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Effect of sample composition on electrophoretic migration Application to hemoglobin analysis by capillary electrophoresis and agarose electrophoresis

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Abstract

The electrophoretic migration, in routine analysis, is crucial for compound identification especially when multiple components are present in the sample. In complex or crude samples, such as those obtained from biological fluids, electrophoretic migration often does not correspond well to that of a pure standard compound. Several factors, related to the sample itself, have been identified as modulating the electrophoretic migration in zone electrophoresis both in gel and capillary electrophoresis (CE): solute mobility and concentrations, salt content, and protein interaction in the sample. Peak shape asymmetry often signals changes in migration especially when comparing samples with wide differences in concentration or those containing high ionic strength. Also, the migration of a protein can be influenced by the presence of a high concentration of another slowly migrating protein in the sample. A weak interaction during the separation between the two proteins which lead to a decreased velocity has been postulated. This was confirmed by finding a curve–linear relationship between the ratio of the two hemoglobin (Hb) variants, hemoglobin F (Hb F) and hemoglobin S (Hb S), and the distance between the two in gel electrophoresis (GE); and also by the observation of formation of a new small peak based on the analysis of hemoglobin F by capillary electrophoresis upon the addition of Hb S to the separation buffer. These factors when present together have an additive effect on the migration. As an example, Hb F, present in low but variable concentration in patients with sickle cell disease (Hb S), migrates in gel electrophoresis slightly slower than it is expected; enough to be confused with other unknown variants. However, the small peaks with different migration distances between Hb S and the adult Hb (Hb A) correlated well ($r = 0.98$) with Hb F performed by an alkali-denaturing assay indicating that these peaks are indeed Hb F in spite of the difference in their migration.

Keywords: Migration time; Isotachophoresis; Peak symmetry; Sample stacking; Capillary electrophoresis; Gel electrophoresis; Hemoglobins; Proteins

1. Introduction

Migration time in capillary zone electrophoresis (CZE) and migration distance in gel electrophoresis (GE) both are used for unknown identification, analogues to the capacity factor in chromatographic systems. A difference in the migration of a compound from that of a standard indicates that the two are not the same. The migration time which represents the velocity of the charged compound is the product of the apparent mobility (true mobility + electroosmotic flow mobility) and electric field strength in a given medium. Mobility reflects the combination of charge, size, shape and hydrophobicity of a compound under a set of analytical conditions. Thus, any changes in the above parameters can affect the overall migration. However, changes in velocity affecting the migration due changes in the electric field strength (directly or indirectly) are more common in practice and more investigated compared to those due to changes in mobility.

In zone electrophoresis (capillary electrophoresis (CE) or agarose), provided the sample size is small (i.e. does not contribute to the total conductance), and the separation conditions are optimum, the peak shape is symmetrical. However, as the sample concentration, volume, or conductance is increased electrodispersion is increased and peak asymmetry can be observed. In other words, as long as longitudinal diffusion is the only mechanism for band spreading and migration at a constant velocity, Gaussian concentration distributions of the peaks are obtained. However, frequently, non-symmetrical peak shapes (concentrations) are obtained in zone electrophoresis [1] for several reasons such as to increase the sensitivity through increase in sample size or to analyze industrial samples directly without cleanup. These non-symmetrical peaks when combined

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with samples varying in concentration lead to differences in migration time/distance. In practice, several factors, separately or in combination, often lead to peak asymmetry in zone electrophoreses: buffer ion mobility [1,2], capillary wall interaction [3–5], ionic strength of the sample [6], micro-heterogeneity of the band (especially for proteins) [7,8] and sample matrix [9]. Conductivity differences between the analyte zones and the pure background electrolyte (BGE) zones can lead to local electric field strength differences which, in turn, can distort the shape of the analyte bands and result in reduced separation efficiencies [10]. Ionic strength of the electrophoresis buffer and adsorption of some ions on the protein affects also the mobility [11,12].

The theory behind some of the above factors causing peak distortion has been investigated. However, the consequence of peak asymmetry, protein interaction and combination of several of the above factors on migration in routine work is not well studied or well appreciated.

In addition to peak shape, we demonstrate here the component interaction in the sample as another important factor which can affect the migration. This involves weak interactions between different proteins present in the sample. Slight differences in migration distances are easily observed visually in gel electrophoresis since usually many samples (or lanes) are analyzed simultaneously side by side. As an example, we show that many small bands, presumably hemoglobin (Hb F), to migrate in hemoglobin gel electrophoresis at several locations different from that of a standard. This can be problematic or of serious consequences in patient diagnosis and treatment since several uncommon hemoglobin variants can also migrate close to Hb F [13].

In routine work, especially in biological and crude industrial samples, the concentration of many analytes can vary a few orders of magnitude between the healthy and the sick individuals or between different samples. In addition to that, these samples can contain variable amounts of salts or differ in conductance. Here, we illustrate that peak asymmetry together with wide differences in concentration has vital implications on compound identification since the apparent migration can shift. This is very important when the sample components contain multiple charges, such as proteins and peptides which can interact or affect each other. The combination of many of the above factors can affect further the apparent migration leading to misinterpretation of the results. In addition to migration, quantification by peak height lacks linearity and analytical efficiency (i.e. decreased plate height). Here we illustrate that these problems occur often in routine work in both capillary and gel electrophoresis.

2. Materials and methods

2.1. Capillary electrophoresis

2.1.1. Instrument

Two CE instruments were used for the separation. Both were set at similar operating conditions. Model 2000 (Beckman Instruments, Fullerton, CA, USA) and Quanta instrument (Waters, Milford, MA, USA) both set at 9.5 kV (normal polarity) with detection at 214 nm. The samples were injected hydrodynamically (anodic injection) for 10 s or as specified. In some experiments, dimethylsulfoxide (DMSO) (1/2000) was added to the sample to indicate the EOF.

2.1.2. Separation buffer

Borate 200 mM, pH 9.0 or as specified.

2.1.3. Capillary

A short untreated fused silica capillary of 30 cm (22.5 cm to the detector) × 50 μm i.d. (Polymicro Technologies, Scottsdale, AZ, USA) was used to achieve fast analysis.

2.2. Agarose electrophoresis

Two systems were used: the commercial automated system (Sebia, Norcross, GA, USA) and a manual laboratory-made system. For the manual system, plates of 3% agarose were prepared in the separation buffer: 30 mmol/l Tris, 45 mmol/l borate and 2 mmol/l EDTA, pH 8.4. The electrophoresis of lysed hemoglobin cells from an adult (Hb A) was performed for 20 min at 200 V and the red color was visualized without staining. The automated Sebia system was used according to the manufacturer instructions using their proprietary reagents.

2.3. Alkali denaturation test for Hb F

Packed red blood cells, 10 μl were added to 2 ml saponin reagent (saponin 5 mg/100 ml water) mixed and left for 30 s to hemolyze and spectra between 400 and 700 nm recorded. NaOH (2 mol/l), 50 μl were added and after 2 min the spectrum was recorded again. Hemoglobin F was measured from the two maxima at 540 and 575 nm relative to a standard.

2.4. Hemoglobin analysis by CE

The sample was injected hydrodynamically for 7 s and electrophoresed at 9 kV for 9 min at 405 nm [14]. The separation buffer was arginine 2.8 g and Tris 2.4 g dissolved in 100 ml water and the pH was adjusted to 8.5.

3. Results and discussion

In routine analysis by CE peak shape often appears not well symmetrical due to several factors [1,15]. High analyte concentration, conductance, and mobility difference between the sample and the co-ions of the separation buffer are major and related factors. Sample constituents that have mobility higher than that of the carrier separation buffer appear to migrate with a concentration distribution that is diffuse at the front and sharp at the rear of the zone. The reverse holds for sample constituents that have mobility

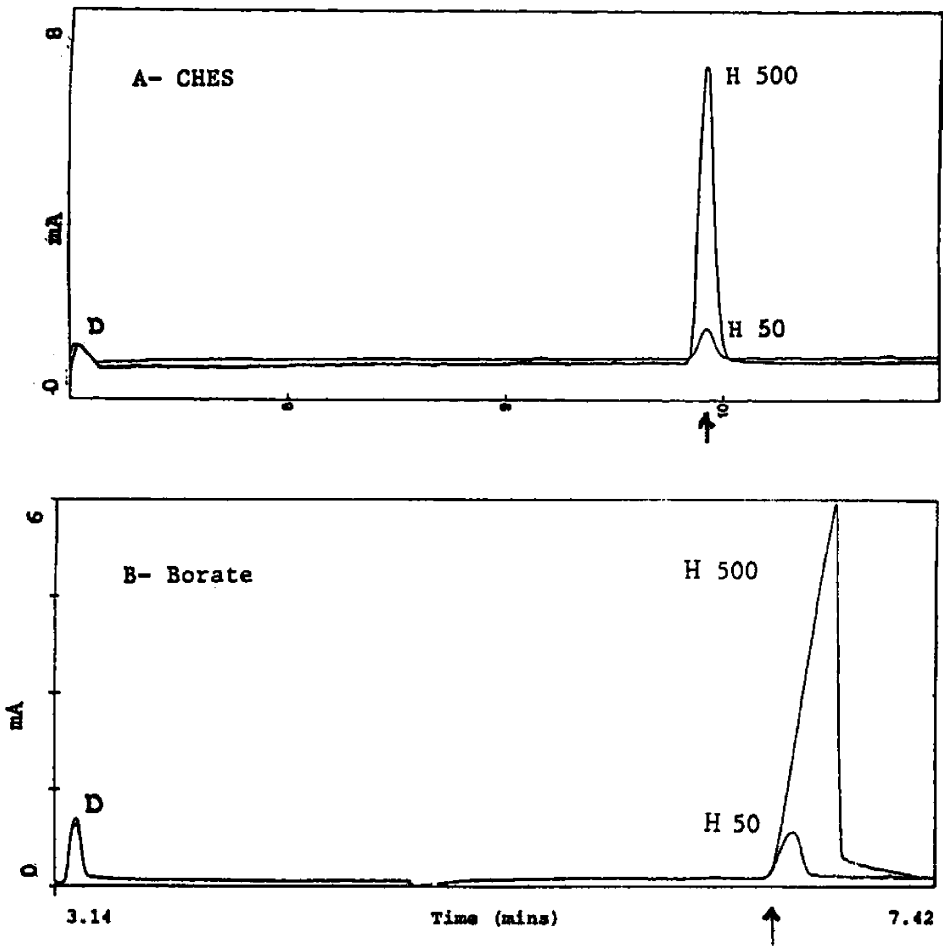

Fig. 1. Effect of both sample concentration and buffer type on migration time for hippuric acid (H): (A) symmetrical peak shape with separation buffer CHES 150 mmol/l, pH 9.0, 214 nm; hippuric acid 500 (top peak) and 50(lower peak) mg/l, and (B) distorted peak shape using separation buffer borate 150 mmol/l, pH 9.0, 214 nm; hippuric acid 500 (top peak) and 50(lower peak) mg/l (D: DMSO; arrow indicates common or constant migration time).

lower than that of the carrier constituent. Skewed peaks especially for samples with wide differences in concentration lead to differences in migration time but within certain limits as shown for hippuric acid in Fig. 1 regardless of the peak is skewed forward, or backward. However, this is absent if the peak is symmetrical. For the asymmetric peak, the slow analyte ions migrate briefly under isotachophoresis (ITP) regulating their speed through a change of their concentration to adjust the field strength in order to keep up moving with same velocity of the leading ion. However, the frontal side of the zone experiences more field strength than the back side of the zone exhibiting distorted peak shape. As the difference in field strength between the two sides of the sample zone (front versus back) increases so does the band diffusion and also the difference in the apparent migration time too. A slow ion analyte in high concentration in the sample leads to an increase in the zone length relative to a one low in concentration. Since this occurs briefly it does not reach equilibrium and the shape of the zone is not typical of isotachophoresis and also not of symmetrical shape. Mikkers et al. [1] have indicated that in the early steps of electrophoresis that both isotachophoresis

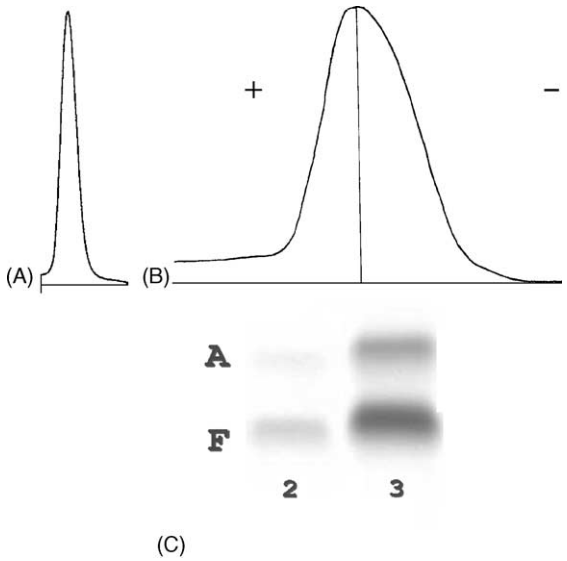

Fig. 2. (A) Peak asymmetry of hemoglobin A in Sebia agarose system with normal X-scale scan; (B) expanded X-scale scan; and (C) (bottom) effect of dilution on Hb F (Sebia system). The sample (AF) of lane 3 was diluted five times in lane 2.

and boundary electrophoresis can occur in conjunction with that of zone electrophoresis. Hippuric acid peak shape (as an example) depends on the buffer type (Fig. 1) being symmetrical in the 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES) and asymmetric in the borate buffer with the apparent migration time in borate buffer fluctuating. For asymmetric peaks, as the concentration of analyte increases, so does the zone width and consequently the apparent difference in migration between a high and a low sample increase too. This effect occurs regardless of the type of diluent in the sample. Samples diluted in low ionic strength buffer show slightly narrower peaks, due to some stacking while those diluted in higher ionic strength show wider peaks due to de-stacking. Other factors can contribute or compound this difference. For example, high ionic strength (excess salts) or very high concentration of the analyte in the sample causes sample de-stacking. As the analyte ions move from high ionic to low ionic strength they accelerate on the front side of the band. Also, low ionic strength separation buffer decreases the tolerance for high salts and also decreases the tolerance for high analyte concentrations in the sample.

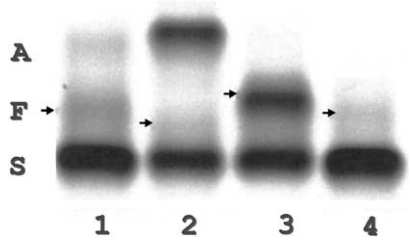

Fig. 3. Affect of hemoglobin S on the migration of hemoglobin F (→). All the lanes contain different amounts of hemoglobin F (→) (Sebia system).

Gel electrophoresis is performed routinely in clinical labs to identify patients with different hemoglobinopathies such as hemoglobin F and hemoglobin S (Hb S). The same factors which affect migration in CE can affect that in GE. The effect of sample concentration on peak shape and consequently on migration is not restricted to CE but also occurs in GE, as illustrated for the separation of hemoglobins in Fig. 2. Using a commercial gel electrophoresis system, hemoglobin A may exhibit initially peak symmetry (Fig. 2A). However, upon careful exami-

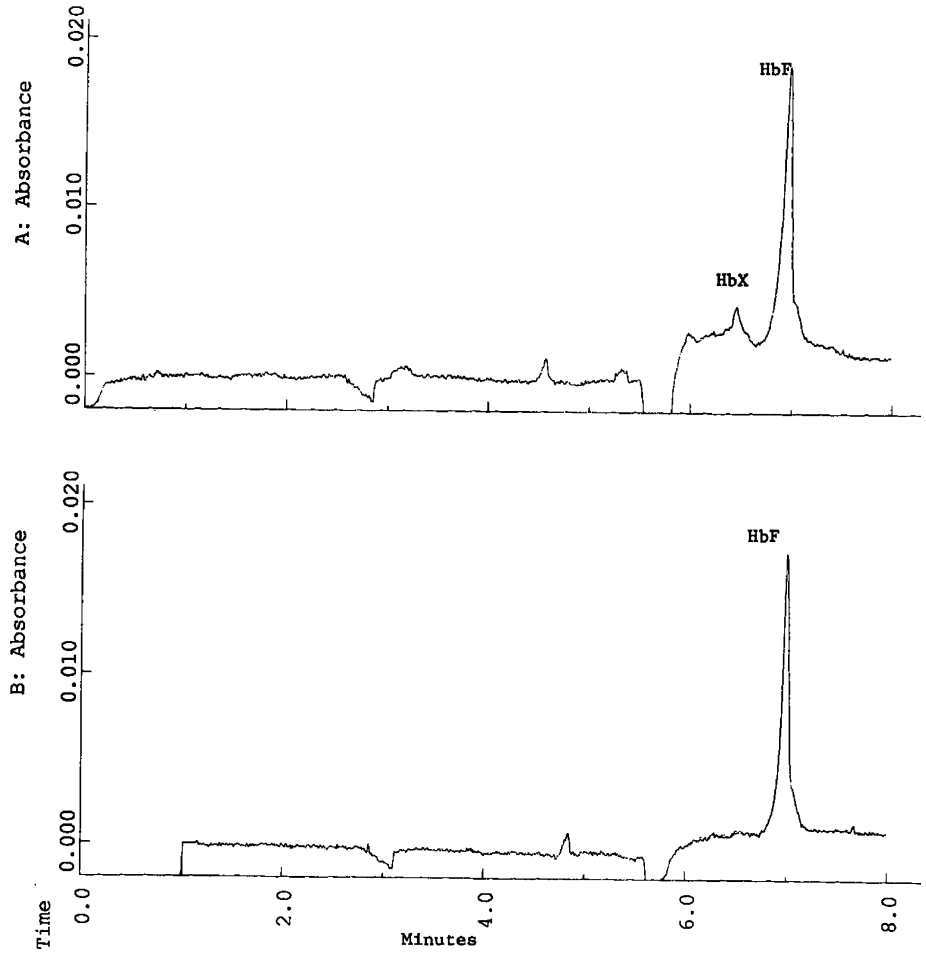

Fig. 4. Separation of hemoglobin F (Hb F) in the sample: (top) in presence, and (bottom) in absence, of an added 100 mg/l of Hb S in the separation buffer (H: new peak). The separation buffer was arginine 2.8 g and Tris 2.4 g dissolved in 100 ml water and the pH was adjusted to 8.5.

nation or upon expanded scale of the *X*-coordinate, peak asymmetry can be detected (Fig. 2B). Samples with low concentrations migrate towards the skewed side compared to the concentrated sample appearing as having a slightly different migration distance (Fig. 2C). This effect can be enlarged if the buffer ions mobility is not well matched with that of the analytes or the sample ionic strength is high. Peak asymmetry and the difference in migration also occurred in a laboratory-made manual gel electrophoresis system where the system was better controlled and defined.

In addition to affecting the migration, wide difference in sample concentration affected two other related and very important parameters, i.e. linearity and plate number. The linearity decreased greatly as measured by peak height as the concentration increased and as the peak deviates from symmetry. Also, in this case the plate number became more inversely dependant on the sample concentration (data not shown).

Another factor is found in this study which can affect the migration time is the composition of the sample. Questionable weak bands with different migration distances between Hb A and Hb S indicated by (→) are observed in Fig. 3. Actually these bands are Hb F. The difference in the migration in the weak mid bands (→) in lanes 1 and 3 or lanes 2 and 3 in Fig. 3 is more dramatic than that observed for the differences due to that by concentration in Fig. 2. The presence of a high concentration of a slow migrating band such as Hb S (in sickle cell disease) in the sample decreases the migration of a minor band like Hb F present in the same sample (Fig. 3). In this case, a weak interaction which occurs during the migration between the molecules of the two bands has to be postulated. Proteins contain several charges and can interact with other compounds such as drugs or other proteins. If the constituents of the sample tend to interact during the migration this affects the migration time. This fact is well studied in drug binding to proteins [16] or antigen–antibody complexes [17,18] where the binding is strong. Protein interaction with buffer ions and its effect on migration have been also reported [11,12]. Multivalent weak ionic species in the buffer can interact with analytes with similar mobilities and result in a strong increase of electromigration. However, here we deal with more subtle or weak interactions which can affect the migration in both CE and GE. This was confirmed here in CE by adding a small amount of Hb S to the separation buffer and observing the formation of a new small but slowly migrating peak (Hb X) when Hb F is separated (Fig. 4). It is difficult without further experiments to determine if this interaction between Hb S and Hb F is specific, due to sample matrix [9] or due to interaction with system peaks [19]. However, if Hb F is present in the sample while the buffer contains Hb F in place of Hb S the small peak is not detected (Fig. 4).

Hb F concentration in blood can vary widely from almost zero to ~90% depending on the age of the individual and, and disease. The presence of small amounts in the range of 1–3% is used to diagnose thalassemia in patients with Hb S Disease. Moreover, hydroxyurea is commonly used to treat patients with sickle disease to increase the Hb F level. Several uncommon Hb variants such as Presbyterian, Deaconess, and Manitoba migrate close to the Hb F location on gel electrophoresis and can confound the identification. The presence of varying amounts of Hb S in the sample affected greatly the migration of Hb F (Fig. 4), which can lead to misinterpretation of the variants.

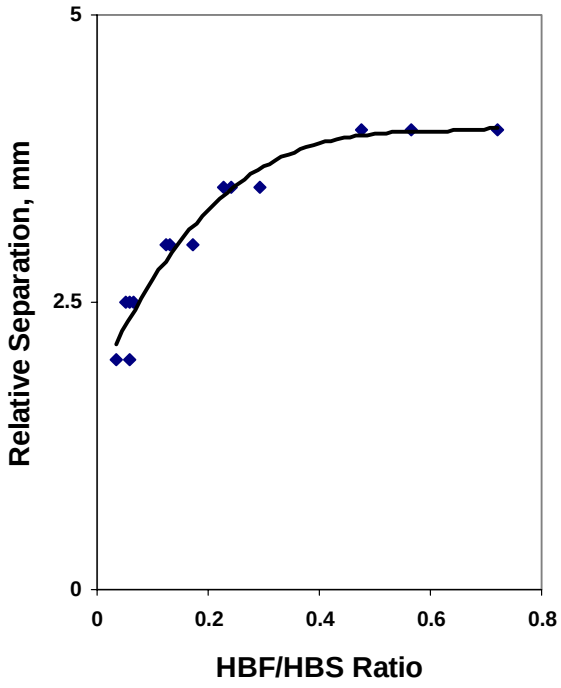

Fig. 5. Ratio of Hb F/Hb S and the relative distance between the two variants (in mm) by agarose electrophoresis (Sebia).

There is a good curvilinear relationship between the ratio of Hb F/Hb S and the distance between these two bands (Fig. 5). This figure illustrates also the importance or the additive effect of more than one factor: the combination of concentration and the weak protein interaction on modulating the migration distance/time further. As the concentration of Hb F relative to Hb S decreases so the difference in mobility due to protein interaction increases. In practice, this relationship is helpful for identification of these peaks. This relationship is not restricted to Hb F. It is observed also for Hb A (Fig. 3, lane 1). The unknown small peaks migrating between Hb S and Hb A but not matching the same migration of that for standard Hb F correlated well with Hb F performed by alkali denaturation test for Hb F (Fig. 6) and also by addition of an added Hb F to the sample. In other words they are indeed Hb F despite the difference in their migration. The presence of a large concentration of Hb S in the sample slowed, to different degrees, the migration of these bands indicating the existence of some interaction.

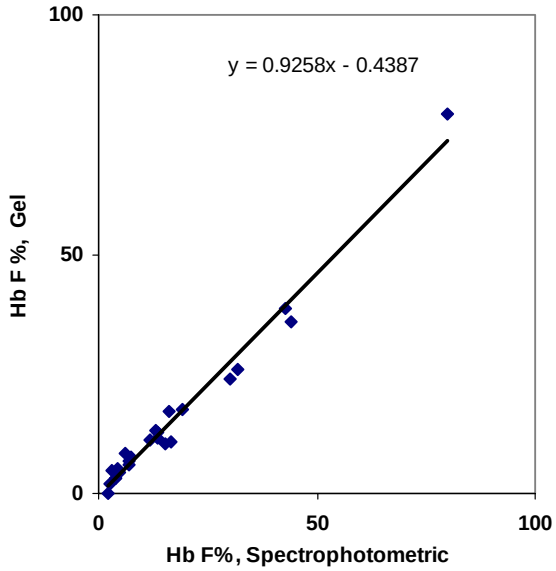

Fig. 6. Correlation of Hb F by gel and by spectrophotometric-alkali denaturation test.

4. Conclusions

The previous data illustrates that the apparent migration of a compound is subject to modification and often encountered in routine analysis. The deviation from a Gaussian distribution for the peak shape is a good indication that the migration is subject for modulation. Several factors can contribute to this deviation such as: sample matrix (proteins and salts), sample concentration, buffer ion mobility and ionic strength all can interact and cause a shift in the analyte migration, which can complicate peak identification, but within certain limits. These factors operate often through changes in the field strength and less frequently through mobility changes. Weak protein interaction in the sample was found in this work to affect mobility. The combination of more than one factor had an additive effect on migration changes. This information should be helpful not just for proper interpretation of hemoglobin analysis but in general for the identification of complex samples.

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