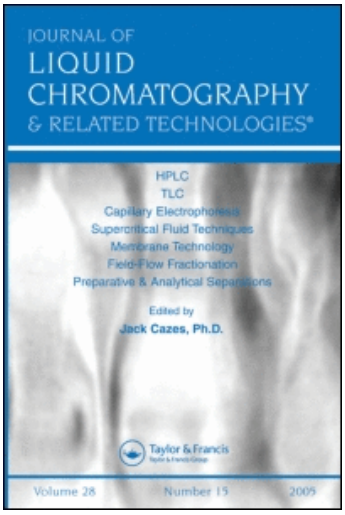

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Analysis of Serum Proteins by Micellar Electrokinetic Capillary Chromatography. Application to a Drug Carrier Evaluation

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ANALYSIS OF SERUM PROTEINS BY MICELLAR ELECTROKINETIC CAPILLARY CHROMATOGRAPHY. APPLICATION TO A DRUG CARRIER EVALUATION

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ABSTRACT

The separation of proteins of fetal calf serum using a MECC method was developed. The influence of pH, sample dilution, nature and concentration of buffer, concentration of surfactant and other conditions affecting the separation were optimized. The usefulness of the addition of two replaceable polymers: hydroxypropylmethylcellulose and dextran to the buffer was investigated. Although their addition had little influence on the separation, an improvement of the resolution of some group of peaks were observed. A 25 mM borate/25 mM phosphate buffer, pH=8.5, with 50 mM SDS allowed to separate the proteins of fetal calf serum, rat serum and healthy and abnormal human

serum with good resolution and short analysis time. The potential of this method in clinical diagnosis is demonstrated. Using standard proteins, an assignment of some peaks in human serum was proposed. Then, the method was applied to the evaluation of a biomimetic drug carrier. The interaction of orosomucoid-coated nanoparticles with serum proteins was evidenced and compared with the one of uncoated nanoparticles. The method allowed to establish a kinetic of desorption of pre-adsorbed orosomucoid by competition with serum proteins.

INTRODUCTION

Various deseases like inflamation, hepatic or blood deseases can be detected by a simple serum protein analysis.[1] Thus, new analytical techniques with high resolution and short analysis time are required to improve separation of serum proteins.

Human plasma is composed of at least 300 different soluble proteins which exhibit different molecular weights and isoelectric points. This results in difficulties to analyse simultaneously all these compounds. Moreover, most of them are glycoproteins which possess, owing to their carbohydrate moiety, a particular behaviour, especially toward their solubilization into micelles.

Several quantitative analysis methods like High Performance Liquid Chromatography (HPLC)[3,4] or electrophoresis were developed for this purpose.[5] Separations of proteins by HPLC using reversed phase, ion-exchange[4] offer poor resolution because proteins tends to adsorb into stationary phases and to form complexes of poor solubility.[3] Electrophoresis is an important separation method for proteins. Although Sodium Dodecyl Sulfate - polyacrylamide gel electrophoresis (PAGE- SDS) and agarose gel electrophoresis are widely used in diagnostic medicine, they remain time-consuming, labor-intensive and unsuitable for automation.[6] Moreover, they are not suitable for accurate quantitative analysis.[7]

Conversely, capillary electrophoresis is a rapid and highly efficient analytical technique which can be automated. Several capillary electrophoresis methods have been developed to analyse serum proteins and to examine the potential of capillary electrophoresis for clinical diagnosis use. Quantitative analyses of serum samples from patients were done by capillary electrophoresis in a uncoated fused-silica capillary and the results obtained were comparable with those obtained by conventional SDS-PAGE[8,9] and by agarose gel electrophoresis.[1,5,10,11] This good correlation has shown that capillary

electrophoresis is a reliable and reproducible technique for clinical diagnostics of serum protein abnormalities.[9,10] A rapid analysis of human serum proteins was reported by Chen[12] in an untreated fused-silica column. The retention times of these proteins correlated well with their isoelectric points. Later, using a buffer system containing a high concentration of phosphate, a serum analysis was achieved rapidly by Chen and Sternberg.[13] More recently, serum proteins were separated into more than ten zones using either a mixture of 0.1 M methylglucamine-0.1 M ε-aminocaproic acid or of 0.1 M methylglucamine-0.1 M γ-aminobutyric acid as running electrolyte.[14] Jenkins and Guerin[11] extended capillary electrophoresis to obtain quantitative separations of paraproteins using a borate buffer. Apolipoproteins were also determined directly in the serum and quantified by Lehmann et al.[9]

However, proteins tend to adsorb to the inner wall of the fused silica capillaries. This phenomenon causes distortion of analyte peaks, resulting in a dramatic loss in the separation efficiency. In fact, at pH>3, fused silica capillary column is negatively charged and proteins have a great tendancy to adhere to the walls of the capillary.[7,15,16]

Many approaches to minimize the adsorption of protein have been described. Protein adsorption can be decreased by using low pH running buffers.[17] At a pH that is near or below the point of zero charge for the fused silica, the silanol groups are not charged and don't attract proteins. Conversely, another approach consists to operate at a pH that is greater than the pI of most basic proteins in the sample, thus causing these proteins to assume a negative charge, resulting in a repulsion between the capillary wall and proteins.[18] Unfortunately, at this extreme pH, most of the proteins are not in their native conformation and some denaturation may occur. Moreover, in many cases, the adsorption of proteins is not completely prevented. Indeed, even though the pH of the running buffer is selected to protonate the silanol groups (pH<3) or to induce a strong negative net charge in the protein molecules in a serum sample (pH>9-10), there still remains some ionised silanols in the first case or some proteins having regions with net positive charges in the other case.

An alternative procedure to eliminate solute-wall interaction is to employ alkali metal salts[19,20] or zwitterions[20,21] to increase the ionic strength of the buffer and to compete for the groups which attract proteins to their surfaces. However, in these conditions, it is necessary to work with narrower capillaries and low voltages to eliminate generated Joule heating. The use of electrolyte additives like ethylene glycol,[1] dextran,[22] hydroxypropylmethylcellulose[23] or organic cations[18] has been also investigated with success to decrease adsorption of proteins onto capillary walls. Corradini et al.[24] have reported the utility of

amines and amino sugars as additives for the capillary electrophoresis of basic proteins. However, in most cases, cationic additives which reduce efficiently the adsorption phenomenon, also decrease the electroosmotic flow resulting in a long analysis time. Moreover, additives can greatly reduce sensitivity and may give rise to distorsed peaks.[6]

Methods to overcome the phenomenon of adsorption have also focused on the modification of the silica surface. Jorgenson and Lukacs[7] reported bonding (3-glycidoxy-propyl)-trimethoxysilane to the capillary wall as early as 1983. Later, Maa et al.[25] have studied the effect of silylation conditions on protein-wall interactions. The capillary walls have also been coated by polyacrylamide,[26] polyvinylpyrrolidone,[17] epoxy-diol,[27] amphiphilic polymers,[28] ionic and non ionic polymers[29] and a hydrophilic coating (CElect-P150).[30] Most of the coatings are not stable in a large range of pH and tend to be expulsed under the effect of the electric field. Moreover, coatings eliminate the electroosmotic flow and resulting separations are not so efficient.

Furthermore, complex protein mixtures, such as plasma, may produce a protein adsorption to the coated layer, which reduces separation efficiency and reproducibility, and rigorous washing must be used to remove the adsorbed proteins while avoiding the alteration of the capillary coating.[13] To reduce protein adsorption, Emmer et al.[31] and Muijselar et al.[32] have proposed the addition of a surfactant to the buffer which form a dynamic coating in capillary.

Another strategy is to employ anionic surfactants which impart a strong negative charge upon the proteins preventing their interaction with the silanol groups.[33] Little work has been devoted to the serum separation using this approach to prevent protein adsorption. In this study, the separation of proteins of fetal calf serum using Micellar Electrokinetic Capillary Chromatography (MECC) was examined. The influence of some factors and various kinds of conditions affecting the separation like pH, sample dilution, buffer composition were optimized. The influence of the addition of a replaceable polymer to the running buffer was investigated.

Analytical conditions were applied to serums from various origins (rat, human). The potential of this method in clinical diagnosis was discussed. Assignment of some peaks in the human serum profile obtained were also carried out by using standard proteins. The method was then applied to study the interaction of a biomimetic drug carrier (orosomucoid-coated nanoparticles) with serum proteins in order to predict its stability after an in vivo administration.

MATERIALS

Sodium dodecyl sulfate, boric acid, human α1-antitrypsin, human α1-glycoprotein 99% pure, human serum albumin (globulin free and fatty acid free) 99% pure, human fibrinogen, human haptoglobin 98-100% pure, human immunoglobulin A, human immunoglobulin G, human prealbumin 95% pure, human C3 protein of complement 95% pure and human transferrin 98% pure were purchased from Sigma (St Louis, US). The purity grades of the proteins are specifications of Sigma. Hydrochloric acid and di-sodium hydrogenophosphate were purchased from Merck (Darmstadt, FRG). Hydroxypropylmethylcellulose was from Seppic (Paris, France) and Dextrans from Fluka (Buchs, Switzerland). All these compounds were used without further purification. Isobutylcyanoacrylate monomer was a gift from Loctite®. Human normal and abnormal serums were from volunteers. Fetal calf serum was obtained from Gibco.

The samples of serums and solutions of standard proteins (Sigma) were prepared with MilliQ® water and stored at - 20°C until used.

APPARATUS AND METHODS

The capillary electrophoresis system Model 2000 (Advanced in Molecular System) was equipped with an automatic injector and a multiwavelength detector. The fused silica capillary (70 cm x 75 µm ID) was thermostated with circulating water at 32°C. Injections were done by pressure (0,2 bar for 2 s). Detection wavelengths were 197, 213, 247 and 280 nm. Voltage was 20 kV. Between each analysis, the capillary was rinsed by pressure with 0,1 N NaOH and distilled water for 5 min and then equilibrated with the running buffer for 5 min.

Orosomucoid-coated polyisobutylcyanoacrylate nanoparticle preparation was described by Olivier et al.[34] Polyisobutylcyanoacrylate nanoparticles were prepared by emulsion polymerization.[35] Briefy : 1 mL of isobutylcyanoacrylate monomer was added to 100mL of a 0.5% (w/v) dextran 70 solution adjusted to pH 2.5 with 3N hydrochloric acid. After polymerization (3 hours), the nanoparticles were filtered and centrifuged. Orosomucoid (2 mg/mL) and nanoparticles suspension (0.5mg/mL) were incubated at 30°C for 24 hours. Then, suspension was centrifuged to eliminate non-adsorbed orosomucoid and the pellet of nanoparticles was redispersed in distilled water. For competition study with serum proteins, 0.5 mL of orosomucoid-coated nanoparticles suspension (10 mg/mL) was mixed with 0.5 mL of human serum. This mixture was incubated at 37°C for various times. For desorption procedure, the mixture

was centrifuged twice and then resuspensed in the running buffer. The mixture was incubated for 16 hours at 20°C and, finally, centrifuged (112,000 g, 30 min, 20°C). The proteins desorbed were monitored in the supernatant by MECC. The same competition study and desorption procedure was employed for uncoated particles.

RESULTS AND DISCUSSION

Influence of Buffer Concentration

In a first part of this study, we have tested a phosphate buffer having a zone of effective buffer action near the physiological pH in order to analyse proteins in their native state. Sodium dodecyl sulfate was employed as the negative surfactant because of its high tendency to bind proteins. Its concentration was set at 25 mM for this preliminary study. Phosphate buffer was reported to decrease protein adsorption onto the capillary walls.[13] When phosphate concentration increased from 10 to 50 mM, we observed more symetrical peaks. This indicates that protein adsorption was effectively reduced. In fact, phosphate ions has been reported to interact with the silica surface[17] and to form complexes with the positive charges of proteins,[13] minimizing the protein to wall interactions. However, this also results in a decrease of electoosmotic flow and consequently in a loss of resolution and in longer analysis times. In order to reduce the analysis time, we selected a phosphate and borate mixed buffer. When borate and phosphate were employed at the same concentration (25/25 mM) in the buffer, the resolution was enhanced and the analysis time was satisfactory. When phosphate and borate concentrations increased from 25 mM to 50 mM, the resolution was not improved while buffer viscosity and electric current rose leading to band broadening and longer retention times. The best compromise was reached using a 25 mM phosphate, 25 mM borate buffer and 25 mM of SDS.

Influence of SDS Concentration

Surfactant concentration is expected to influence the size and the number of micelles and thereby the solubilization of proteins into the micelles. As a consequence, the influence of the concentration of SDS on the separation must be studied. SDS concentrations tested ranged from 0 to 100 mM. Without the addition of surfactant, separation was not satisfactory. The presence of broad tailing peaks indicated that protein-column interaction still occured.

Peak resolution and analysis time increased with SDS concentration showing a more efficient solubilization of proteins into micelles. But at concentration over 100 mM, zone broadening was observed probably due to the high buffer viscosity. A 50 mM concentration of SDS was so chosen.

Influence of pH

The effect of pH was then studied. Separations were performed at pH values ranging from 7.0 to 9.0. Below pH 8.0, a high voltage (30 KV) had to be applied to obtain a short analysis time. However, at this voltage peak deformation was observed probably because of the Joule heating generated by the high current.

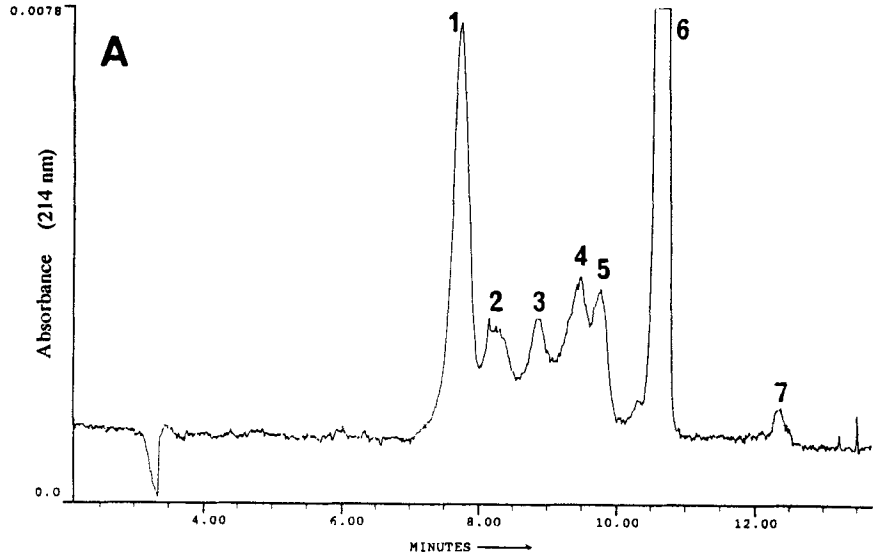
At pH above 9.0, current was also increased, zone broadening was observed and proteins denaturation was probable. The pH 8.5 and 9 gave the best results and pH = 8.5 was selected because it is closer to the physiological pH.

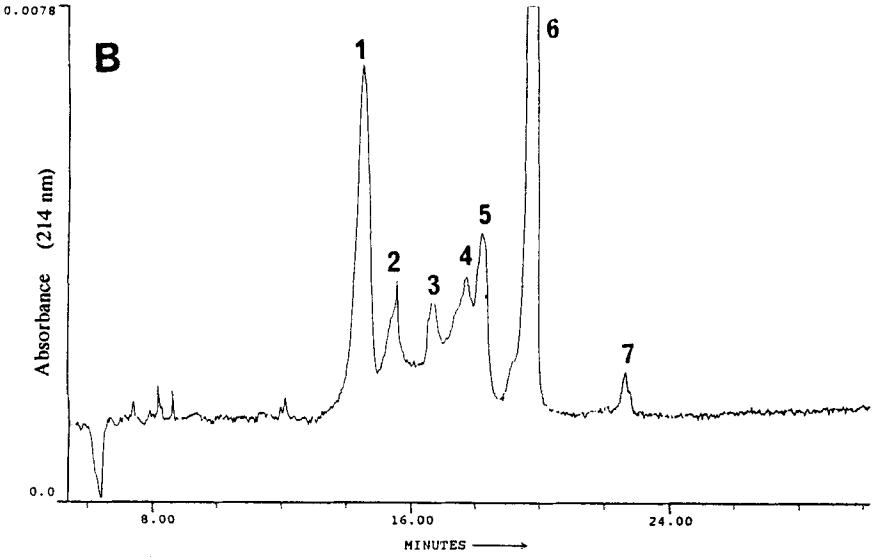
Influence of Sample Dilution

Sample concentration is a critical parameter to optimize separations.[7] In fact, when samples are too concentrated, poor peak shapes are observed resulting in poor resolution. On the contrary, as serum consists in a mixture of proteins which are present at very different concentrations, a sample too diluted will not afford a detection of the minor compounds. That is why a compromise had to be found.

Owing to the high viscosity of the serum preparation, we have diluted serum in distilled water to favour the stacking effect. No other sample purification was needed. This is an important advantage on using this method. Within the series of sample dilution tested, the best result was obtained with a 1/80 dilution (v/v).

Finally, Figure 1 shows the separation of fetal calf serum in the optimum experimental conditions which were : 25 mM of phosphate buffer, 25 mM of borate buffer, 50 mM of SDS, pH 8.5, and sample dilution at 1/80. The electropherogram profile displays seven major fractions. Within these fractions, some separations are observed, especially for fractions 2 and 7, while fractions 4 and 6 exhibit a shoulder indicating the presence of a mixture of compounds within each fraction.





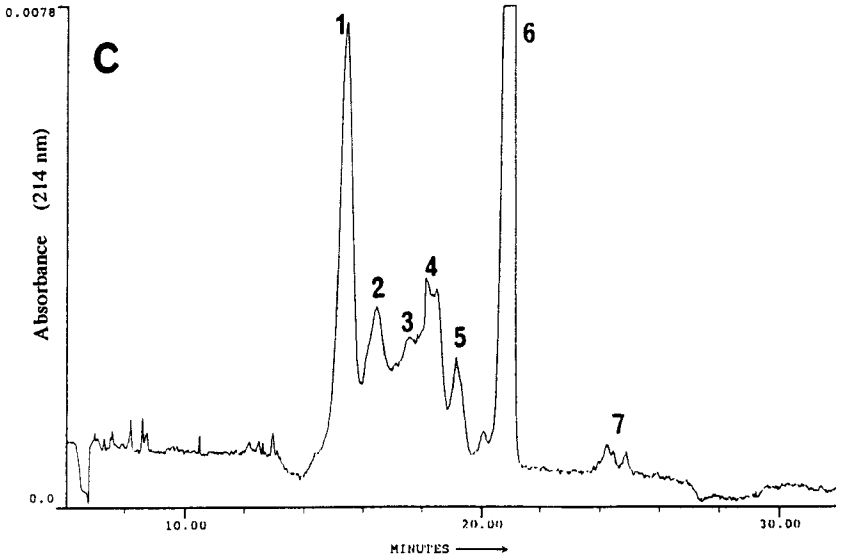
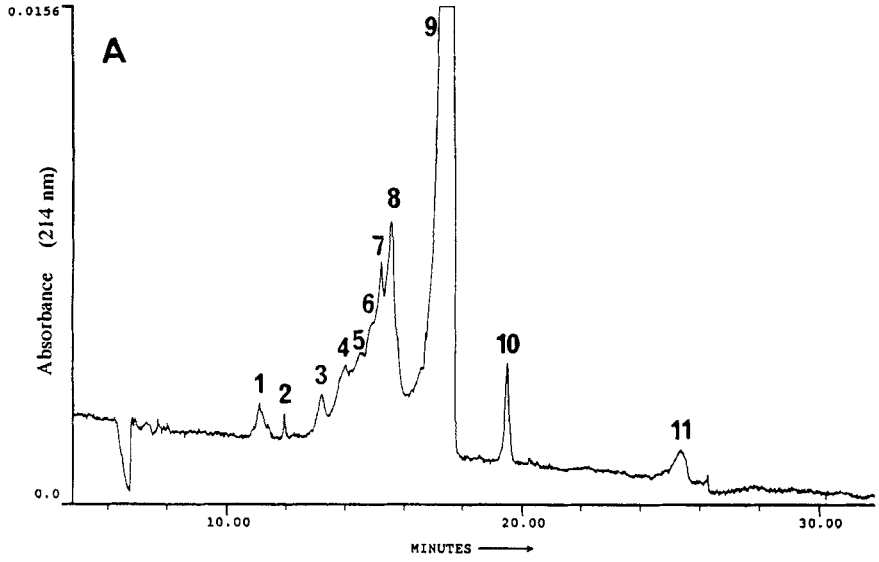


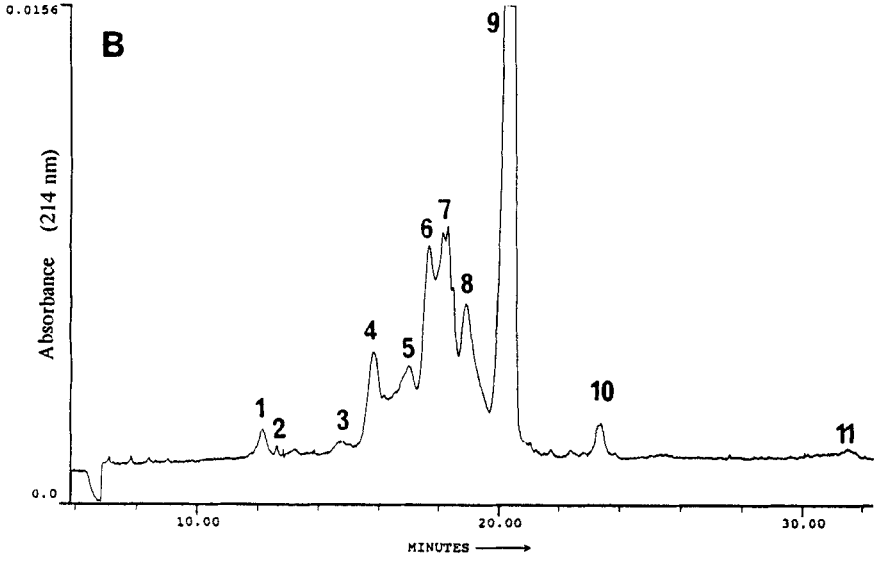
Figure 1. (above and left) Electropherograms of fetal calf serum (dilution 1/80). Capillary : 70 cm x 75 µm i.d. ; buffer : 25 mM borate, 25 mM phosphate, 50 mM SDS ; pH 8.5 ; hydrodynamic injection : 2 sec at 0.2 bar ; temperature : 32°C ; detector : UV at 213 nm ; applied voltage : 20 KV. (a) without polymer, (b) HPMC 0.05%, (c) dextran 0.5%.

Influence of Polymer Addition

In order to improve this separation, we have investigated the influence of the addition of replaceable and UV-transparent polymers to the running buffer. Several polymers have already been tested for the separation of proteins : cellulose derivatives, dextran, polyacrylamide and polyethylen glycol.[22,33,36] We selected two different hydrophilic polymers : hydroxypropyl-methylcelluloses (HPMC) and dextrans which have already been employed as sieving polymers for the capillary electrophoresis separation of SDS-proteins complexes.[22,37] They may also form complexes with silanol groups to decrease adsorption phenomenon.

To facilitate the preparation of the buffer and to ensure a better homogeneity of the buffer, a HPMC with medium viscosity (6cps for 2%) was employed. HPMC concentrations between 0.01% to 1% were tested. We expected higher concentrations of HPMC to produce longer analysis time. However, when HPMC concentration decreased, retention time increased and





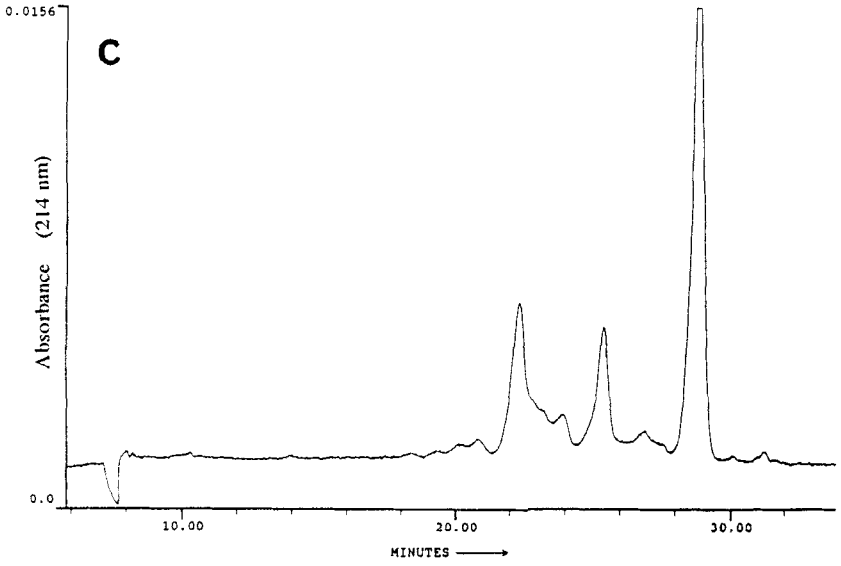


Figure 2. (above and left) Electropherograms of serums from different origin (dilution 1/80). Experimental conditions : same as Figure 1. (a) normal human serum, (b) abnormal human serum, (c) rat serum.

selectivity was enhanced. An explanation for this is that HPMC which is ionised in presence of borate, probably interacts with proteins and prevents proteins inclusion in SDS micelles from occuring ; thus resulting in a faster migration of the proteins. The best resolution could be achieved with 0.05% of HPMC. Thereafter, dextran concentrations from 0.25% to 10% were also tested. At a dextran concentration of 10%, we observed that the number of peaks was lower and that the baseline was unstable. As for HPMC, a low percentage of dextran (0.5%) gave the higher number of peaks and a better stability of the baseline.

Figure 1 displays the serum profiles using buffers containing either 0.5% of dextran or 0.05% of HPMC. At these optimized concentrations, the addition of the polymers had little influence on the separation. With HPMC, we observed several peaks in the fractions 2 and 4, a shoulder in fraction 6 and a new region before fraction 1. On the other hand, a shoulder in fraction 1 and numerous peaks in regions 4 and 5 are observed with the addition of dextran. These polymers modify in a different manner the separation but the order of

elution remains unchanged. They influenced proteins separation probably more by making interaction with proteins than by acting like sieving polymers. Finally, they only improved resolution of some fractions of peaks and we concluded that their addition may be useful to analyse some particular proteins.

Application to Other Serums

The method was then applied to the analysis of proteins in human serum and rat serum. In both cases, the addition of polymers did not markedly affect the separation. That is why to facilitate the preparation of the running buffer and to avoid problems in the reproducibility of the analyses, polymers were not used in the further study.

Figure 2 shows electropherograms of healthy human, abnormal human and rat serums. As a function of the species, profiles were markedly different. The profile obtained with a serum of a patient suffering from a gammopathy was qualitatively similar to that obtained from a normal serum. The differences relied mainly in the intensity of fractions 4, 6, 7 and 8 which were increased in the abnormal serum profile (Figure 2 b). However taking into account the variability of serum between people, only the fraction 6 which was dramatically increased could be considered to correspond to a protein abnormality. As we will discuss later, this fraction contains mostly the immunoglobulins G and A (IgG, IgA).

Assignment of Some Human Proteins

Next, a tentative assignment of the most important proteins in the profile was carried out. To establish a protein map, human standard proteins were used. Proteins migrated as follows : first prealbumin and orosomucoid, then haptoglobin, followed by IgG and IgA which were not resolved. α1 antitrypsin gave, surprisingly, two peaks, the former eluting with transferrin and fibrinogen, the second eluting later, together with the C3 protein of the complement (first peak of standard) and albumin. The positions of these proteins on the human serum profile are indicated in Figure 3.

The standard C3 protein of complement gave two peaks, the first having the same elution time as albumin and the second eluting later. This second peak did not correspond to any fraction in human serum profile. Our hypothesis was that the standard protein has been cleaved in two fractions in contrast to the native serum protein which probably elutes as a single peak close or within the albumin fraction.

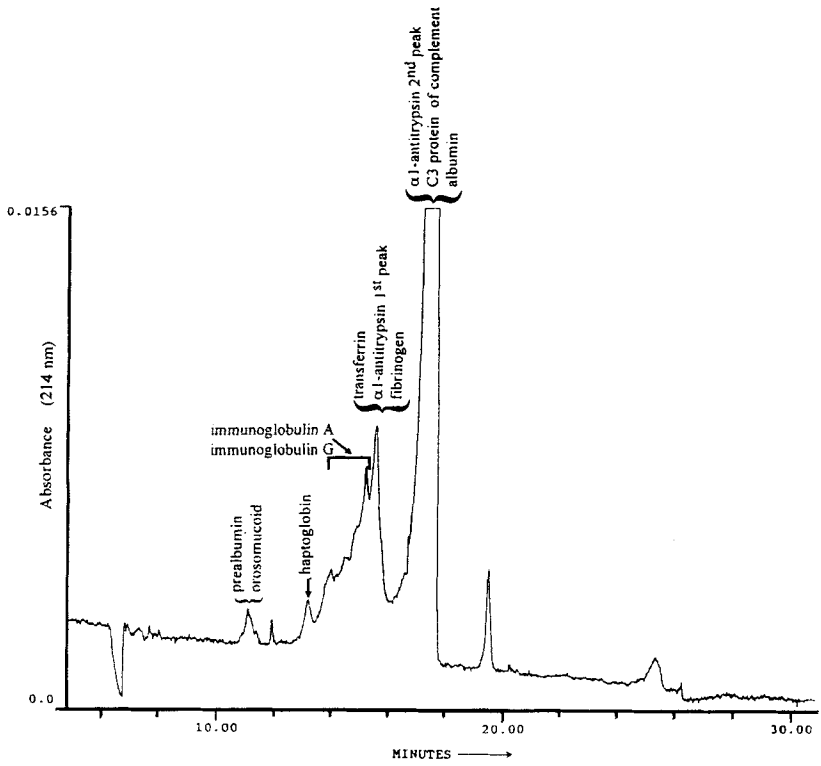


Figure 3. Identification of some human serum proteins in the capillary electrophoresis profile. Experimental conditions : same as Figure 1.

The elution order of all these proteins was quite different from those reported by other methods like gel electrophoresis[5] or capillary zone electrophoresis.[5,10,11,12] The glycoproteins are expected to exhibit a particular behaviour in MECC compared to proteins. In this study, one of the major parameters affecting the migration of the glycoproteins contained in the serum seems to be their percentage of carbohydrate. We observed as a general trend that protein having a higher carbohydrate content are eluting earlier : first orosomucoid with 40% of carbohydrate, then haptoglobin with 16.4%, transferrine with 5.9%, α1 antitrypsin with 12.2%, fibrinogène with 4% and later C3 protein with 2.7%. This was readily explained by the fact that the carbohydrate moieties of glycoproteins prevent the interaction of proteins with micelles and, consequently, glycoproteins highly glycosylated solubilize into micelles to a lesser extent than proteins. There were exceptions to this rule : immunoglobulins G (2.9%) and A (7.5%) which eluted between haptoglobin

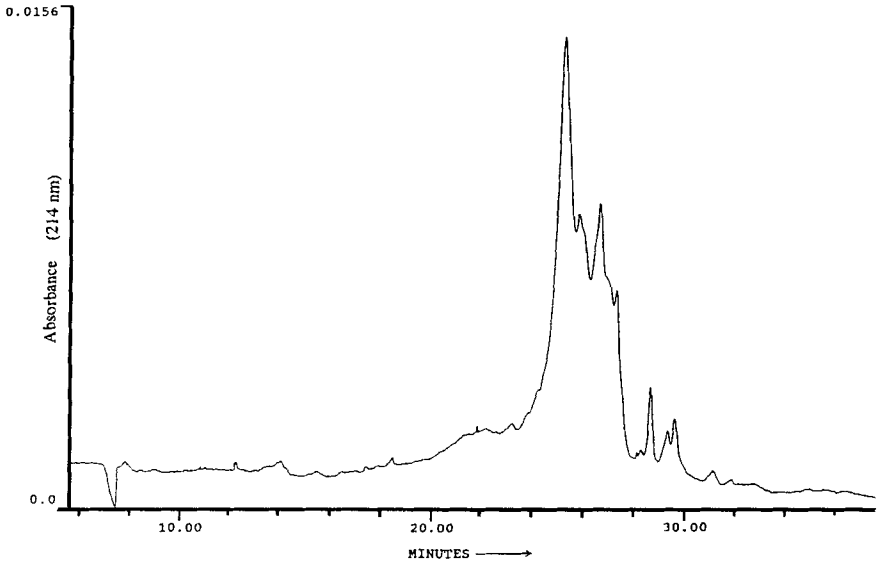


Figure 4. Electropherograms of human serum after 8 days of storage at ambiant temperature. Experimental conditions : same as Figure 1.

and transferrine. But immunoglobulins have a particular structure and a high molecular weight which may explain their electrophoretic behaviour. Moreover, α1 antitrypsin (12.2%) and transferrin (5.9%) would have migrated in the reverse order if the percentage of carbohydrates was the unique parameter influencing the order of elution. This indicates that other parameters affect the separation of glycoproteins in this system. The elution of the other proteins is managed by a combination of their hydrophobicity, their size and their molecular weight which are the other parameters affecting a separation in MECC.

Analytical Method Validation

Serum is a complex mixture which can greatly vary between people. For these reasons we have first carried out the validation procedure on one unique serum sample. We observed that after several freezing and defreezing periods the profile was modified. To better understand this variation, we analysed every day a fresh human serum stored at ambiant temperature. Figure 4 shows the profiles obtained the first day and after 8 days of storage. The profiles are

very different. We concluded that serum samples are not enough stable to be used for a validation procedure. Then, the repeatability of the migration times and of the peak areas was investigated with five consecutive runs using a mixture of 3 human standard proteins : orosomucoid, IgA and albumin.

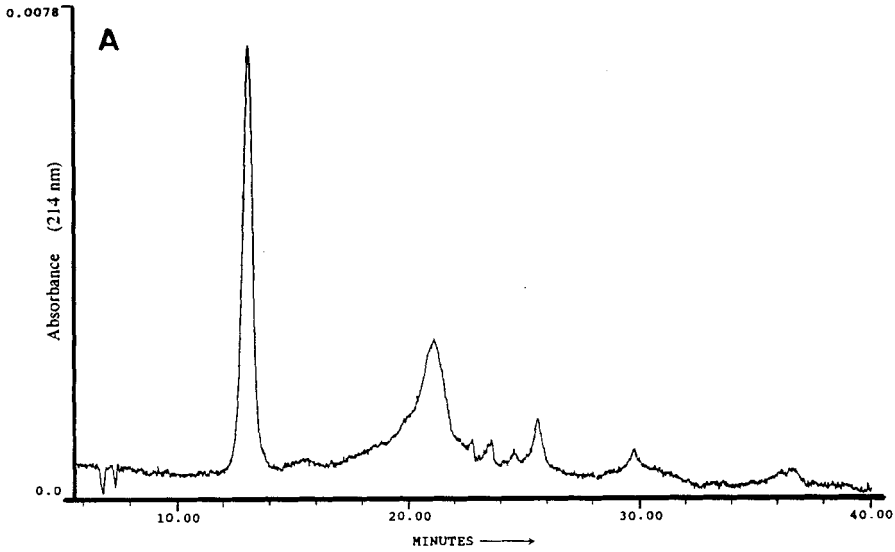
Proteins pass the detector at different velocity according to their electrophoretic mobility. Thus, proteins migrating slower will produce a higher peak area. For this reason, corrected peak areas were used to study the repeatability of the peak areas. With regard to the relative standard deviations of the peak areas (6.9% for orosomucoid, 6.1% for IgA and 4.2% for albumin) and of migration times (5.3% for orosomucoid, 6.2% for IgA and 7.0% for albumin), the repeatability is relatively satisfactory. We have explained these results by a residual adsorption of proteins onto capillary walls, which reduced the electroosmotic flow after successive analyses. This resulted in increasing migration times and peak deformations. To obtain a better repeatability of the peak areas and of the migration times, we improved the capillary rinsing method by remplacing the 0.1 N sodium hydroxide solution by a 0.5 N sodium hydroxide solution. Moreover, an efficient cooling of the capillary by a more performant system would probably have allowed a better reproducibility.

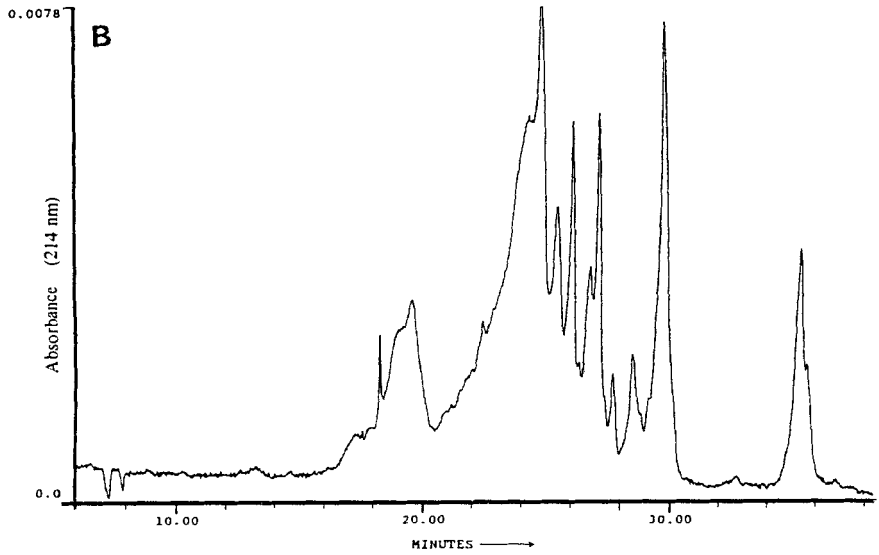
Application To Orosomucoid-Coated Nanoparticles

Finally, the optimized method was applied to study the stability of the drug carrier. After the incubation of orosomucoid-coated polyisobutyl-cyanoacrylate nanoparticles in the presence of human serum, the analysis of the supernatant was performed by MECC.

Incubation times were tested between 5 min and 2 hours. As shown in Figure 5, when incubation time increases, orosomucoid was progressively desorbed, whereas serum proteins adsorbed more and more on nanoparticle surface. The profile of the proteins adsorbed onto the nanoparticle surface was composed of 6 major fractions : orosomucoid (fraction 1), immunoglobulins (fraction 2), albumin (fraction 3), C3 protein of complement (fraction 5) and, in fractions 4 and 6, other proteins have not been identified. As immunoglobulins, complement and albumin are well known to participate in the opsonization of drugs, we speculated that the desorption of orosomucoid occured because of the opsonisation of the nanoparticles.

Figure 6 shows the kinetic of desorption/adsorption of serum proteins into coated nanoparticles. Orosomucoid pre-adsorbed onto particles slightly protects the drug carrier against adsorption of other serum proteins by comparison with uncoated nanoparticles. In fact, there is less proteins adsorbed





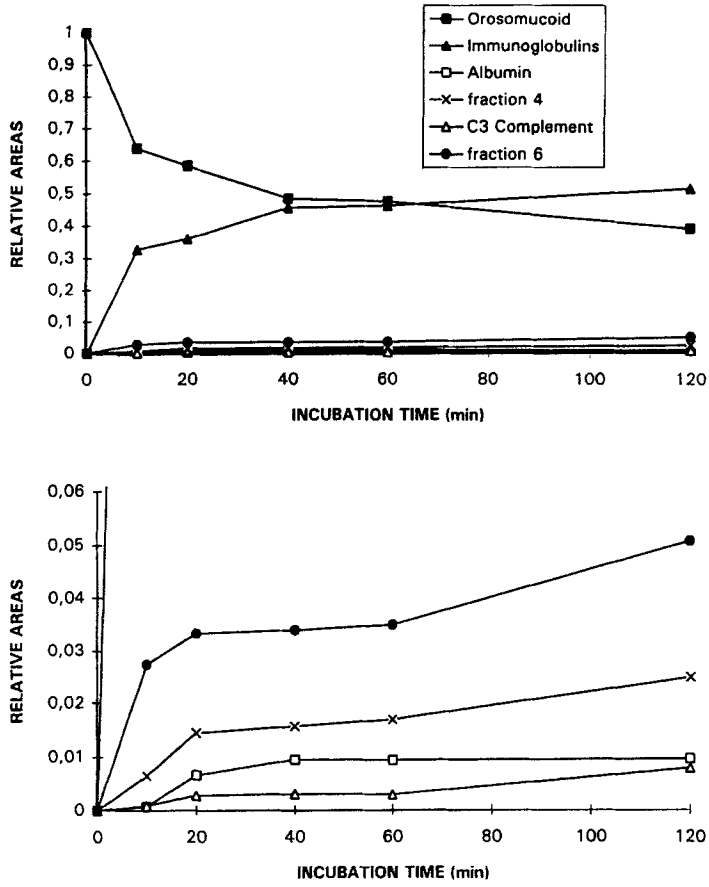


Figure 6. Kinetic of desorption or adsorption of serum proteins onto the orosomucoid-coated nanoparticles.

(no transferrin for example) and in less amount when the particles are initially coated with orosomucoid. These results show that orosomucoid allows to slow down the phemonenon of opsonisation which represent one of the major problem encountered in the development of a drug carrier.

Figure 5. **(left)** Electropherograms of adsorbed proteins on surface nanoparticles after 20 minutes of contact with human serum. Experimental conditions : same as Figure 1. (a) orosomucoid-coated nanoparticles, (b) uncoated nanoparticles.

Proteins migrating in fractions 4 and 6 had migration times comprised between the 2 peaks of the standard C3 protein of the complement. Moreover, complement is known to be activated by contact with particles and with container surface where serum is stored. Figure 4 showed that, after 8 days, the profile of serum was composed of peaks with retention times near those of non-identified proteins. Our hypothesis was that non identified proteins might belong to complement activation products.

In view of these results, it appeared that the orosomucoid adsorbed onto nanoparticles allowed to decrease the number and the quantity of serum proteins which tend to adsorb onto particles but only during a few minutes. Then, we concluded that orosomucoid is displaced, at least in part, by competitive serum proteins which may be complement fractions and other proteins involved in the opsonization of particles. However the possible erosion of surface nanoparticle in the presence of human serum may not be neglected as it can constribute to this displacement.

CONCLUSION

As a conclusion, with MECC, numerous parameters have to be studied in order to achieve good separations. This optimization is long but necessary and allows high efficiency of this technique. After this stage, it is clear from the above results that, MECC can be applied to complex mixtures of proteins like serums with good resolution and short analysis time. With the described method, it is possible to assign the position of the major proteins in the separation pattern and to establish a protein map of a serum. The potential of MECC for clinical diagnostic applications is promising. In this study, the method allowed to study a new drug carrier stability in human serum and to point out that a desorption of the orosomucoid pre-adsorbed onto nanoparticles occured when nanoparticles were incubated with human serum.

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