

Study of cerebrospinal protein fluids by SDS polyacrylamide gel electrophoresis

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ABSTRACT

A simplified sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) method was applied to unconcentrated cerebrospinal fluids (CSF) and detected significant protein patterns. Small amount of samples were required in this method. The normal protein patterns show at least 5 bands in fresh specimens which were coincided in mobility and in staining pattern with serum and saliva proteins sample which was used as marker.

Keywords: *human, PAGE, profile, Saliva, SDS*

Abbreviations: CSF, cerebrospinal fluid. EDTA, ethylene diamine tetra-acetic acid. PAGE, polyacrylamide gel electrophoresis. rpm, revolution per minute. SDS, sodium dodecyl sulfate. TEMED, N,N,N,N'-tetramethylethylenediamine.

INTRODUCTION

Great variety of neurological diseases require investigation of the cerebrospinal fluid (CSF) to prove the diagnosis or to rule out relevant differential diagnoses (1). Analysis for proteins in cerebrospinal fluid can be useful for diagnosing various neurological diseases (2).

A discontinuous SDS polyacrylamide gel electrophoresis method that introduced by Laemmli at 1970 (3) provides a high resolution technique for electrophoretic separation of the protein components of various biological fluids. Though many variations are commonly being used, but the facility of this method for use with small volumes of dilute protein solution makes it ideal for investigating of cerebrospinal fluid as long as many other proteins for many researchers(4). In 1964, Cunningham published five examples of electrophoretic separation of CSF proteins by disc gel electrophoresis (5). Monseu and Cuminutesgs (6) applied the column technique in a qualitative and semi-quantitative analysis of CSF specimens.

The present study was designed to explore the possibility of using discontinuous SDS PAGE gel electrophoresis as a diagnostic aid in the laboratory.

MATERIALS AND METHODS

Reagents: acrylamide was from Thomas Baker, India. Ammonium persulfate, bromophenol blue, glycine, and N,N'-Methylenebisacrylamide were from BDH, England. Boric acid was from LUDECO, Belgium. Coomassie brilliant blue G-250 was from Sigma, USA. Deionized distilled water was from Iraqi company of medical treatment, Iraq. Disodium ethylene diamine tetra-acetic acid (sodium EDTA) was from Scharlau, European Union. Ethanol and 2-mercaptoethanol were from Riedel, USA. Glacial acetic acid was from G.C.C., England. Glycerol was from Watania, Jordan. Sodium dihydrogen phosphate was from Laboratory Rasayan – India. Isopropanol was from Thomas Baker, India. Sodium thiosulfate and Sodium dodecyl sulfate (SDS) were from Carlo Erba reagents, India. TEMED (N,N,N,N'-

tetramethylethylenediamine) was from SERVA, Germany. Tris-base was from Fluka, Switzerland.

Apparatus: the equipment used for one-dimensional gel electrophoresis and the preparation of polyacrylamide was from Cleaver Scientific Ltd., UK. This device was described elsewhere (10).

Spinal fluid: 100 μ l of 3 normal cerebrospinal fluid (CSF) specimens were collected by lumbar puncture for routine clinical-diagnosis tests from patient of various ages. The untested remainder of the specimens was used in this study. CSF specimens were centrifuged at 9000 rpm for 5 minutes at 4°C in a micro-centrifuge (Hermle, Germany), the supernatant (spinal fluid) was frozen immediately at -20°C until required. Because of the danger of in-appropriate storage under these conditions electrophoretic separation was undertaken after only a couple of days after collection.

Electrophoresis:

The electrophoresis was taken place in the dual vertical electrophoresis unit (10 \times 10 cm gel plates with 1mm spacers). Gel dimensions were 10-cm wide by 8-cm length and 0.1-mm thickness. Laemmli discontinuous method was considered with some modifications as the following:

1. Gel preparation:

Resolving gel preparation and pouring: The resolving gel concentration used in these experiments was 10%. In which 3.34 ml of 29/1 acrylamide – bisacrylamide (30%) stock solution was mixed with 6.66 ml 1 x of resolving gel buffer (0.1% SDS, 0.375 M tris-HCl, pH 8.8, 0.1%) and filtered by Whatman filter paper (No. 1). Freshly prepared 100 μ l (10%) ammonium persulfate and 10 μ l TEMED were added to the gel solution. The mixture was mixed briefly (that depends on the quality of ammonium persulfate and TEMED used). 10 ml syringe was used, which was inverted, to expel any air entered syringe barrel. Using the same syringe, the acrylamide gel solution was expelled into the space between the two glass plates carefully on the margin of the glass plates to avoid generating air bobbles formed. Gel pouring was continued until the gel level reached 1 cm below where

the comb teeth finish (which was provided enough space for pouring of stacking gel). 1 ml distilled water was added at the top of the gel to exclude oxygen from the surface. The acrylamide gel solution was allowed to polymerize for 30 minutes at room temperature. Before pouring stacking gel, distilled water and any remaining un-polymerized gel was drew.

Stacking gel preparation and pouring: The stacking gel (0.125 M, pH 6.8, 0.1% SDS) concentration used in these experiments was 4 %. In which 0.4 ml acrylamide – bisacrylamide (30%) stock solution was mixed with 2.6 ml (1x) stacking gel buffer (0.1% SDS, 0.125 M, pH 6.8,) and filtered by Whatman filter paper (No. 1). Freshly prepared 30 μ l (10%) ammonium persulfate and 5 μ l TEMED were added to the gel solution. The acrylamide gel solution was mixed briefly. 5 ml syringe was used, which was inverted. Using the same syringe, the acrylamide gel solution was expelled into the space between the two glass plates carefully. Once the acrylamide gel solution reached its estimated level at the top of the notched glass plates, the comb teeth was inserted carefully from one end of the glass plates (45°) to avoid generating air bubbles. Since the stacking gel polymerization takes longer time compared to resolving gel counterpart, the acrylamide gel solution was allowed to polymerize for 50 minutes at room temperature.

2. Loading samples:

After removing the comb, 10 μ l (4X) loading buffer (40% glycerol, 20% 2-mercaptoethanol, 12% SDS, 50% 4x stacking tris, 0.3% bromophenol blue) was added to 30 μ l spinal fluid. The mixture was mixed briefly, and boiled immediately for 2 minutes at 90°C. To remove the precipitants, CSF samples were centrifuged for 1 minutes at 13000 rpm in a cool microcentrifuge at 4°C. 25 μ l of each sample was loaded into alternative wells of the gel.

3. Gel electrophoresis conditions:

Electrophoresis was taken place in mini-slab gel (purchased from CleaverScientific – UK) and performed at room temperature using pre-cold electrophoresis buffer (stored in refrigerator) at 75 V / 10 mA for stacking gel at the beginning of run (0 hour), 75 V / 5 mA for stacking gel – resolving gel

attachment point (30 minutes), and 100 V / 12 mA for resolving gel until the tracking dye reached the bottom of the resolving gel. When electrophoresis was completed, the gel slab and the reservoir buffer were relatively warm, but Rodger and Holmes (1979) mentioned that Such warming did not appear to be harmful to the resolution (8).

4. Staining by Coomassie brilliant blue G-250:

The Standard staining method of Sambrook and Rushell (9) was considered with some modifications correlated with the dilution of Coomassie brilliant blue G-250 dye concentration to 10 folds according to Fraij, 1989 (10). Polyacrylamide gel was placed in Coomassie staining solution (0.025 % w/v Coomassie brilliant blue, 45 % methanol, 10 % glacial acetic acid) and gently rotated (revolution per one second) at room temperature on a rotary shaker (Karl Kalb – Germany) for 30 minutes. During shaking, stacking gel was discarded. When intensely stained bands appeared against a clear background, the resolving gel was submerged in Coomassie destaining solution (45 % methanol, 10 % glacial acetic acid) and rotated in the same conditions for 30 minutes. Gel was positioned in a white plate and picture was taken immediately by 7.2 M.P. digital camera (Sony – China).

RESULTS AND DISCUSION

Out of many electrophoretic methods, SDS-PAGE was very useful because many kinds of proteins in CSF can be easily fractionated and detected without concentration of the original sample (11). As shown in figure 1, CSF specimens were never previously concentrated before undergoing SDS-PAGE. In addition, Mashige *et al.* (16) mentioned that despite of the multitude of detection method of CSF samples, SDS-PAGE was considered to be very useful in analyzing many kinds of proteins in these samples, and bands could be easily fractionated and detected without concentration of the original samples (12).

Routine methods for protein analysis of CSF require either (a) extensive concentration, which necessitates high sample volume and risks protein

loss/modification (12); or (b) technically demanding detection methods such as silver staining (13). These results revealed that complex, high-resolution electrophoretic patterns, revealing both protein content and distribution, can be obtained with unconcentrated CSF samples. SDS-PAGE was claimed to demonstrate IgG banding patterns characteristic of demyelinating diseases (14) but some researchers were described that as “inappropriate”, because the method separates according to molecular size rather than charge or isoelectric point (15). SDS discontinuous PAGE offers significant advantages in spinal fluid protein separation over other methods: 1- The volume of sample (total protein concentration) required was small, 2- the unconcentrated samples were resolved in acceptable resolution, 3- the technique was simple, and working solutions were relatively stable. On the other hands, there was certain disadvantages: 1- because of asymmetry of the bands, which occasionally occurs (as in figure 1), band comparison might not be advantageous, 2- time consuming gel buffers preparations, and 3- the acrylamide reagent was neurotoxic (12) .

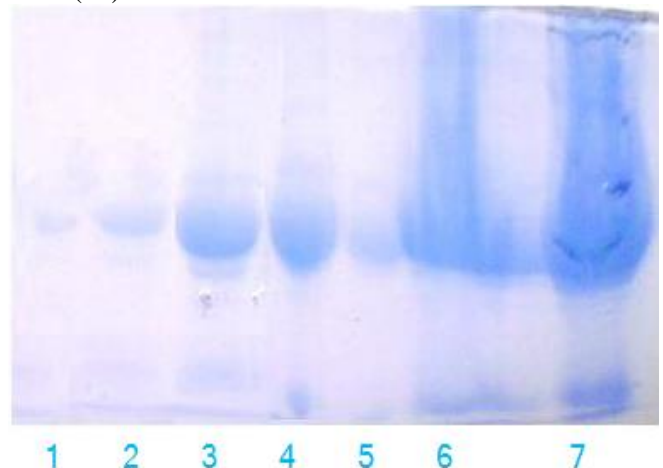


Figure (1): Electrophoresis of three CSF specimens on SDS-discontinuous polyacrylamide gel, 5% stacking gel (discarded) and 10% resolving gel. All lanes were run at 100 V/15mA for resolving gel. CSF were made visible after staining with 0.025% Coomassie brilliant blue G-250. Lane 1 and 2: long time non-properly stored CSF specimens, lane 3: a fresh and properly stored CSF specimens, lane 4: saliva specimen taken from apparently normal volunteer, lane 5: bovine albumin as a size marker, lane 6-7: two serum specimens taken from apparently normal volunteers. The direction of electrophoresis was from top to bottom.

It should be noted that the number of electrophoretic specimens in present study were fewer than being considered as a typical pattern, that because of the rarity of clear CSF specimens in such away it was difficult to obtain samples without visual evidence of erythrocytes made the collector committed to store each specimen under -70°C for long periods of times (more than one month). Therefore, some suitable precautions were taken in which no more than three specimens were stored under -20°C and analyzed by this method.

It was found that even $6\mu\text{l}$ of CSF sample could be seen on polyacrylamide gel when it was stained with Coomassie brilliant blue (figure 2). The possibility to visualize this too little amount of CSF sample by SDS polyacrylamide gel electrophoresis was encouraged me to emphasize that unique pattern of CSF specimens could be seen if someone infected with neurological disease or any malady related with spinal cord.

The possibility of introducing SDS PAGE as indicative tool for CSF specimens was opened the door widely toward more elaborate diagnosis, included the pattern of bands variations of the separated samples. Although protein concentration in CSF samples was not determined in this paper but the direct involvement of CSF samples in SDS PAGE without previous concentration was greatly facilitated pre-handling procedure that precede electrophoresis precluding the ability of electrophoresis CSF samples even without any previous determination of protein concentration, which in some methods waste the sample when the withdrawn amount of the sample is low.

Since the sensitivity and rarity of clear CSF specimens I recommend the interested researchers whom in need to more specimen numbers of that kind to made sure of the existence of stable -70°C storage conditions, because inappropriate storage may reduce band resolution (not shown results). It was recommended to use two dimensional gel electrophoresis techniques as well for more elaborate details of the nature of the separated bands (17). In addition, there was prerequisite necessity to quantify

the obtained results by a spectrophotometer as long as densitometer.

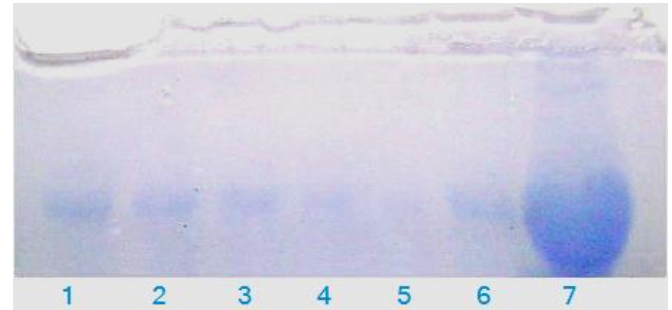


Figure (2): Electrophoresis of graduated concentration of the same CSF specimen on SDS-discontinues polyacrylamide gel, 5% stacking gel (discarded) and 10% resolving gel. All lanes were run at 100 V/15mA for resolving gel. CSF were made visible after staining with 0.025% Coomassie brilliant blue G-250.. Lane 1: $12\mu\text{l}$, lane 2: $10\mu\text{l}$, lane 3: $8\mu\text{l}$, lane 4: $6\mu\text{l}$, lane 5: $4\mu\text{l}$ of CSF the same CSF specimen. Lane 6: bovine albumin marker (67KD). Lane 7: serum. The direction of electrophoresis was from top to bottom.

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