Introduction

Protein profile in patients with neurological diseases is one of the crucial diagnostic criteria, as well as a tool in the prognosis and treatment of the disease. Therefore, it is of great importance to analyze the protein content and characteristic profile in the patients suffering from conditions such as multiple sclerosis, epilepsy, pareses, dementias, brain strokes.
Materials and Methods

Materials

Serum samples were obtained from the biocological laboratory in the Clinic of Neurology (Faculty of Medicine), and were labeled according to the Declaration of Helsinki ethical committee. High molecular mass (HMW) and low molecular mass (LMW) protein standards were obtained from BioRad Laboratories. Denaturing agents, SDS, β-mercaptoethanol (BME) and urea were obtained from Sigma-Aldrich. Lab-on-a-chip electrophoresis was carried out on the Agilent 2100 Bioanalyzer from Agilent Technologies, Santa Clara, USA. Protein 200 Plus LabChip kit was used in the analysis.

Methods

Serum samples were collected from whole blood by venipuncture, and stored at −80 °C until analyzed. Before handling the serum samples, total protein, albumin and immunoglobulin G (IgG) concentrations need to be measured. The total protein concentration in the serum was determined by a spectrophotometric method, using the Alcyon 2100 Bioanalyzer. Turbidimetry was used to analyze albumin and immunoglobulin according to the prescribed procedure. All chips were prepared according to the Agilent protocol provided. The samples were set using the proposed procedure, as well as the optimized procedure. The gel and the gel dye mix were prepared as proscribed in the Agilent manual (5, 6).

When optimization of the denaturing agent was performed, samples were prepared using 79 μL deionized water, 4 μL sample and 7 μL of BME. Protein standard was included in every run, therefore providing comparison between different runs. The influence of the additional denaturing agents (SDS and urea) was determined using the same procedure of sample preparation, and the signals of the proteins were observed. Afterwards, different concentration of the denaturing agent was used in order to obtain the optimal sample: denaturing agent ratio. When the temperature influence was studied, sample incubation was done at five different temperatures: 5 °C, 22 °C (room temperature), 37 °C, 60 °C and 90 °C. After sample preparation, samples were loaded onto a chip. The chip was placed in the bioanalyzer and run. In the process of electrophoretic separation in the bioanalyzer, the electric current provides ideal conditions for staining and destaining within 45 s per sample. The analysis chamber itself is heated to 30 °C to ensure the constant conditions. After the separation, the signals are detected by laser-induced fluorescence detection (670–700 nm) (6–8). The Protein 200 Plus software assay was used to obtain the complete numerical analysis. The reproducibility and statistical analysis were performed using Statsoft Statistica v7.0.61.0.

Results and Discussion

Lab-on-a-chip analyses have proven to be more precise and less troubleshooting when analyzing complicated protein mixtures, in comparison to conventional electrophoresis. The analyses of serum samples from the patients were performed in order to determine their protein profile. Both control group samples...
and patient samples were analyzed and compared to the protein standard (Figure 1).

Determination of the protein molecular mass was done, as well as band-shape and peak analysis. Protein sizing with the chip-based analysis was performed by running a protein standard consisting of 10 proteins (HMW plus LMW standard in optimal ratio) and was analyzed in the terms of examining reproducibility (data not presented). Results have shown that the protein profile differs in samples with neurological diseases in the range of appearance of different additional protein fractions. Concentration changes have also been noted for the immunoglobulin fractions in the patients suffering from systemic disorders, and for the alpha-globulin fraction in patients with inflammation diseases.

We used this method to analyze the influence of several denaturing agents and their concentration on the sample preparation. The effects on the electrophoretic protein profile were observed and explained using the method of laser-fluorescence quenching. The samples were prepared under reducing conditions, using SDS, urea and BME as reducing agents. It was noted that incubation with BME gives best resolution (Figure 2).

Further, BME concentration in the sample (total protein: denaturing agent) was optimized. We have used different concentrations varying in ratios TP: BME (1:1; 1:1.5; 1:2; 1:2.5; 1:3; 1:3.5 and 1:5). The results have shown that the optimal ratio for serum sample analysis is 1:3.5 (Figure 3). Additional increase in the BME concentration does not influence the resolution and signal of protein peaks.
It is noted that the fluorescence signal increases when the denaturing agent concentration increases to a certain level. This effect can be explained when the fluorescence mechanism is considered. In the native state, the fluorescence from the dye molecules is severely quenched. When proteins are denatured they unfold, and the fluorescence increases. Such a dramatic change is measurable even at the single molecule level, permitting the study of folding dynamics of protein molecules in real time (8, 9).

When analyzing protein mixtures, temperature is a very important parameter due to its effects onto the protein conformation and affinity to bind with the dye, and therefore to increase the signal of detection. For providing denaturing conditions, sample preparation should be done by boiling with the denaturing agent. In Disc-PAGE, however, it is noted that lower temperatures provide better results. We have analyzed this effect at 5 temperatures of incubation using the optimized denaturing agent, BME, in the optimal concentration within sample preparation. It was noted that the optimal temperature for generating denaturing conditions is 90 °C (Figure 4).

Five peaks with notable changes in intensity (peak 2, 3, 4, 5 and 6) were observed, and the fluorescence quenching effect was noticed (Figure 5).

Significant changes in the peak intensity were observed. The effect is similar to when denaturing agents are considered. Because of the samples’ complicated structure and the three-dimensional structure of the protein constituents, the dye molecules are in close proximity with each other and their fluorescence is severely quenched. When heated and denatured, proteins unfold, swell and reduce the proximity among dye molecules, thus leading to an increase of the fluorescence.

Lab-on-a-chip is more efficient technique when analyzing complicated protein mixtures in comparison to conventional electrophoresis. However, its usage in routine analyses is still limited, due to the need of an optimized method which is easy to handle, cheap and follows certain procedure. The serum proteins are a complicated mixture and are very sensitive to changes in sample temperature and denaturing agents. Significant change of the fluorescence intensity is observed probably due to fluorescence quenching. In some cases, the band intensity was changed several times. Therefore, further analyses are necessary in order to ensure implementation of the technique in routine laboratory practice.

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