and the coordinators of the Department of Physical and Macromolecular Chemistry at the Faculty of Chemistry (Charles University in Prague), PhD Bohuslav Gaš and PhD Eva Tesarova are gratefully acknowledged.

References

1. Mitrevski A. Prilog kon ispiyuvanjeto na proteini vo likvor so elektroforeza. Doktorska disertacija, Skopje 2000.
2. Rifai N, Gillette MA, Carr SA. Protein biomarker discovery and validation: the long and uncertain path to clinical utility. Nat Biotechnol 2006; 24: 971–83.
3. Trenčevska O, Aleksovski V, Stojanoski K. Temperature and denaturing substances influence on lab-on-a-chip electrophoresis. Journal of Medical Biochemistry 2009; 28: 36–40.
4. Floriano PN. Microchip-Based Assay Systems, Methods and Application. Humana Press, Totowa, New York, 2007.
5. Nakashima I, Fujinoki M, Fujihara K, Kawamura T, Nishimura T, Nakamura M, Itoyama Y. Alteration of cystatin C in the cerebrospinal fluid of multiple sclerosis. Ann Neurol 2006.
6. Nelson RW, Krone JR, Bieber AL, Williams P. Mass-Spectrometric Immunoassay. Anal Chem 1995; 67: 1153–8.
7. Desai S, Barthmaier P. Evaluation of albumin removal using the Agilent 2100 bioanalyzer. Agilent tech.
8. Bousse L. Protein sizing on a microchip. Anal Chem 2001; 73: 1207–12.
9. Nedelkov D, Shaik S, Trenčevska O, Aleksovski V, Mitrevski A, Stojanoski K. Targeted Mass Spectrometric Immunoassay for Detection of Cystatin C Isoforms in Cerebrospinal Fluid. The Open Proteomics Journal, 1, 54–58, 2008.
10. Fishman RA, Cerebrospinal fluid in diseases of the nervous system. 2nd ed., Philadelphia, W.B. Saunders comp., 1992.
11. Daskalovska V. Multipla skleroza, Skopje, 2000.
12. Fishman RA, Cerebrospinal fluid in diseases of the nervous system. 2nd ed., Philadelphia, W.B. Saunders comp, 1992.
13. Nedelkov D. Mass spectrometry-based immunoassays for the next phase of clinical applications. Expert Rev Proteomics 2006; 3: 631–40.
14. Irani DN, Anderson C, Gundry R, Cotter R, Moore S, Kerr, DA, McArthur JC, Sacktor N, Pardo CA, Jones M, Calabresi PA, Nath A. Cleavage of cystatin C in the cerebrospinal fluid of patients with multiple sclerosis. Ann Neurol 2006; 59: 237–47.

Received: June 10, 2009

Accepted: July 11, 2009