ELSEVIER

Journal of Chromatography A, 709 (1995) 99–110

JOURNAL OF CHROMATOGRAPHY A

Capillary zone electrophoresis of serum proteins: study of separation variables

Vladislav Dolník

Institute of Analytical Chemistry, Academy of Sciences of the Czech Republic, Veveří 97, CZ-61142 Brno, Czech Republic

Abstract

Electrophoresis of serum proteins is one of the traditional applications of zone electrophoresis. Whereas electrophoresis in supporting media uses usually 5,5'-diethylbarbiturate at pH 8.6 as the buffer, in capillary zone electrophoresis with on-line UV detection, this electrolyte is of little use because of its high UV absorbance. For that reason, a number of operational electrolytes differing in composition were tested for use in capillary electrophoresis of serum proteins. The influence of the pK_A of co-ions and counter ions and the concentration of the operational electrolyte was examined. If 0.1 *M* methylglucamine–0.1 *M* ϵ-aminocaproic acid or 0.1 *M* methylglucamine, –0.1 *M* γ-aminobutyric acid is used as the operational electrolyte, capillary electrophoresis separates serum proteins into more than ten zones.

1. Introduction

Electrophoresis of serum proteins has been among the classical methods in clinical chemistry for many years. Usually paper, agar or cellulose acetate served as the separation bed, and serum proteins were separated into about five fractions: albumin, α_1-globulin, α_2-globulin, β-globulin and γ-globulin. More than 20 years ago, agarose brought a significant improvement, enabling more protein zones to be separated [1,2]. The method has since been modified [3–9] and the separated zones were assigned to the individual proteins: prealbumin, albumin, α_1-lipoprotein, α_1-antitrypsin, α_2-macroglobulin, haptoglobulin, β-lipoprotein, transferrin, C_3-complement and γ-globulin [2,5,7]. The clinical interpretation of electropherograms is based on variation in their content [6,7,10,11]. In all these methods, veronal (5,5'-diethylbarbiturate) buffer has been used. Since this is a US Drug Enforcement Administration controlled drug, Monthony et al. [12] replaced veronal with Tricine and proposed the Tris–Tricine buffer for the electrophoresis of serum proteins. However, with this newly formulated buffer, α_1-fraction co-migrated with albumin in agarose electrophoresis. The better resolution of α_1-fraction and albumin in the barbital buffer seems to be due to the formation of a veronal–albumin complex and this complex exhibits higher mobility than albumin itself because of the higher charge caused by ionization of veronal. An increase in the ionic strength of the buffer by adding NaCl helps to separate albumin and α_1-fraction; however, addition of salicylate or hippurate, which also form complexes with serum albumin, leads to a more pronounced effect [13].

Several papers have been devoted to the electrophoresis of serum proteins in capillary format with on-line detection [14–19]. Whereas Gordon et al. [14] and Reif et al. [19] used

0021-9673/95/$09.50
SSDI 0021-9673(95)00161-1

sodium borate as the operational electrolyte, Chen and co-workers [15–18] did not specify the composition of their electrolyte and used the description Beckman protein analysis buffer.

This paper shows the effect of some separation variables, especially the pK_A of both the co-ion and counter ion, on the separation of serum proteins by capillary electrophoresis.

2. Experimental

Bis-Tris and Tricine were purchased from Fluka (Buchs, Switzerland), vinylmagnesium bromide from Aldrich (Milwaukee, WI, USA) and γ-aminobutyric acid, ε-aminocaproic acid and N-methyl-D-glucamine from Sigma (St. Louis, MO, USA). All other chemicals were purchased from Lachema (Brno, Czech Republic).

Analyses were performed with a Crystal CE System Model 310 (ATI Unicam, Cambridge, UK) with UV detection using a Spectra 100 variable-wavelength detector (Thermo Separation Products, Palo Alto, CA, USA) at 200 nm. The data were collected by using software 4880 (ATI Unicam).

A fused-silica capillary (J&W Scientific, Fulsom, CA, USA) of dimensions 75 μm I.D. and 360 μm O.D. was used. The total length was usually 55 cm with 45 cm to the detection window. The detection cell was made by cutting off ca. 1 mm of the polyimide layer [20]. In some experiments, coated capillaries were used where the coating was made with polyacrylamide after vinylation of the capillary surface with vinylmagnesium bromide [21].

Whole blood was drawn from healthy volunteers (members of this laboratory) and used to prepare serum. Serum was used immediately after centrifugation; however, in some cases it was kept at −26°C before its use.

3. Results and discussion

The aim of this work was the experimental testing of a series of various operational electrolytes. The particular zones were identified according to the generally known migration order of serum proteins in agarose gels. Analysis of serum proteins from different individuals was helpful in this respect. Staining of lipoproteins with Sudan Black with subsequent electrophoresis and detection at 570 nm showed only limited possibilities for the identification of lipoproteins. If the fractions were resolved into several subfractions, α_1-antitrypsin, α_2-macroglobulin and transferrin were considered as the main components of α_1-globulin, α_2-globulin and β-globulin, respectively, and their mobilities were used to express the mobility of the whole fraction. (Proteins behave as anions under given conditions and the negative values of the mobility correspond to this fact. However, for sake of simplicity, "higher mobility" here means a higher absolute value of mobility.) Substances differing in pK_A suitable as both counter ion and co-ion were sought. Finally, the co-ions were chosen according to their pK_A to cover the pH range which preliminary experiments had shown to be the most important, i.e., $pK_A \approx 8–11$. Three counter ions were used in these experiments: Na^+, Tris and methylglucamine. The pK_A values of the substances in question, and also the pH of the operational electrolytes, which were prepared by simple mixing of weighed substances without any further pH adjustments, are given in Table 1.

In the first series of experiments, operational electrolytes containing 50 m*M* NaOH and 0.1 *M* co-ion were used. As NaOH is a strong base, the pH of the electrolyte is equal to the pK_A value of the individual co-ions. The dependence of absolute value of mobility of the main serum protein fractions on the pK_A of the co-ions is given in Fig. 1: all fractions of serum proteins migrate with a higher velocity with increasing pK_A of the co-ion, i.e., with increasing pH of the operational electrolyte. The separation of the albumin–α_1-fraction pair is crucial, as pointed earlier. With increasing pH, the mobility difference between albumin and α_1-fraction increases, whereas the β-globulin and α_2-globulin mobility window is reduced even when the β-globulin–α_2-globulin separation is not deteriorated.

Table 1
pH of operational electrolytes

Component	Na^+	Tris ($pK_A = 8.1$)	MGA ($pK_A = 9.9$)
Tricine ($pK_A = 8.1$)	8.12	8.06	9.10
Asparagine ($pK_A = 8.72$)	8.91	8.41	9.47
Boric acid ($pK_A = 9.24$)	9.24	8.17	9.20
Glycine ($pK_A = 9.78$)	9.86	8.84	9.92
GABA ($pK_A = 10.46$)	10.58	9.27	10.26
EACA ($pK_A = 10.80$)	10.93	9.36	10.62

Fig. 2 shows the sample plot when the counter ion Na^+ is replaced with Tris. If an equimolar mixture of Tris and the appropriate acid is used, the pH of the operational electrolyte is theoretically equal to the mean of the pK_As of Tris and the co-ion, as Tris is a weak base. The ionization also influences the ionic strength of the solution. That is why it is more reasonable to plot mobilities vs. pK_a instead of the traditional pH. The real values of pH for the prepared operational electrolytes are given in Table 1. We can observe similar effects as with electrolytes containing

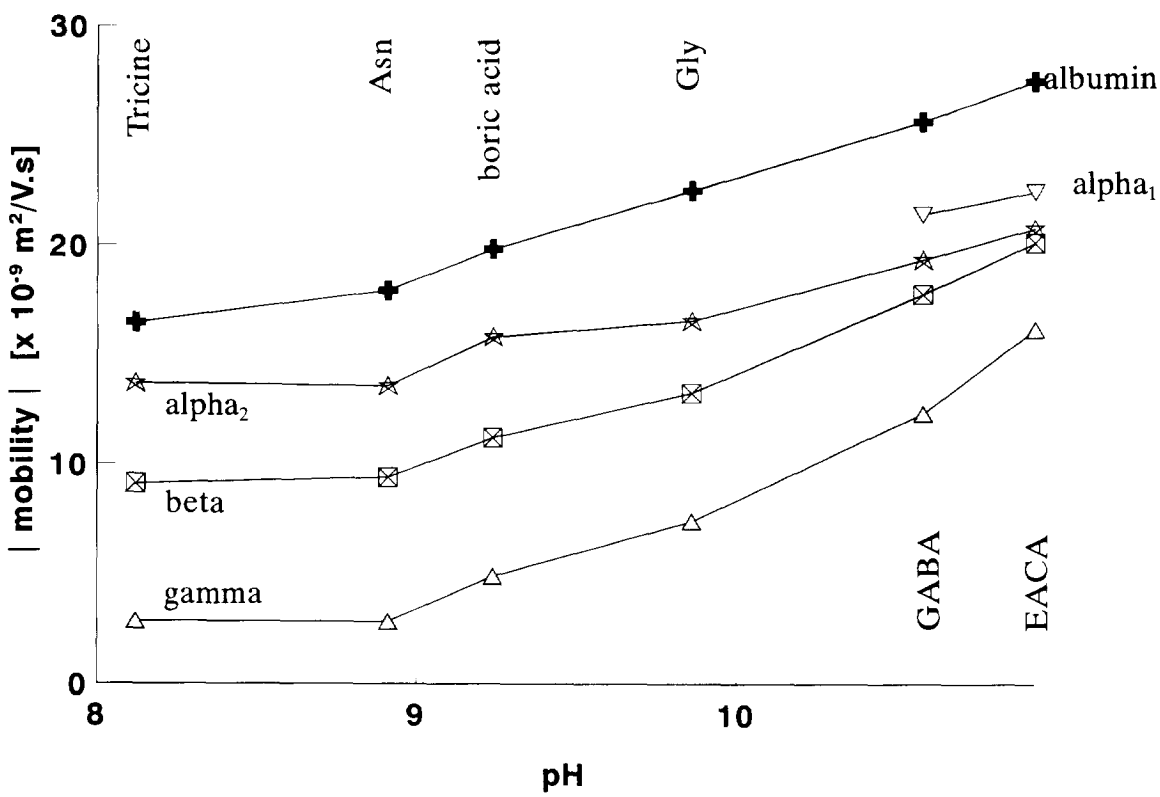

Fig. 1. Effect of pH on the mobility of serum proteins. Experimental conditions: uncoated capillary, 75 μm I.D. × 360 μm O.D., total length 550 mm, effective length 450 mm; operational electrolyte, 50 m*M* NaOH–100 m*M* co-ion; voltage, 11 kV. Fourfold diluted serum sampled with a pressure of 10 mbar for 3 s.

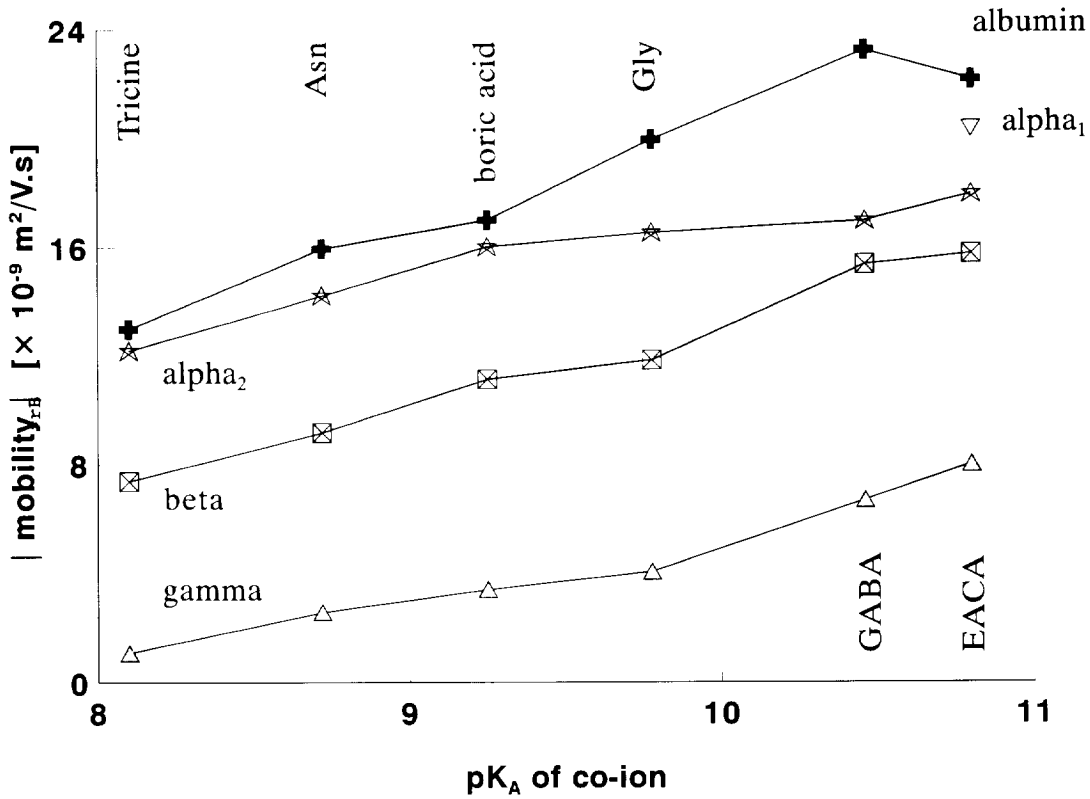

Fig. 2. Effect of pK_A of co-ion on the mobility of serum proteins with Tris as counter ion. Operational electrolyte: 0.1 *M* Tris–0.1 *M* co-ion. Other experimental conditions as in Fig. 1.

sodium, i.e., an increase in the co-ion pK_A or pH results in a better resolution between albumin and α-fractions and a lower resolution of β- and γ-globulins. However, in this case, the α_1-globulin peak is obtained with Tris–ϵ-amino-caproic acid exclusively.

The best results were obtained when methylglucamine (MGA) was used as the counter ion in the operational electrolyte formulations. Therefore, the corresponding electropherograms are discussed individually (Fig. 3). In 0.1 *M* MGA–Tricine (Fig. 3a), the most remarkable effect is the low mobility of γ-globulin, which results in co-migration of this zone with the system peak indicating zero mobility. This is surprising, since in Na–Tricine, γ-globulin migrates anodically to the full extent, although the pH is significantly lower than in 0.1 *M* MGA–Tricine. This is probably caused by the interaction of the counter ion with γ-globulins forming ion associates and/or ion pairs, which results in charge reduction and hence a decrease in mobility. In the α_2-globulin–β-globulin area, there is a good resolution of the appropriate subfractions. The peak close to albumin (t_m = 22.98 min) does not correspond to α_1-globulin, as α_1-globulin provides in our sera a significantly smaller peak than the α_2-fraction.

The use of 0.1 *M* MGA–asparagine provides an electropherogram similar to that with 0.1 *M* MGA-Tricine (Fig. 3b). A smaller portion of γ-globulins appears on the cationic side of the electropherogram. The UV absorption of asparagine results in a significant decrease in the signal-to-noise ratio.

Use of the popular boric acid as the co-ion failed in the separation of serum proteins in conjunction with MGA (Fig. 3c). This is caused

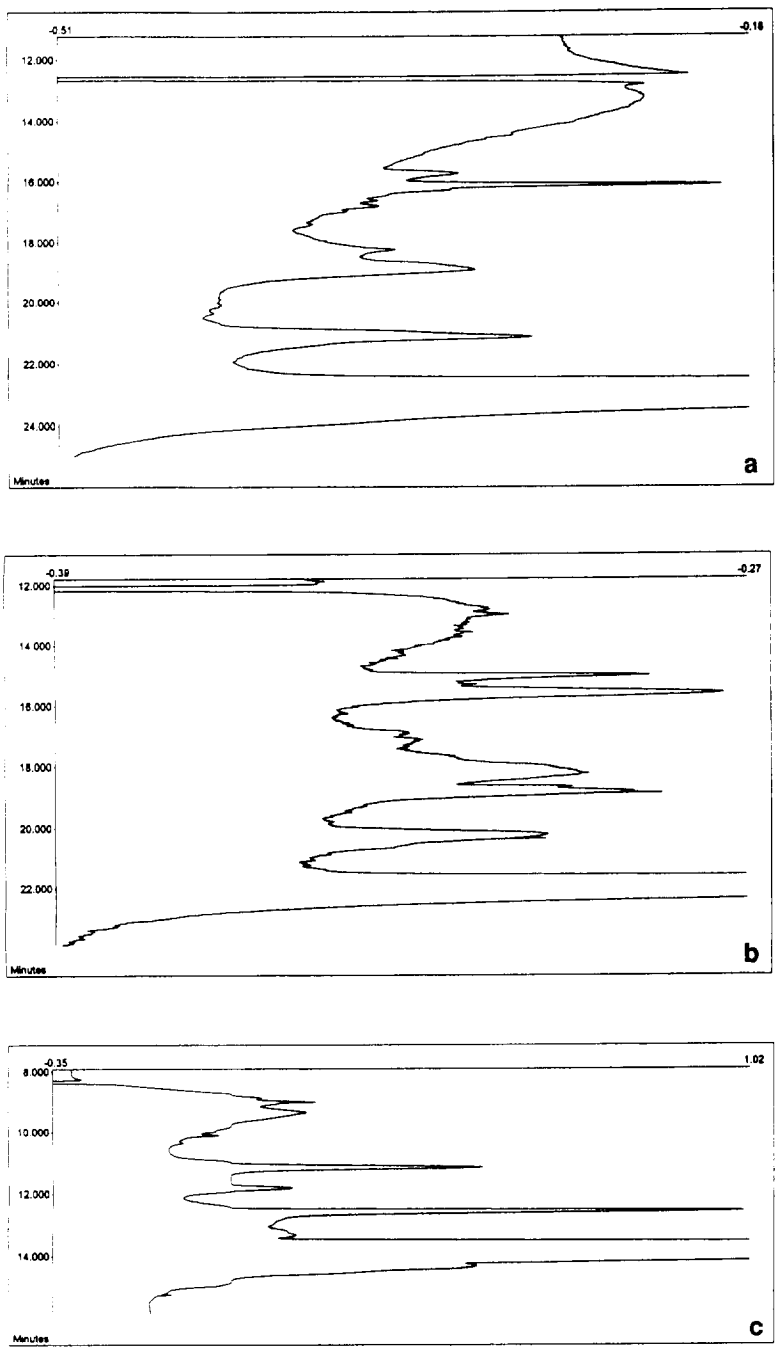

Fig. 3. (*continued on p. 104*).

by the complexation of boric acid with hydroxy groups of MGA, which results in a shift of the pK_A of boric acid and a complicated acid–base equilibrium manifested in a significant deviation of the real pH of the operational electrolyte from the theoretical value.

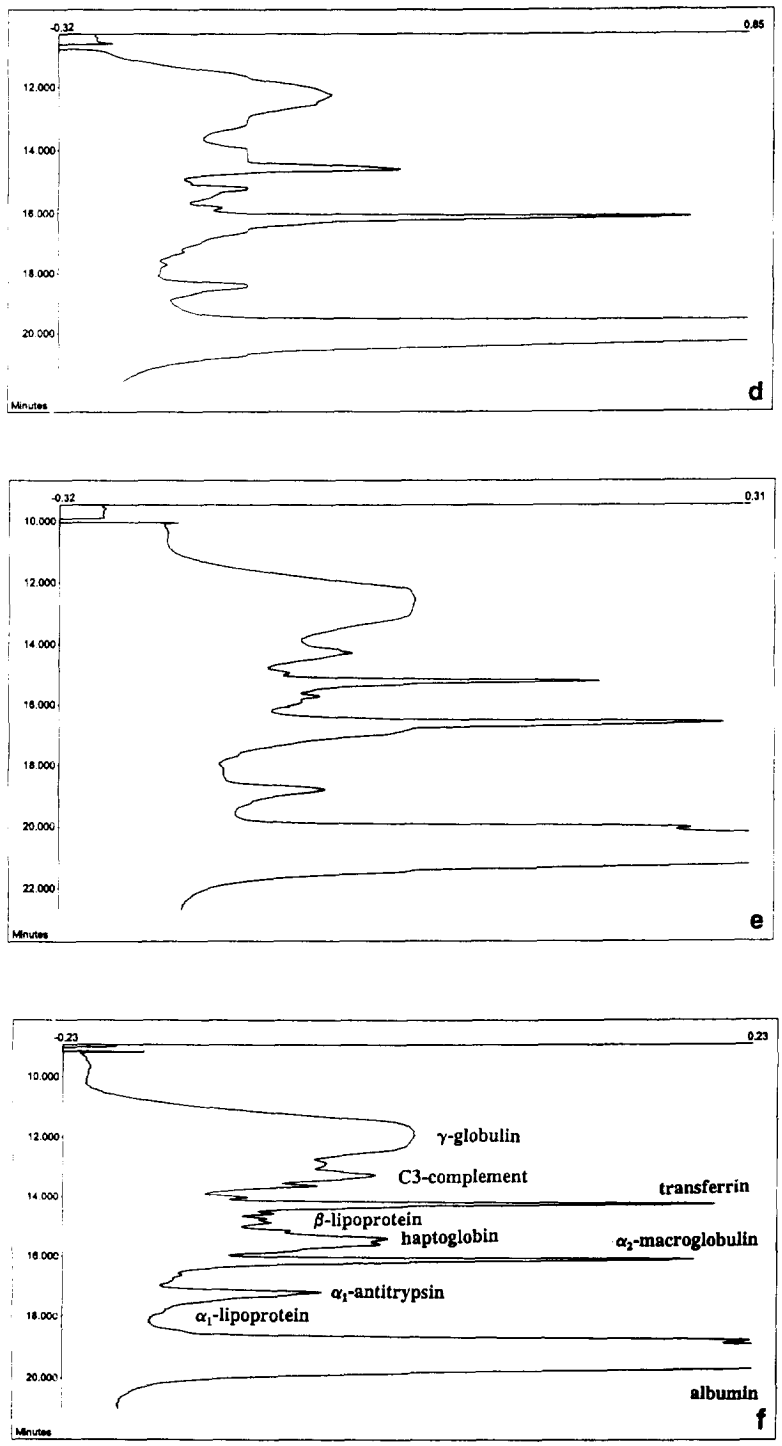

Fig. 3. Electropherogram of serum proteins in operational electrolyte containing 0.1 *M* methylglucamine as counter ion. Co-ion: (a) 100 m*M* Tricine; (b) 100 m*M* asparagine; (c) 100 m*M* boric acid; (d) 100 m*M* glycine; (e) 100 m*M* γ-aminobutyric acid (GABA); (f) 100 m*M* ε-aminocaproic acid (EACA). Other experimental conditions, as in Fig. 1. Ordinate: absorption at 200 nm.

A further increase in pK_A or pH by using glycine as the co-ion leads to an improvement in resolution, primarily by the independent migration of α_1-fraction (Fig. 3d). All the zones, which are known from electrophoresis in agarose, can be found in the electropherogram obtained with 0.1 *M* MGA–glycine.

Similarly, all fractions were found if γ-aminobutyric acid (GABA) (Fig. 3e) and ε-aminocaproic acid (EACA) (Fig. 3f) were used as the co-ion. With the highest mobility, i.e., with the longest migration time, prealbumin appears in the electropherogram. (At least one UV-absorbing non-protein peak with a mobility higher than that of prealbumin can be found in the electropherograms if the separation is performed for a sufficient time. As it was not the aim of this study to analyse all peaks, such as uric acid, no significant attention was devoted to these peaks.) There was a regularly appearing shoulder on the peak of albumin, the identity of which is unknown. In α_1-fraction, the main peak of α_1-antitrypsin can be observed with at least one shoulder, which may correspond to α_1-lipoprotein. Experiments with Sudan Blue-stained serum were not successful in identifying the α_1-lipoprotein peak. With a mobility smaller than that of α_1-globulin, small peaks of group-specific proteins migrate. In α_2-globulin fraction, haptoglobulin and α_2-macroglobulin peaks can be seen. It is not easy to distinguish them, as their migration order varies with the phenotype of the individuals. There are three peaks (series of peaks) in β-fraction: β-lipoprotein, transferrin and C_3-complement. Whereas transferrin migrates as an individual sharp peak, the other subfractions provide several peaks. Both C_3-complement and β-lipoprotein are unstable in serum and decompose. Hence the heterogeneity of these subfractions is at least partially caused by the decomposition.

If we compare the mobilities of the main protein fractions obtained with methylglucamine as the counter ion, a fairly similar plot is obtained as for sodium and Tris (Fig. 4). The mobilities of proteins increase with increasing pH or pK_A; however, the presence of the α_1-fraction makes a significant difference. As methylglucamine is a weak base, the pH of the operational electrolytes is not identical with the pK_A of the co-ions again. Because the ionic strength varies in the given range, it is advantageous to plot mobility vs. pK_A. The real values of pH for the operational electrolytes are given in Table 1.

When the electroosmotic coefficient from the measurements mentioned above is plotted vs. pK_A (Fig. 5), an interesting plot is obtained. The electroosmotic flow is virtually independent of pH in the given pH range when sodium is used as the counter ion with constant ionic strength. When sodium is replaced with Tris or methylglucamine, a dependence of the electroosmotic coefficient is found, which is more pronounced for methylglucamine. This effect seems to be caused by the decreasing ionization of these organic bases with increasing pH, which results in reduced inter-ionic interaction of these bases with the capillary wall and thus in a higher value of the electroosmotic coefficient.

Further, we were interested in knowing the concentration dependence of the mobilities of serum proteins, with the aim of increasing the operational voltage and hence decreasing the separation time. The dependence of the mobilities of the main serum protein fractions on the concentration of methylglucamine–EACA is plotted in Fig. 6. The plot shows that α_1-fraction disappears from the electropherogram at concentrations below 50 m*M*. The operational electrolyte 10 m*M* methylglucamine–EACA enabled an electric field strength of 545 V/cm to be used without any overheating, thus shortening the analysis time significantly. However, it did not help α_1-fraction to appear in the electropherogram and therefore 0.1 *M* methylglucamine–EACA is recommended as a suitable operational electrolyte for the electrophoresis of serum proteins.

Because of the known effect of hydrophobic acids on the resolution of albumin–α_1-fraction, we decided to investigate this effect in capillary electrophoresis. Unfortunately, the acids, the improving effect of which is known from slab gel electrophoresis, absorb UV radiation and therefore their use in capillary electrophoresis with

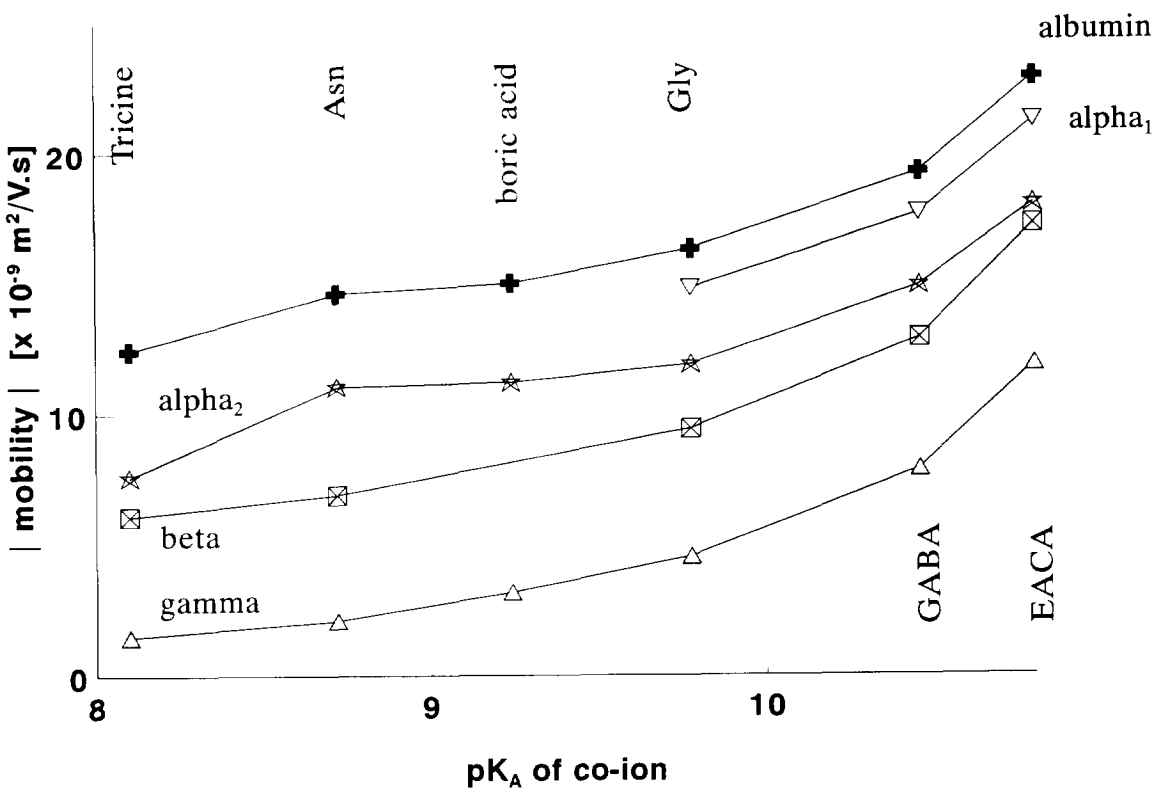

Fig. 4. Effect of pK_A of co-ion on the mobility of serum proteins with methylglucamine as counter ion. Operational electrolyte: 0.1 *M* methylglucamine–0.1 *M* co-ion. Other experimental conditions as in Fig. 1.

on-line UV detection is impossible. For that reason, the influence of fatty acids was investigated. Attention was paid primarily to the effect of lauric acid when (i) lauric acid was the only co-ion in the operational electrolyte and (ii) when lauric acid was used in a mixture with another co-ion.

When the operational electrolyte containing 5 m*M* methylglucamine and 2.5 m*M* lauric acid was used, α_1-fraction was not resolved from albumin and appeared as a shoulder on the albumin peak. When the concentration of operational electrolyte was doubled, the α_1-globulin–albumin resolution was improved, probably by forming a laurate–albumin complex which exhibits higher mobility than albumin itself. The low ionic strength of this electrolyte enables a high operational voltage to be applied. In the given system, a maximum voltage of 30 kV could be successfully used to shorten the analysis time whilst maintaining the separation of the main fractions of serum proteins in about 3 min (Fig. 7).

If the concentration of the operational electrolyte is increased further to 60 m*M* methylglucamine–30 m*M* lauric acid, the electrophoretic profile of serum proteins is dramatically changed (Fig. 8). Albumin migrates as two split peaks and a number of new peaks appear in the electropherogram. The number of new peaks suggests a good potential for this electrophoresis of serum proteins. However, our optimism is moderate, as a lot of work, primarily in identification and clinical interpretation of electropherograms, must be done in order to introduce this particular analysis into the family of practical applications of capillary electrophoresis. At the given concentration, laurate forms micelles, which should be borne in mind by those who distinguish zone electrophoresis from micellar

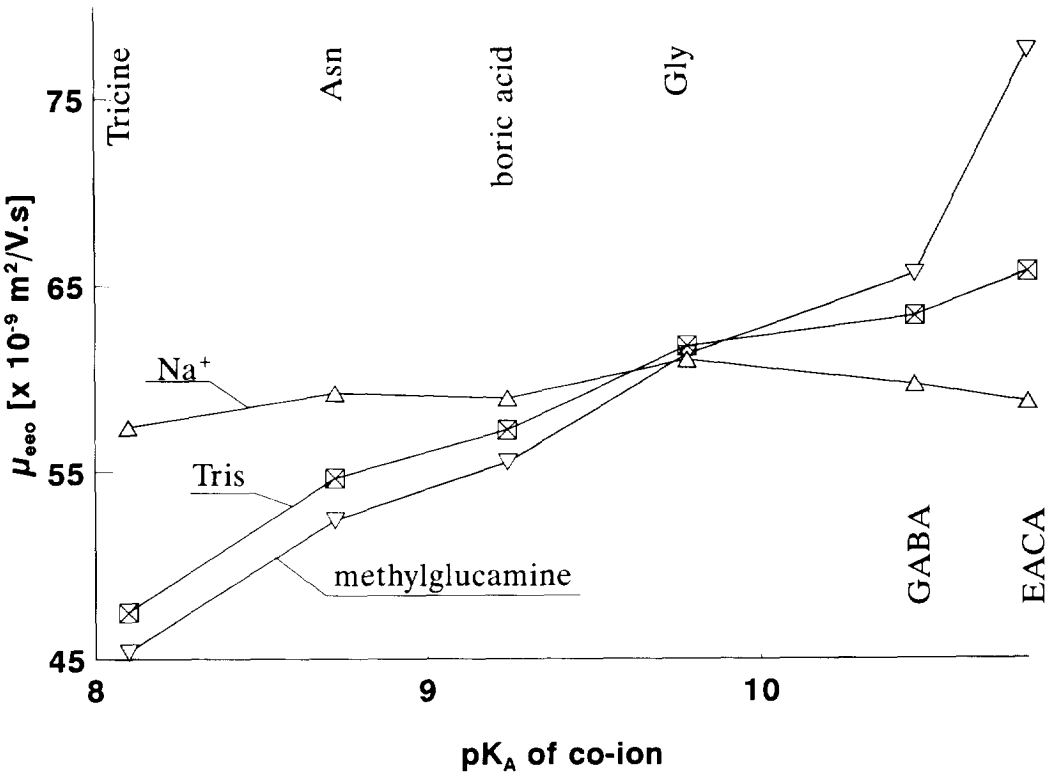

Fig. 5. Effect of pK_A of co-ion on the electroosmotic coefficient with sodium, Tris and methylglucamine as counter ion. Experimental conditions as in Figs. 1, 2 and 4.

electrokinetic chromatography. In our approach, it is sufficient to speak about zone electrophoresis of the protein complexes with laurate. Indeed, the dimensions of protein molecules do not support the simplistic model of spherical micelles with separands incorporated in.

If the operational electrolyte contains lauric acid in the presence of other ions, the detrimental effect of laurate on the integrity of serum proteins starts at lower concentrations. This effect is obvious with an operational electrolyte containing 100 m*M* methylglucamine–90 m*M* EACA–5 m*M* lauric acid (not shown), i.e., at a concentration of lauric acid which, in the absence of other anions did not attack the integrity of serum proteins. This effect is easy to explain: an increase in ionic strength results in increased hydrophobic interactions, and that is exactly what happened in this case. If the content of lauric acid is further increased, the albumin and γ-globulin peaks are preserved, but a number of new peaks with mobilities higher than that of albumin appear in electropherogram (Fig. 9).

4. Conclusions

Good resolution of serum proteins is achieved by capillary electrophoresis when the operational electrolyte 0.1 *M* methylglucamine–0.1 *M* EACA or 0.1 *M* methylglucamine–0.1 *M* GABA is used. An operational electrolyte containing 10 m*M* methylglucamine and 5 m*M* lauric acid permits the separation of serum proteins with high voltage and the main serum protein fractions can be separated in about 3 min. Capillary electrophoresis in the presence of 60 m*M* methylglucamine–30 m*M* lauric acid or 100 m*M* methylglucamine–80 m*M* EACA–5 m*M* lauric acid provides the separation of a large

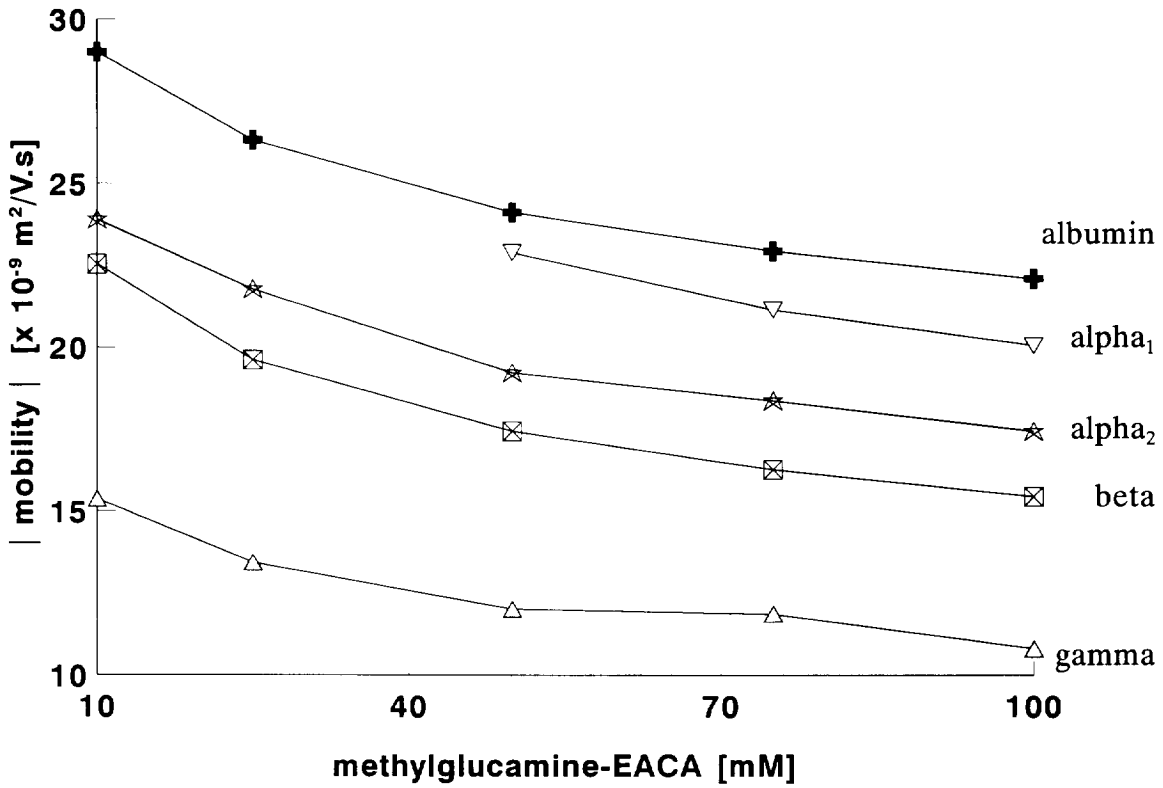

Fig. 6. Effect of concentration of equimolar methylglucamine–EACA on the mobility of serum proteins. Operational electrolyte: 0.01–0.1 *M* methylglucamine–0.01–0.1 *M* co-ion. Other experimental conditions as in Fig. 1.

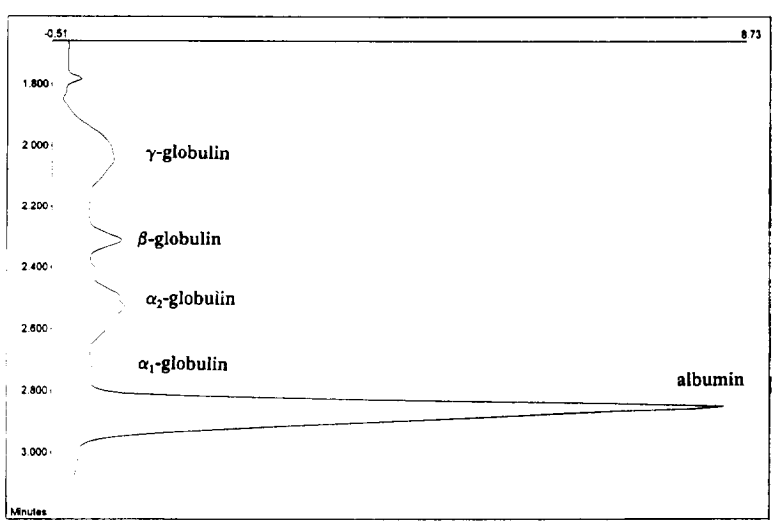

Fig. 7. Electropherogram of serum proteins in operational electrolyte containing 10 m*M* methylglucamine and 5 m*M* lauric acid. Voltage, 30 kV. Other experimental conditions, as in Fig. 1. Ordinate: absorption at 200 nm.

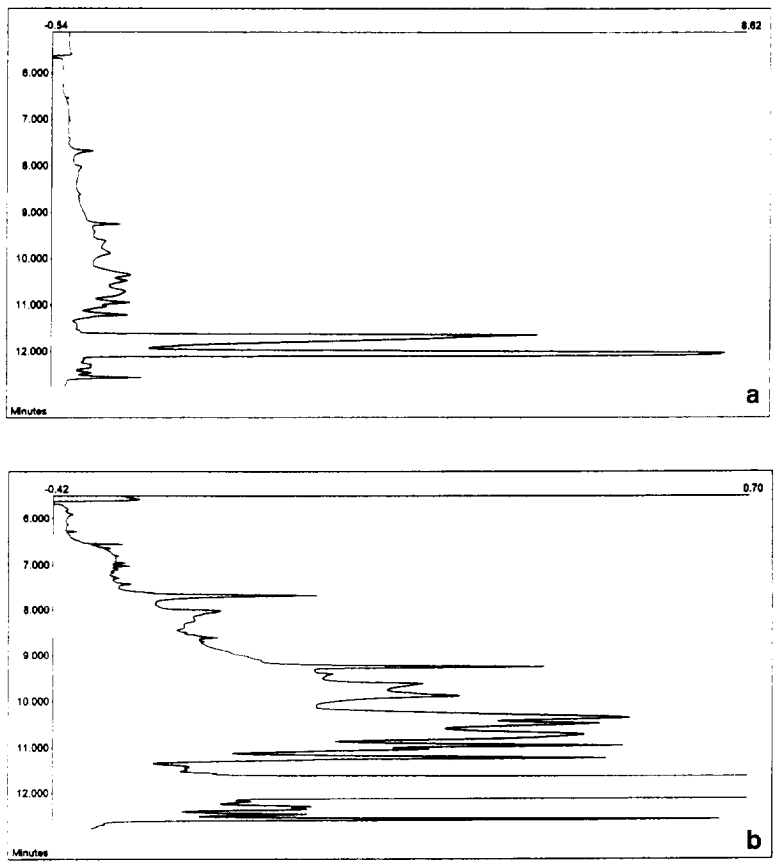

Fig. 8. Electropherogram of serum proteins in operational electrolyte containing 60 m*M* methylglucamine and 30 m*M* lauric acid. Experimental conditions, as in Fig. 1. Ordinate: absorption at 200 nm. (a) and (b) differ by ordinate scale.

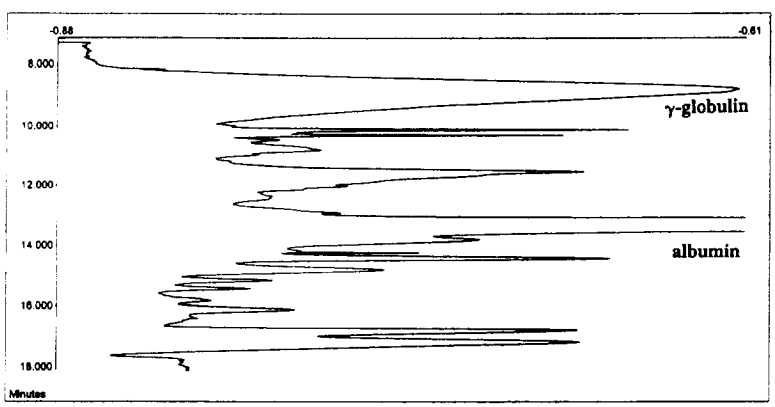

Fig. 9. Electropherogram of serum proteins in operational electrolyte containing 100 m*M* methylglucamine, 80 m*M* EACA and 10 m*M* lauric acid. Experimental conditions as in Fig. 1. Ordinate: absorption at 200 nm.

number of peaks as a result of the disintegration of serum proteins by hydrophobic interactions with laurate. This gives new potential to the electrophoresis of serum proteins.

Acknowledgements

The author thanks ATI Unicam (Cambridge, UK) and Chromspec (Prague, Czech Republic) for providing the full instrumentation for capillary electrophoresis. A gift of a silica capillary from J&W Scientific (Folsom, CA, USA) is also acknowledged.

References

[1] B.G. Johansson, Scand. J. Clin. Lab. Invest., 29, Suppl. 124 (1972) 7.
[2] C.B. Laurell, Scand. J. Clin. Lab. Invest., 29, Suppl. 124 (1972) 71.
[3] J.F. Heremans and P.L. Masson, Clin. Chem., 19 (1973) 294.
[4] R.F. Ritchie and R. Smith, Clin. Chem., 22 (1976) 497.
[5] T. Sun, S.-K. Chan and S. Gross, Am. J. Clin. Pathol., 67 (1977) 247.
[6] T. Sun, Y.Y. Lien and S. Gross, Ann. Clin. Lab. Sci., 8 (1978) 219.
[7] J.-O. Jeppson, C.-B. Laurell and B. Franzén, Clin. Chem., 25 (1979) 629.
[8] K. Ojala and T.H. Weber, Clin. Chem., 26 (1980) 1754.
[9] M. Doren, D. Conlon and L. Blankstein, Clin. Chem., 27 (1981) 200.
[10] M. Engliš, in Z. Deyl (Editor), Electrophoresis, Part B, Elsevier, Amsterdam 1983, p. 201.
[11] E. Gianazza and P.G. Righetti, J. Chromatogr., 569 (1991) 43.
[12] J.F. Monthony, E.G. Wallace and D.M. Allen, Clin. Chem., 24 (1981) 1825.
[13] J. Ambler and M. Rodgers, Clin. Chem., 26 (1980) 1221.
[14] M.J. Gordon, K.-J. Lee, A.A. Arias and R.N. Zare, Anal. Chem., 63 (1991) 69.
[15] F.-T.A. Chen, C.-M. Liu, Y.Z. Hsieh and J.C. Sternberg, Clin. Chem., 37 (1991) 14.
[16] F.-T.A. Chen, J. Chromatogr., 559 (1991) 445.
[17] F.-T.A. Chen, Clin. Chem., 38 (1992) 1651.
[18] F.-T.A. Chen and J.C. Sternberg, Electrophoresis, 15 (1994) 13.
[19] O.W. Reif, R. Lausch and R. Freitag, Int. Lab., June (1994) 10.
[20] V. Dolník and M. Novotny, Anal. Chem., 65 (1993) 563.
[21] K.A. Cobb, V. Dolník and M. Novotny, Anal. Chem., 62 (1990) 2478.