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Review

Biomedical applications of capillary electrophoresis

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Abstract

After having grown through the stages of technique development and instrumentation availability, capillary electrophoresis has reached the stage of applications. This review attempts to show the potential of this technique for biomedical analysis. Rather than going into a detailed description of the technical details of the separation conditions suitable for the separation of a particular category of compounds, the focus is on the general principles and areas in which this technique can be applied and the prospects for the future. Particular emphasis is placed on the separation of complex matrices and their simplification, a daily task in biomedical laboratories. In addition, methods for the optimization of separation conditions are considered. Considerable prospects for capillary electrophoresis lie in profiling. The applicability of the technique in peptide and protein mapping is discussed in some detail. Finally, three other topics are dealt with, namely enzymic activity microassays, drug–protein binding assays and monitoring of drugs in body fluids.

Contents

1. Introduction

Capillary electrophoretic methods are currently undergoing a booming expansion. They have already passed the initial stage during which the conditions for the separation of a number of compounds were established along with general rules for separating small molecules, macromolecules, diastereomers, compounds poorly soluble in water, etc. The start was slow, partly because

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of the lack of commercially available equipment. Today, at least six different producers have put their products on the market. Step by step, interest is slowly shifting from instrumental research towards applications.

Capillary electrophoresis and related capillary techniques can offer a number of advantages that are welcome in the biomedical field. Not only to they have high separation power, offering separations with a large number of theoretical plates, and sufficient selectivity under properly chosen conditions, but also they are versatile and can separate the most diverse types of compounds. Nevertheless, it is true that particular equipment should be used for separating one category of compounds at a time. Moreover, capillary electromigration methods need extremely small amounts of samples, a fact that could open up entirely new analytical perspectives in clinical biochemistry (*e.g.*, microanalyses on biopsies).

A number of reviews have appeared recently in an attempt to make the scientific community outside separation science more familiar with this technique [1–29] and bodies have started to appear (see, *e.g.*, refs. 1 and 2). It is therefore assumed that the reader is familiar with the technique as such, or can acquire the necessary information from these sources. Rather than presenting general information about equipment, buffer systems, operating conditions, etc., we have attempted here to summarize conditions under which capillary electrophoretic techniques can be used for biomedical purposes.

At the very beginning we faced a problem, namely the definition of biomedical applications. Any protein found in nature can be exploited at least in biomedical research, if not further. The same holds true for, *e.g.*, nucleic acids and their fragments. An exhaustive treatise in this direction would be exceedingly voluminous indeed. We have therefore limited ourselves to selected areas in which we have tried to indicate not only the procedures that are already available now, but also to indicate the possibilities which, we believe, will be used in the biomedical field in the years to come.

2. Analysis of complex sample matrices. Simple anions and cations

In biomedicine it may be necessary to determine more than one category of compound in natural material. Complex mixtures of anions, cations or mixtures of anions with other analytes present, *e.g.*, in tissue culture media [30], and the analysis of cations present as impurities in raw drugs or tissues may serve as typical examples.

In the separation of inorganic cations and anions [30–35] and also organic carboxylic acids [36,37], let us consider capillary ion electrophoresis (CIE) [38], which is a rapidly growing technique covering the various classes of ions that were for years the domain of ion chromatography. The separations are carried out in fused-silica columns with detection at 185, 214 or 254 nm. A list of compounds that can be separated by this procedure is given in Table 1. The separation times are very short, being 1.8–3.0 min for a set of lanthanides, alkali metals and alkaline earths on the one hand, and a set of organic and inorganic anions on the other. The separations obtained by Jones and Jandik [38] are best exemplified by the electropherogram shown in Fig. 1.

A complex matrix to analyse is the culture media used for tumour cell cultivation. Ma *et al.* [30] reported that a rapid separation of polyamines, some amino acids and simple cations (potassium and sodium) can be achieved within 10 min in a 60 cm × 75 μm I.D. fused-silica capillary and using quinine sulphate (8 m*M*) in 20% ethanol at pH 5.9 as the running buffer. A typical separation obtained is shown in Fig. 2.

As not all solutes separated here or coming into consideration for assay absorb at the specified wavelengths, negative detection is applied. This means that a highly UV-absorbing compound is added to the background electrolyte and the zones of individual solutes appear as negative peaks when passing the detector window. Frequently 5 m*M* chromate is used for this purpose, but there are other compounds (*e.g.*, quinine sulphate or salicylic acid) that can also be used for this purpose. Sometimes one can

Table 1
Anions and cations that have been characterized by CIE with UV detection

Inorganic anions	Organic anions	Organic anions	Metals
Arsenate	Acetate	Isocitrate	*Alkali metals*
Arsenite	*trans*-Aconitate	α-Ketoglutarate	Lithium
Azide	Ascorbate	Lactate	Sodium
Borate	DL-Aspartate	Maleate	Potassium
Bromate	Benzoate	Malonate	Rubidium
Bromide	Butanesulphonate	Methanesulphonate	Caesium
Carbonate	Butyrate	Nonanesulphonate	
Chlorate	4-Carboxybenzaldehyde	Octanesulphonate	*Alkaline earth metals*
Chloride	Chloroacetate	Orotate	Beryllium
Chlorite	Citrate	Oxaloacetate	Magnesium
Chromate	Crotonate	Oxalate	Calcium
Cyanide	Decanesulphonate	Pentanesulphonate	Strontium
Fluoroborate	Dodecanesulphonate	*o*-Phthalate	Barium
Fluoride	Dichloroacetate	Propanesulphonate	
Hypochlorite	Ethanesulphonate	Propionate	*Transition metals*
Iodide	Formate	Pyridinedicarboxylate	Manganese
Metasilicate	Fumarate	Pyruvate	Iron
Metavanadate	Galactarate	Quinate	Cobalt
Molybdate	D-Galacturonate	Salicylate	Nickel
Monofluorophosphate	D-Gluconate	Shikimate	Copper
Nitrate	Glucuronate	Sorbate	Zinc
Nitrite	L-Glutamate	Succinate	Cadmium
Orthovanadate	Glutarate	Tartarate	Mercury
Perchlorate	Glycerate	Terephthalate	Lead
Persulfate	Glycolate	Trichloroacetate	
Phosphate	Glyphosate	Trifluoroacetate	*Lanthanides*
Phosphite	Heptanesulphonate	Trimesate	Lanthanum
Selenate	Hexanesulphonate	*p*-Toluate	Cerium
Selenite	α-Hydroxybutyrate	Valerate	Praseodymium
Sulphate	Hydroxymethylbenzoate		Neodymium
Sulphide	2-Hydroxyvalerate		Samarium
Sulphite			Europium
Thiocyanate			Gadolinium
Thiosulphate			Terbium
Tungstate			Dysprosium
			Holmium
			Erbium
			Thulium
			Ytterbium
			Lutetium
			Non-metal cation
			Ammonium

The anions are divided into two categories, inorganic and organic, and are listed alphabetically. The cations are listed according to their class and in order of increasing atomic number. From ref. 38, with permission.

meet commercially available products the composition of which is not declared (e.g., UV CAT available from Waters, Milford, MA, USA). An extensive study on using this type of background electrolyte modifier was published by Weston *et al.* [39].

Indirect detection can be used as a universal detection scheme [40] without the need for

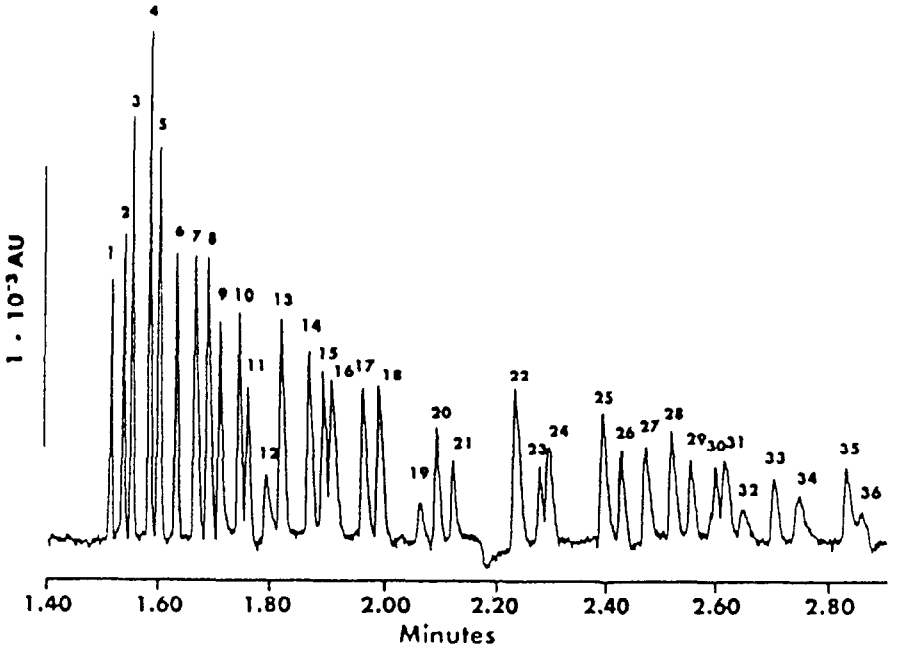


Fig. 1. Electrophoregram of a complex mixture of anions. Peaks: 1 = thiosulphate; 2 = bromide; 3 = chloride; 4 = sulphate; 5 = nitrite; 6 = nitrate; 7 = molybdate; 8 = azide; 9 = tungstate; 10 = monofluorophosphate; 11 = chlorate; 12 = citrate; 13 = fluoride; 14 = formate; 15 = phosphate; 16 = phosphite; 17 = chloride; 18 = glutarate; 19 = *o*-phthalate; 20 = galactarate; 21 = carbonate, 22 = acetate; 23 = chloroacetate; 24 = ethanesulphonate; 25 = propionate; 26 = propanesulphonate; 27 = aspartate; 28 = crotonate; 29 = butyrate; 30 = butanesulphonate; 31 = valerate; 32 = benzoate; 33 = L-glutamate; 34 = pentanesulphonate; 35 = D-gluconate; 36 = D-galacturonate. Electrolyte, 5 m*M* chromate and 0.4 m*M* OFM anion BT adjusted to pH 8.0; applied potential, 30 kV (negative polarity); capillary, 60 cm (52 cm to the detector) × 50 μm I.D., fused silica. Indirect UV detection, injection by electromigration at 1 kV for 15 s. From ref. 38, with permission.

complicated precolumn or postcolumn derivatization (although, *e.g.*, amino acids are best accessible to analysis as UV-absorbing and fluorescent derivatives) (see refs. 41–49). Certainly the most attractive indirect detection methods are those that exploit indirect laser-excited fluorescence, but other indirect detection methods can also be successfully applied for biomedical purposes. Virtually all charged compounds subjected to analysis can be detected in this way [50]. Indirect detection methods can be used also with micellar electrokinetic separations [51,52] and need not necessarily be limited to optical measurements only (it is also possible to use, *e.g.*, indirect amperometric measurements). Foret *et al.* [53] pointed out that the highest sensitivity can be achieved for sample ions having a mobility close to that of UV-absorbing ions. They also worked out theoretically some disadvantages of indirect UV detection, particularly the limited linearity range (two orders of magnitude only). The upper limit is adversely influenced by concentration overload and, as indicated already, these negative influences can be diminished by choosing conditions such that the effective mobilities of the background electrolyte and sample ion do not differ too much. A thorough theoretical investigation of the indirect UV detection conditions with respect to sensitivity can be found in the paper by Bruin *et al.* [40].

In complex mixtures another frequent problem is to simplify the sample matrix and/or to concentrate the analytes to be assayed. A combination of isotachophoresis and capillary zone elec-

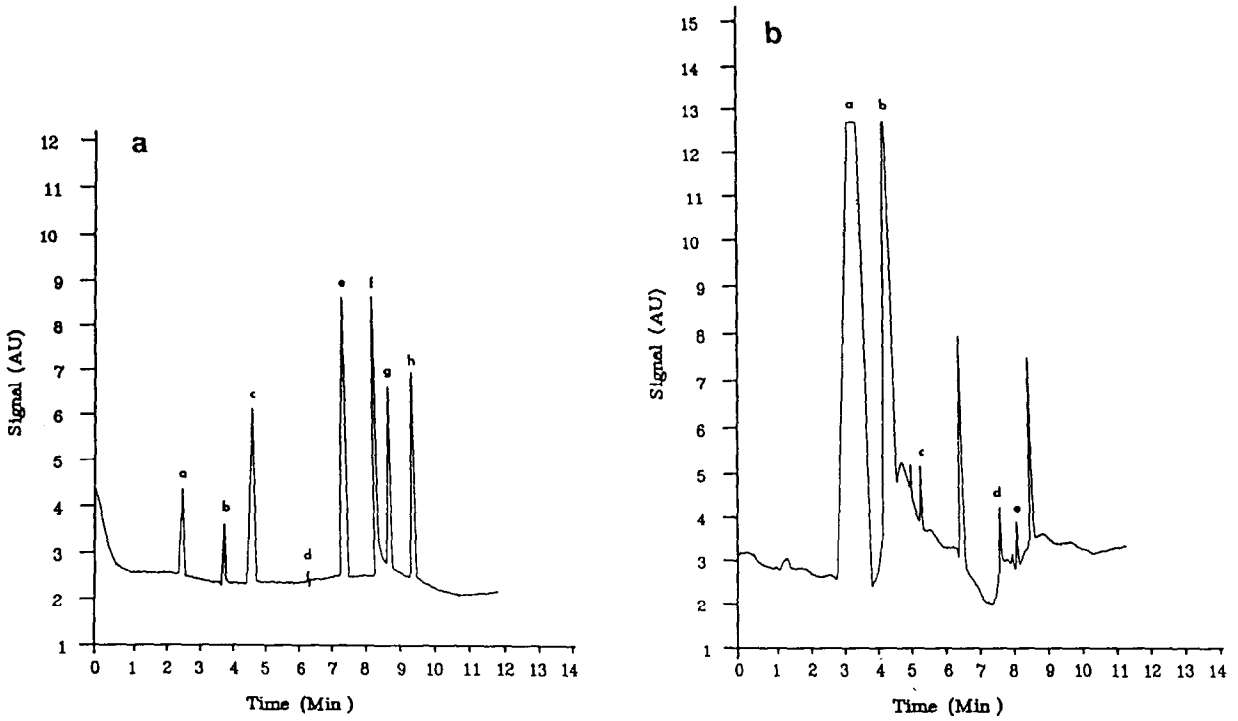


Fig. 2. (a) Separation of three polyamine standards and other components co-existing in the cell culture media in 0.8 m*M* quinine sulphate in 20% ethanol (pH 5.9) containing 0.5% hydroxymethylcellulose. Indirect photometric detection. A 3-s, 30-kV injection of 100 μM each was followed by electrophoresis at 30 kV in a 60 cm × 75 μm I.D. capillary. Pretreated column; injection volume, 26.1 nl; detection wavelength, 236 nm. Peaks: a = potassium; b = sodium; c = putrescine; d = L-histidine; e = spermidine; f = spermine; g = L-lysine; h = arginine. (b) Separation of polyamines in a tumour cell culture in the same background electrolyte as in (a). Separation conditions as in (a). Peaks: a = potassium; b = sodium; c = putrescine; d = spermidine; e = spermine. From ref. 30, with permission

trophoresis (CZE) can be used; as shown by Kaniansky *et al.* [54] isotachophoresis can be used to remove the most abundant sample constituent(s) while simultaneously concentrating the sample zones migrating between the leading and terminating electrolytes. In this way, the sample is made ready for the subsequent capillary electrophoresis run. In another mode proposed by Kaniansky *et al.* [54], isotachophoresis was used for maximum sample clean-up. Here only the analyte(s) with a minimum of the matrix constituents was transferred for the final capillary zone electrophoresis assay. The zone to be transferred was defined by using appropriate spacers added to the sample that identified the isotachopherogram section to be transferred to the capillary electrophoresis run. In both of these approaches the authors arbitrarily used urine as the sample matrix, thus proving the suitability of their method for biomedical purposes.

Another frequent demand in clinical biochemistry, especially in the analysis of drugs or other low-molecular-mass compounds in serum or plasma, is deproteinization. For this purpose acetonitrile and ethanol were successfully used by Shihabi [55] and Tagliaro and co-workers [56–58], respectively. Unexpectedly, many compounds exhibited larger plate numbers in the presence of 60% acetonitrile. Shihabi [55] ascribed this effect to stacking elicited by the low resistivity of acetonitrile in the sample. It was also emphasized that acetonitrile improves the solubility of some compounds and increases the

sample volume that can be introduced into the capillary. The applicability of the method was shown with serum theophylline.

3. Optimization of separation conditions for endogenous compounds

Of the numerous endogenous compounds, we discuss proteins and enzymes in more detail in separate sections. A detailed discussion of endogenous compounds separated by electrokinetic methods is precluded owing to lack of space. The reader is therefore directed to the book by Li [1], where more information on different separation approaches can be found (see also ref. 59).

Instead, we would like to show here how to rationalize the optimization of compounds normally occurring in the body and how to minimize the number of experiments needed for a successful result. The approach represents a further development of already existing methods and has been worked out by Yao *et al.* [60] for the separation of porphyrins.

As the naturally occurring porphyrins have two to eight ionizable COOH groups, their separation should be carried out at alkaline pH and Yao *et al.* [60] selected a CAPS (3-cyclohexylamino-1-propanesulphonic acid) buffer at pH 10.8. The difficulty separating porphyrins is that while the members of the family carrying different numbers of carboxyl groups per molecule are easy to separate and their migration is governed by their negative charges, the separation of the four dicarboxyporphyrins is difficult to perform if the conditions for the electrophoresis are not optimized. Moreover, in repeated runs the peak shapes deteriorate and increased migration times were observed, suggesting absorption of the analytes on the capillary wall. In order to overcome these effects, sodium dodecylsulphate (SDS) was added to the background electrolyte. Although the peak shape and retention times were improved with 100 m*M* SDS, it was also observed that SDS alone does not provide sufficient selectivity and solubility of dicarboxyporphyrins. Therefore, an organic modifier, N,N-dimethylformamide (DMF), was introduced into the running buffer. Porphyrins are readily soluble in DMF and thus the presence of this modifier in the electrolyte improved the affinity of the analytes in the aqueous phase. On the other hand, if the concentration of DMF is too high, the retention times of the peaks increase substantially owing to slowing down of the electromigration flow. In other words, what was needed was the optimization of the concentrations of DMF, CAPS and SDS in the running buffer.

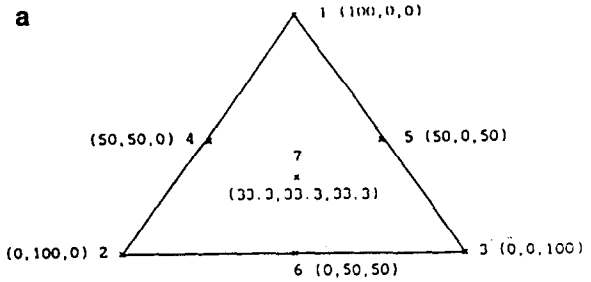
Yao *et al.* [60] applied the ORM scheme to optimize the conditions. The first step in this scheme is to determine the working range of each of these three parameters. This choice was based on the requirement to obtain reasonable retention times. It has to be kept in mind that although increased retention times allow more time for resolution of individual peaks, in reality long separations are frequently distorted owing to peak spreading. Consequently, the separation time was set to 30 min (which is a value generally applicable to almost any separation). Based on these limits, seven experiments were planned on the strategic points of the triangle. The conditions are depicted in Fig. 3. From the runs obtained under these conditions, the resolution values were calculated for adjacent peaks:

$$R = \frac{2\Delta t}{W_1 + W_2} \tag{1}$$

where Δt is the difference in migration times and W_1 and W_2 are peak widths at the baseline. The resolution values were then fitted into the following equation:

$$R = \alpha_1 x_1 + \alpha_2 x_2 + \alpha_3 x_3 + \alpha_{12} x_1 x_2 + \alpha_{13} x_3 + \alpha_{23} x_2 x_3 + \alpha_{123} x_1 x_2 x_3 \tag{2}$$

where α_i are the coefficients and x_i the percentages of each parameter as shown in Fig. 3. By using a computer program, the coefficients were calculated and once they were known the resolutions could be calculated for any buffer composition within the triangle. In all the test runs hexacarboxy- and heptacarboxyporphyrin and uroporphyrin were always well resolved



Expt	CAPS, mM	SDS, mM	DMF, %
1	80	20	4
2	20	50	4
3	20	20	10
4	50	35	4
5	50	20	7
6	20	35	7
7	40	30	6

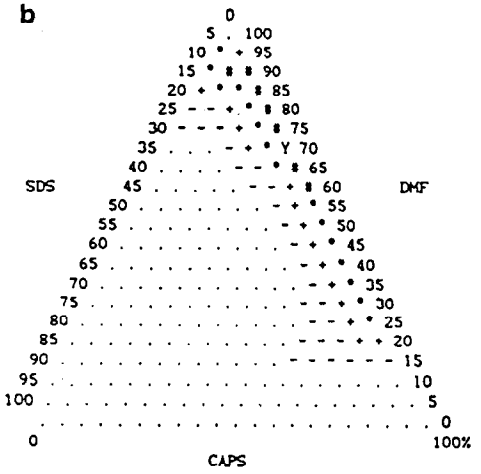


Fig. 3. (a) Experimental design and conditions for the seven experiments; the coordinates at points on the triangle ($x1$, $x2$, $x3$) denote the percentages of 3-cyclohexylamino-1-propanesulphonic acid, SDS and DFM, respectively. (b) Overlapped resolution map for the six porphyrins (peak) pairs. ● = $R < 0.8$; − = $0.8 < R < 1.2$; + = $1.2 < R < 1.5$; * = $1.5 < R < 1.8$; # = $R > 1.8$. For point Y, see Fig. 4. From ref. 60, with permission.

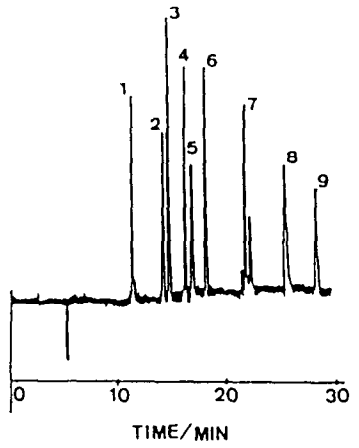


Fig. 4. Separation of the nine porphyrins using the optimum conditions corresponding to point Y within the # region in Fig. 3b. 38 mM 3-cyclohexylamino-1-propanesulphonic acid (pH 10.8)–20 mM SDS–8.2% DMF. Detection by fluorescence, 405/615 nm; 18 kV, 18 μA. Peaks: 1 = deuteroporphyrin; 2 = ZnPP; 3 = coproporphyrin; 4 = mesoporphyrin IX; 5 = PP; 6 = pentacarboxylporphyrin; 7 = hexacarboxylporphyrin; 8 = heptacarboxylporphyrin; 9 = uroporphyrin. From ref. 60, with permission.

from the others. Therefore, further attention was directed only to the solutes causing some separation problems. For the remaining six porphyrins five resolution maps were created, overlapped, and the lowest resolution amongst all resolution maps was plotted. In this way regions defining buffer compositions with which the minimum desired resolution can be achieved for all solutes was identified (for the final resolution map, see Fig. 3b). Next, the validity of the results was tested by running a separation in 38 mM CAPS–20 mM SDS–8.2% DMF, the result of which is shown in Fig. 4.

This approach can be applied to virtually any set of compounds to be analysed. When combined with cutting methods and/or a suitable preconcentration step (see Section 2) it can virtually guarantee good results.

4. Separation of biomedically important proteins

It is beyond the scope of this review to discuss in detail all the problems related to the capillary electrophoresis of proteins. Optimization of the separation conditions and modification of the capillary wall represent the two basic approaches to prevent irreproducible sorption of proteins to

the capillary wall. For more information the reader is referred to more specialized reviews or books (see Introduction). Here we summarize methods and approaches that are clearly of biomedical relevance.

Recently Zhu *et al.* [61] evaluated capillary isoelectric focusing and free zone capillary electrophoresis for the separation of native haemoglobins and globin chains. Analysis of the haemoglobin composition of human blood is of major clinical interest because of the number of disorders associated with abnormal haemoglobins. High-resolution separations of adult human haemoglobin A, foetal human haemoglobin F and haemoglobin variants S and C are now possible. Isoelectric focusing of haemoglobins was achieved by using a 17 cm × 25 μm I.D. capillary coated with a non-specified hydrophilic polymer. Water-purged capillaries were washed with 10 m*M* phosphoric acid between separations. Haemoglobin samples were mixed with pH 3–10 ampholytes to a final ampholyte concentration of 2%. Samples were pressure injected and focusing was carried out at 7 kV for 5 min using 40 m*M* sodium hydroxide as catholyte and 20 m*M* phosphoric acid as anolyte. Cathodic mobilization was effected by replacing the catholyte with a proprietary zwitterionic solution (Bio-Rad, unspecified). For capillary zone electrophoresis a 35 cm × 25 μm I.D. coated capillary was used. The separation was run in 100 m*M* sodium phosphate buffer (pH 3.9) containing 7 *M* urea and 1% of reduced Triton X-100. Samples were loaded electrophoretically at 8 kV for 8 s and separated at 8 kV. Zhu *et al.* [62] also recently evaluated capillary isoelectric focusing with respect to abnormal haemoglobins associated with beta-thalassaemia. The technique can easily differentiate haemoglobins Bart's and H.

Whereas with capillary electrophoretic separation it is possible to detect the separated haemoglobins at, *e.g.* 210 nm, the same approach is not possible with focused haemoglobins. The reason is that the ampholytes used show a strong absorbance at this wavelength and obscure the results.

Besides diagnostic purposes as just described, capillary electrophoresis proved to be a versatile method for the determination of proteins, peptides and amino acids in pharmaceutical formulations. Some practical hints and dangers resulting from the application of capillary electrophoresis to this field were recently published by Guzman *et al.* [63]. Significant errors may result if the analysis is performed under unsuitable conditions. The peak area response for protein analytes (which is generally low if UV detection is used) may depend considerably on the nature of the background electrolyte used. For optimum peak area response and reproducibility, protein derivatization with, *e.g.*, fluorescamine was recommended. This separation was carried out in the presence of a moderate ionic strength buffer containing lithium chloride, tetramethylammonium chloride or trimethylammonium propylsulphonate. Humanized monoclonal antibodies were used as solutes tested for purity.

Another broad field in the separation of proteins is the separation of complex protein mixtures. In this area single-dimension techniques can be applied with only limited success. Although the theory of two-dimensional separations has been fully explained [64–67], practical applications are coming into practice only slowly. The first attempts at coupling capillary electrophoresis with HPLC involved analysis of only certain regions of interest from the first dimension by the second-dimension separation. The main problem in coupling CZE with HPLC lies in the incompatibility of the time constants of the two systems. Because of this, HPLC (or column gel chromatography) should always be the first separation step followed by the electrokinetic separation. Recently, a system for two-dimensional separations combining gel chromatography and capillary zone electrophoresis that exhibits practical applicability features has been developed by Lemmo and Jorgenson [68]. Size-exclusion chromatography is carried out in a 1 m × 250 μm I.D. microcolumn. The effluent from this size-exclusion chromatographic microcolumn fills a sample loop on a computer-controlled six-port valve. The contents of this loop are transferred past the grounded end of the electrophoresis equipment capillary for electromigra-

tion injection. Detection is done by UV absorbance at 214 nm. The system was shown to be applicable for analysing human, horse and bovine sera (Fig. 5).

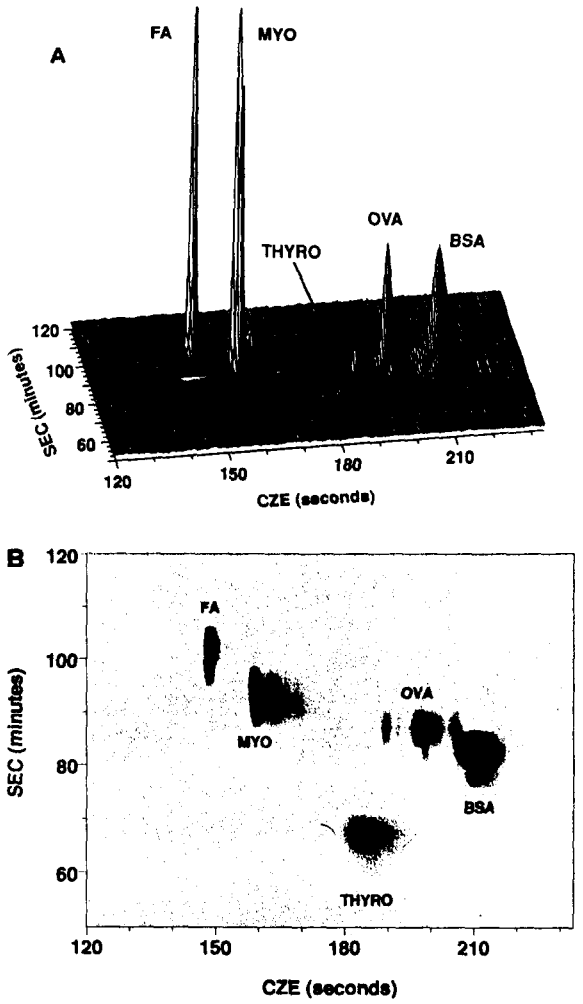


Fig. 5. Separation of protein standards by two-dimensional size-exclusion chromatography–capillary electrophoresis. Size-exclusion injection was for 10 min at 7 bar. A head pressure of 55 bar was applied to produce a flow-rate of 360 nl/min (for the chromatographic separation). The electrophoresis capillary was 38 cm long, 20 cm to the detection window. Conditions: 5-s electromigration injection at −3 kV and 4-min runs at −8 kV. Data collection two points/s. Top, surfer-generated 3D chromatoelectropherogram; bottom, spyglass-generated greyscale image. Peaks: FA = foetal serum albumin; MYO = myoglobin; THYRO = thyroglobulin; OVA = ovalbumin; BSA = bovine serum albumin. From ref. 68, with permission.

The success of this arrangement is based on extremely fast separations in the capillary electrophoresis section (the overall electrophoresis run is mostly around 4 min, but in any case less than 10 min). Of course, the size-exclusion separation is still long, of the order of 120 min. However, the construction of the device and the short electrophoresis running times allow fast sampling for the electrophoresis part and the creation of useful two-dimensional maps.

Another area of concern is silica-based size-exclusion chromatography of proteins, which is done under conditions of high salt concentration, often of the order of 0.5 *M*. Such salt concentrations are not compatible with the electrophoresis system, which in most instances is run with about 50 m*M* buffers. Therefore, Lemmo and Jorgenson [68] used Zorbax GF 450 as the packing material for the size-exclusion step because it has a relatively low excess surface charge. Thus they were able to carry out the size-exclusion separation at a salt concentration that was compatible with the electrophoresis step. On the other hand, this set-up is limited to separating proteins with p*I* less than 8.23. If the p*I* is higher, adsorption of proteins both to the size-exclusion column packing and the capillary wall of the electrophoresis equipment takes place, distorting the results. Another constructional problem is the interface between the size-exclusion column and the electrophoresis capillary. It is this interface that determines the operational conditions of the whole system.

As stated [68], an ideal two-dimensional size-exclusion–capillary electrophoresis system would allow operation near the optimum flow-rate for the chromatographic column with a sampling frequency for CZE high enough to prevent recombination of components separated in the first (chromatographic) step. To meet these conditions, the size-exclusion chromatographic step should operate near a flow-rate of 50 nl/min with capillary electrophoresis runs of 2–3 min. Under these conditions only 100–150 nl of sample would be generated for injection into the capillary electrophoresis part. In the apparatus described by Lemmo and Jorgenson [68], the sampling loop, however, was 900 nl; collection of

900 nl takes about 18 min at a size-exclusion chromatographic flow-rate of 50 nl/min. Although an 18-min CZE run would result in a considerably better resolution, gross undersampling of the size-exclusion column effluent would distort the final result. Consequently, the operating conditions are a compromise between the optimum conditions for size-exclusion chromatography and capillary electrophoresis.

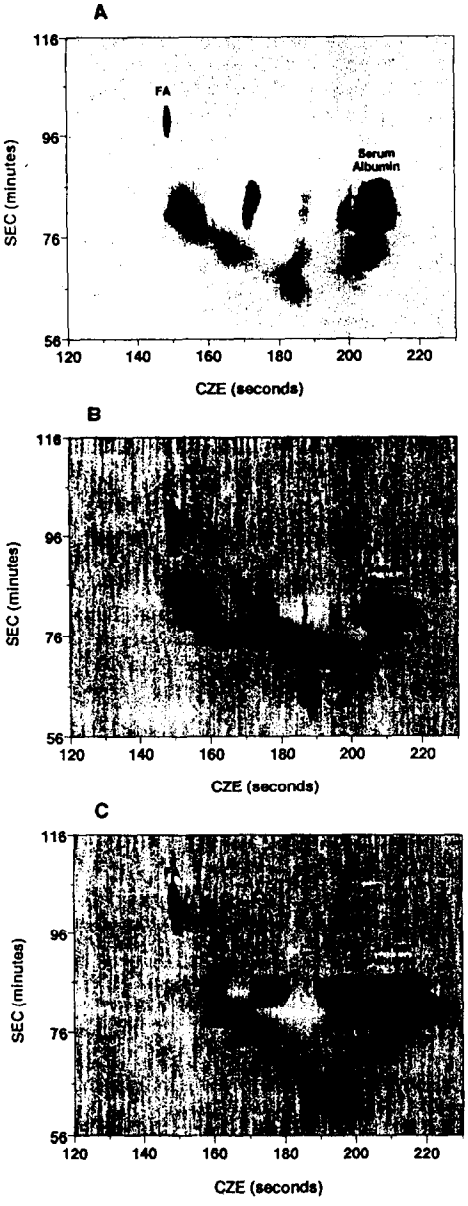


Fig. 6. Separation of serum samples by two-dimensional size-exclusion chromatography–capillary electrophoresis. Conditions as in Fig. 5. Spyglass-generated greyscale images are presented for (A) human serum, (B) horse serum and (C) bovine serum. From ref. 68, with permission.

In spite of all the compromises and problems, acceptable results were obtained as shown in Figs. 5 and 6. Nevertheless, the prospects for this approach for *e.g.*, looking for abnormal proteins in tissues and tissue or cell protein profiling is high.

There is at least one other category of frequently studied proteins of biomedical importance, apolipoproteins: the influence of various detergents on the capillary electrophoretic behaviour of these proteins was studied by Tadey and Purdy [69]. It was demonstrated that electrophoretic mobility increased in the presence of anionic detergents and decreased in the presence of non-ionic Triton X-100. Plasma apolipoproteins exhibited different affinities to SDS and sodium deoxycholate (anionic detergents). Optimum resolution of HDL and LDL apolipoproteins was obtained with high-pH buffers containing SDS. VDLD apolipoproteins were separated either in SDS-containing electrolytes or in buffers containing cetyltrimethylammonium bromide. Particularly for VLDL better results were obtained with polyacrylamide-coated capillaries.

5. Enzyme activity assay by capillary electrophoresis

A frequent task in biomedically oriented laboratories is the determination of enzyme activities. Although not widely used at the moment, capillary zone electrophoresis offers the possibility of ultramicroanalyses of enzymes in different naturally occurring samples. The idea of these microassays was developed by Bao and Regnier [70] and demonstrated in their work with glucose-6-phosphate dehydrogenase.

This approach starts with understanding the familiar mechanism of enzyme-catalysed reactions, in which a substrate S reacts with an enzyme E to form an enzyme–substrate complex ES. This complex decomposes with the forma-

tion of a product P and regenerates the enzyme. Because different members of this family may have different charges, they may also exhibit different electrophoretic mobilities. Bao and Regnier [70] took as a hypothetical example an enzyme with net charge +10, a substrate with net charge −2 and a product with net charge −1. Apparently the mobility of the enzyme would be much greater than that of the substrate or the product and it will occur in the opposite direction. It is possible to arrange the whole system in such a way that the band of the enzyme can be made to overrun the band of the substrate by applying an electric potential across the capillary. Thus Bao and Regnier [70] introduce the idea of electrophoretic mixing. They argued that there is no dilution in such a system, turbulence is not required and, consequently, spreading of the zone will be minimal. The only band-spreading force here is a simple diffusion. All this, however, is true in the absence of the endo-osmotic flow. Finally, they emphasized that such a mixing can be fast, of the order of seconds.

When the substrate and the enzyme are electrophoretically mixed, most of the enzyme will appear in the enzyme–substrate complex. If we take the example used by the authors, the net charge of the enzyme–substrate complex will be $10 - 2 = 8$. As in practice enzyme activity measurements are carried out under substrate saturating conditions, we can assume that the mobility of the enzyme is actually the mobility of the ES complex. The next step is the incubation. This can be done in the stopped-flow mode at zero potential. In this case, neither electrophoretic nor electrokinetic transport takes place and all the components of the mixture remain within a single, narrow zone. The other possibility is to run the mixture under a constant potential. In this case the reactants and products will be separated and mixed continuously. At first glance it may seem that in the latter case an assay would be precluded by continuously separating the enzyme from the product. This, however, is not so, as in practice the enzymic reaction occurs orders of magnitude faster than the separation. Bao and Regnier [70] calculated that product formation could be 100–100 000 times greater than the amount of the enzyme, depending on the turnover number of the enzyme.

Finally, when the product is formed in the system and separated from the parent compound in some electrophoretic mode, it has to be detected. Clearly, at this stage, the product has to be transported to the detector by a combination of electrokinetic and electrophoretic processes.

A practical arrangement could be visualized as follows: a surface-deactivated capillary is filled with an enzyme saturating concentration of the substrate. Buffer and all other ingredients needed are also added. Enzyme is introduced as in any other capillary electrophoretic system, *e.g.*, either electrokinetically or hydrodynamically. When the potential is switched on, the components will be mixed and the reaction will start. With few exceptions, the product and the substrate–enzyme complex will be transported at different velocities. Product formation will continue until the enzyme leaves the system. The theoretical elution profile will be as shown in Fig. 7. Here it is assumed that the enzyme–substrate complex moves with a higher velocity than the product (panel A). The first product detected at point A corresponds to the enzyme as it migrates past the detector. The spike at position B is that of the product formed during the short interval between introducing the enzyme into the system and switching on the potential. If the situation is such that the transport velocity of the product is higher than that of the enzyme–substrate complex, the electropherogram will be reversed as shown in Fig. 7B. At point A we observe the artificial spike caused by the existence of the interval between introducing the enzyme and switching on the potential. From the position of the artificial peak on the electropherogram, it is possible to determine the relative migration velocities of the enzyme–substrate complex and the product. The level of the plateau indicated as C in Fig. 7A and B is directly proportional to the enzyme concentration at constant potential. In Fig. 7C the situation that may occur with an isoenzyme mixture and a common product is depicted.

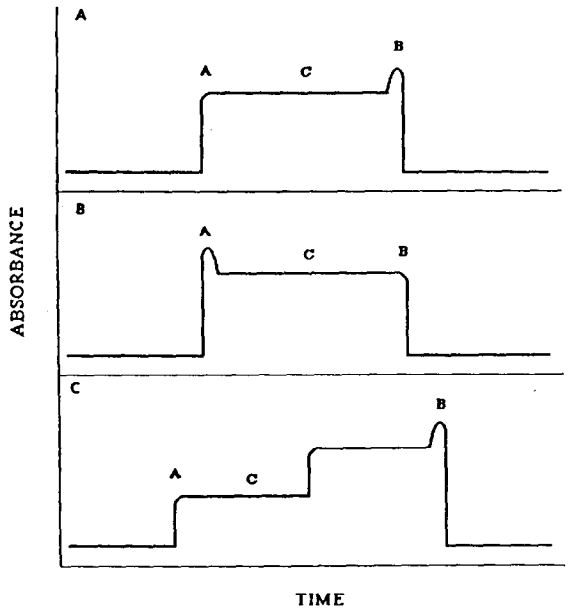


Fig. 7. Predicted models showing various electropherograms in capillary electrophoretic enzyme assay. The moving velocities are (A) ES > P and (B) P > ES. A multiple isoenzyme form is shown in (C) with the moving velocity of the common product smaller than those of these isoenzymes. From ref. 70, with permission.

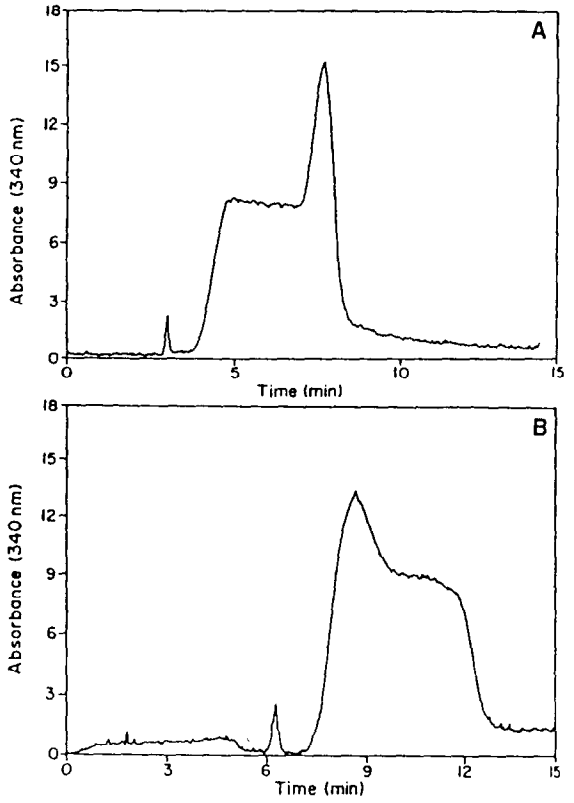


Fig. 8. Electropherograms showing accumulated peaks resulting from the parked reaction at different running times. (A) NADPH accumulated at the beginning before electrophoresis; (B) NADPH accumulated just before G-6-PDH passed the detection window. From ref. 70, with permission.

Switching the system to zero potential for a fixed period of time within the time window when the enzyme passes the detector would allow for the product accumulation. When the power is switched on again the enzyme will be separated from the product and, if the product has suitable detection properties, it will appear as a peak on the enzyme plateau. This approach is sometimes referred to as "parked reaction".

A practical application referring to the activity assay of D-glucose-6-phosphate:NADPH oxidoreductase is shown in Fig. 8.

Of course, a less sophisticated approach can also be used for enzyme activity determination, namely fractions can be collected and enzymatic activity measured in these fractions. The practical realization involves a normal run before fraction collection to detect the migration time of the peak, which should be collected. The time T_c when the start of the peak is going to leave the capillary can be estimated as retention time multiplied by the length of the capillary divided by the capillary length to the detector. When fractions are to be collected, the potential is switched off just before T_c. A new vial is placed at the end of the separation capillary, the potential is switched on again and the peak is forced to move to the sample collection vial. In practice, in this step the potential was set to 7.5 kV, which made the enzyme peak move into the fraction collection vial within 0.6 min. The substrate is added to the vial of the collected peak, the vial is incubated for a defined period of time and the product of enzymatic reaction may be assayed, *e.g.*, in a second capillary electrophoresis run. This approach was used for assaying proteolytic activity in fermentation broth, but the principle is applicable to any other enzyme source with slight modifications [71]. In this

particular case, undiluted fermentation broth was injected directly into the capillary in order to obtain as much material as possible. Also, the volume of the injected sample was as much as 30 nl, which, on the other hand, decreases the separation efficiency. For separation 33 m*M* phosphate buffer (pH 9.5) was used and the separation was carried out at 15 kV.

Finally, if the product of the enzymatic reaction is known, the activity of the enzyme can be assayed on the basis of determining the product "off line". Thus, for instance, glutathione peroxidase activity can be measured by determining the oxidized and reduced form of glutathione by capillary electrophoresis as demonstrated by Pascual *et al.* [72]. The electrophoretic separation buffer used was 100 m*M* tetraborate (pH 8.2) containing 100 m*M* SDS.

6. Protein–drug binding assays

It is well known that drugs bind to plasma proteins, particularly to serum albumin and alpha acid glycoprotein, and only the protein-unbound fraction is responsible for the pharmacological effect. Three variants of size-exclusion chromatography, namely the Hummel and Dreyer method [73], the vacancy peak method [74] and frontal analysis [75], represent the methods of choice today. Recently, Kraak *et al.* [76] tested all three variants in the capillary zone electrophoresis version and showed that the frontal analysis procedure is the preferred approach.

Size-exclusion chromatographic methods exploit the difference in the exclusion of the drug and drug–protein complex from the column packing. In capillary electrophoresis, separation of the bound and unbound drug is based on the charge and size differences. Because the protein molecule is much larger and carries much more charge than the molecule of any drug, it is reasonable to assume that by binding the drug to the protein, neither its charge nor its molecular mass will be significantly altered. Consequently, both the drug and the drug–protein complex will have the same electrophoretic mobility and similar methods to those developed for size-exclusion chromatography can be used with capillary electrophoresis, provided that the protein and the drug have different electrophoretic mobilities.

When applying the Hummel and Dreyer method in its capillary zone electrophoretic version, the capillary is filled with the background electrolyte containing the drug, which causes a large detector background. Then a small sample containing the drug, buffer and the protein is injected. The total concentration of the drug in the sample is the same as that in the background electrolyte, but part of it is bound to the protein. If the potential is switched on, the protein–drug complex starts to move to the cathode, leaving a local deficiency in the drug concentration in the direction of the anode. This deficiency causes a negative peak, which moves with the mobility of the drug and the size of which corresponds to the amount of the bound drug. Because during the migration the protein–drug complex is always in equilibrium with the free drug in the buffer, the protein–drug complex will give a positive peak. As stressed by Kraak *et al.* [76], if at the detection wavelength the absorbance of the binding protein were zero, and the molar absorptivities of the drug and drug–protein complex were the same, then the areas of the positive and negative peak must be equal. This, however, is a situation that almost never occurs in practice (Fig. 9).

When applying the vacancy peak method, at the beginning the capillary is filled with the background buffer containing both the protein and the drug. The situation is similar to the Hummel and Dreyer method described above and the background signal in the detector is high. Next a small plug of the buffer only is applied and the power is switched on. Assuming that the mobilities of the protein and the protein–drug complex are higher than the mobility of the drug itself, the following effects are seen at the front and rear edge of the buffer plug: at the front edge the drug is migrating more slowly than the protein and therefore it stays behind. At the rear end the protein migrates faster than the plug. This process continues until both fronts reach each other. Then, in the middle of the

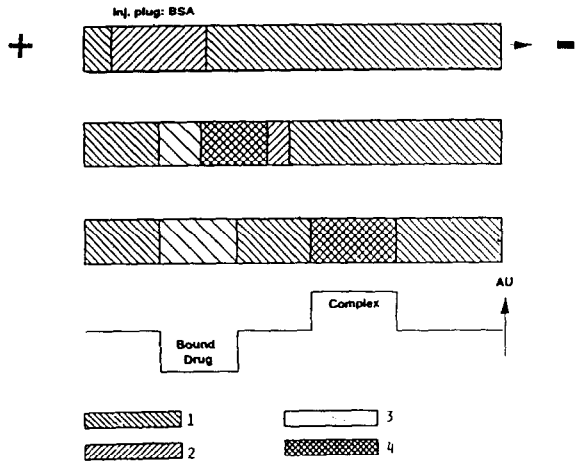


Fig. 9. Schematic representation of the Hummel and Dreyer capillary zone electrophoretic method. Zone identification: 1 = drug; 2 = BSA; 3 = buffer; 4 = BSA–drug complex. From ref. 76, with permission.

plug, the protein starts to absorb drug molecules again as the whole process is reversible. This reabsorption continues until an equilibrium is reached and the drug is reconcentrated to its original level. From this moment on, a steady state is reached and two negative bands appear on the electropherogram: the first corresponds to be the bound drug and the second represents the free drug zone (Fig. 10).

The last approach, also applicable to the capillary zone electrophoresis arrangement, is analogous to frontal analysis. Here the capillary at the beginning of the experiment is filled with the background electrolyte only and a very large sample plug containing buffer, protein and the drug is injected into the capillary. Because of the differences in electrophoretic mobility between the drug and the protein, the free drug starts to leak out at the rear edge of the plug. A plateau corresponding to the free drug concentration is formed. At the front edge of the plug another plateau is created representing the free protein. Thus the result of this run is a three-step profile in which the free protein plateau is followed by the zone of protein–drug complex and the profile is terminated by a plateau corresponding to free drug. The height of the drug plateau is taken as a measure of the free drug concentration. As stressed by Kraak *et al.* [76], depending on the detection wavelength used, the first plateau is frequently not seen by the detector (Fig. 11).

Schematic representations of the three approaches to drug-binding assays are presented in Figs. 9–11. Kraak *et al.* [76] testing the binding of warfarin by bovine serum albumin (BSA) used 0.067 *M* potassium phosphate buffer (pH 7.4) as the background electrolyte. For the practical evaluation of data by the Hummel and Dreyer method, the simplified approach presented by Pinkerton and Koeplinger [77] was applied. In this approach two injections at a given warfarin concentration were applied, the sample consisting of buffer and protein and the blank buffer. From the peak areas the bound

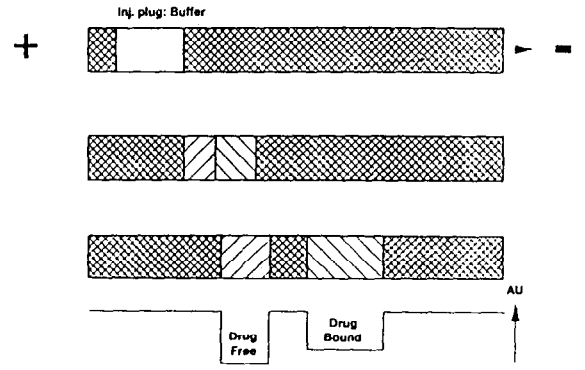


Fig. 10. Schematic representation of the vacancy peak capillary zone electrophoresis method. Zone identification as in Fig. 9. From ref. 76, with permission.

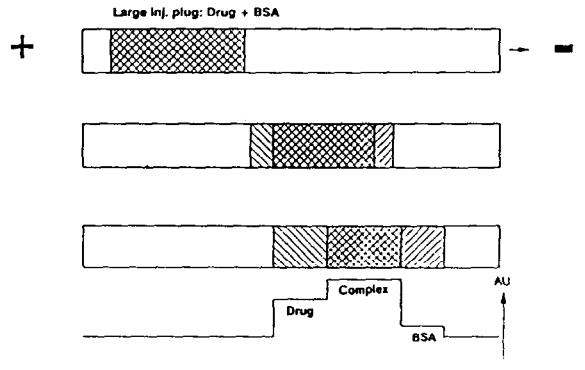


Fig. 11. Schematic representation of the frontal analysis zone electrophoretic method. Zone identification as in Fig. 9. From ref. 76, with permission.

drug concentration (D_b) can be calculated according to the equation

$$D_b = \frac{A_p - A_e}{A_e} \cdot C_d \quad (3)$$

where A_p is the area of the sample peak, A_e is the area of the buffer peak and C_d is the drug concentration in the buffer.

7. Diagnosis of metabolic disorders by capillary electrophoresis

A number human diseases, particularly metabolic disorders, may result in the accumulation of characteristic metabolites in serum, urine or tissues. In addition to HPLC, GC and related coupled techniques (*e.g.*, LC–MS), capillary electrophoresis can also be used to reveal metabolic disorders related to the metabolism of amino acids, nucleotides, peptides and, perhaps, various others [78].

Homocystinuria is a metabolic disorder related to a number of causes, all of which lead to the accumulation of homocystine or homocysteine in serum and urine. Clinical symptoms include dislocation of the eye lens, lengthening of bones and a tendency to thrombosis. The underlying biochemical defect seems to be cystathionine beta synthetase deficiency. Homocystine (homocysteine) assays can be carried out in a 40 cm × 75 μm I.D. fused-silica capillary after derivatization with FMOC reagent (9-fluorenylmethyl chloroformate): standard solutions are prepared at a concentration of 5 mmol/l in 0.05 mol/l phosphate buffer (pH 7.5). To prevent oxidation, DTT (dithiothreitol) is added to a final concentration of 0.8 mmol/l to 250 μl of the standard solution and the standard solution is injected into the CZE device after 10 min of reaction. The buffer used for dissolving the standards is also used as the running buffer. For urine, the samples are reduced with DTT (2.5 mmol/l urine) for 15 min, diluted with equal parts with the running buffer, adjusted to pH 7.5 and filtered through a 0.22-μm filter. To an aliquot (250 μl) of each filtrate 50 μl of Thiolyte MB (monobromobimane) are added and the sample is allowed to react for 10 min before injection into the capillary. A similar procedure can also be used for red blood cells. The cells are centrifuged from citrate blood, diluted with water (equal part), further lysed by freezing and thawing, proteins are precipitated by the addition of perchloric acid (1 ml/l) and removed by centrifugation. The subsequent procedure is the same as with urine.

Figs. 12 and 13 show typical examples of urinalysis of patients with homocystinuria and cystinuria, respectively. The laboratory diagnosis of cystinuria is, according to Jellum *et al.* [78], carried out in two steps: first a colour test is performed using sodium nitroprusside after the addition of a reducing agent to the urine. Following a positive test (red colour), quantitative amino acid analysis is required to differentiate between cystinuria and homocystinuria.

Phenylketonuria is a disease caused by phenylalanine hydroxylase deficiency. The deficit of this enzyme is at the basis of several biochemical changes such as reduction in melanin, serotonin and γ-aminobutyric acid, increased excretion of phenylketone and indole compounds in phenylketonuria patients. The main change is an increase in phenylalanine in serum. This plays a prominent role in the development of progressive mental retardation, which is typical of this disease. For the serum phenylalanine determination a capillary electrophoretic method was worked out by Tagliaro *et al.* [57] (Fig. 14). Separations were carried out with ethanol-deproteinized samples in a 65 cm × 50 μm I.D. capillary (45 cm to the detector) using 25 m*M* borate buffer (pH 10, adjusted with 1 *M* NaOH). The samples were run routinely at 20 kV with UV detection at 214 nm.

Glutathione synthase deficiency or pyroglutamic aciduria is typical in massive urinary excretion of 5-oxoproline (pyroglutamic acid). The disease is typical in severe metabolic acidosis and defective central nervous system functions. The biochemical background is the deficiency of glutathione in cells and tissues with a high production of 5-oxoproline in a modified γ-glutamyl cycle. Laboratory diagnosis of this

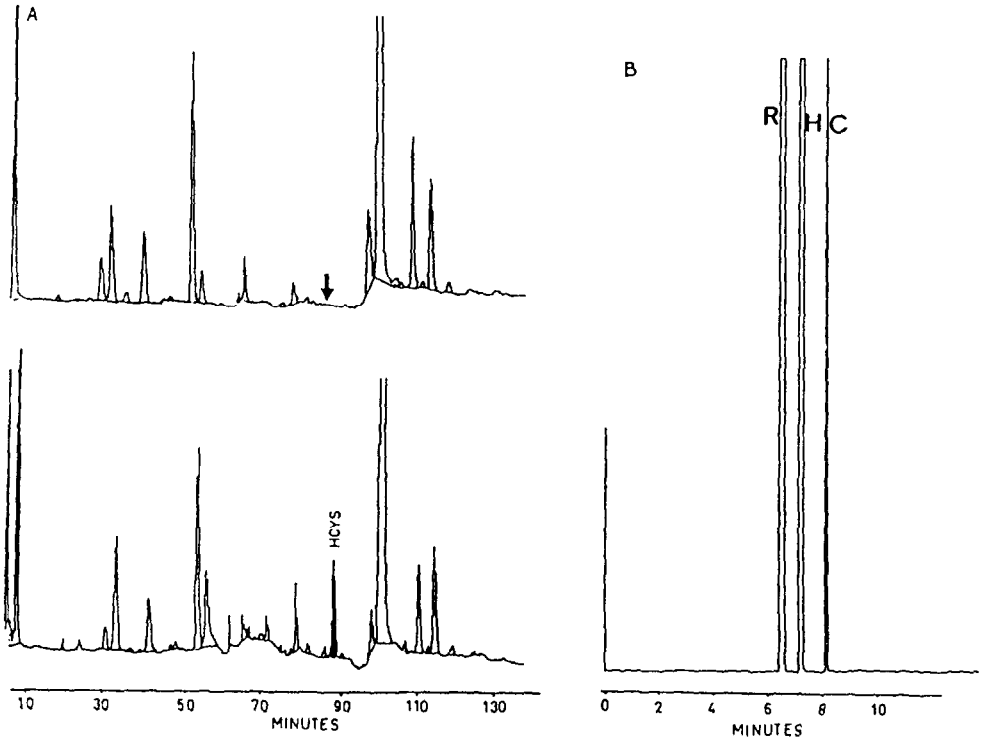


Fig. 12. Amino acid analysis by classical ion-exchange chromatography of urine from a control (A, top) and a patient with homocystinuria (A, bottom). (B) Capillary electrophoresis of urine from a patient. The sample was reduced with dithiothreitol and derivatized before separation. Peaks: R = reagent (monobromobimane); H = homocysteine; C = cysteine. From ref. 78, with permission.

disorder includes the identification of oxoproline in urine, which may be done by GC–MS or, more conveniently, by CZE. A typical example is presented in Fig. 15. Separation can be carried out under similar conditions to those described for cystinuria. It must be borne in mind that glutathione is normally present at high concentrations in cells (red blood cells are often the material of choice) but is absent in urine or serum. Laboratory diagnosis is done in two steps: in the first step high concentrations of glutathione are found in urine, while the second step is aimed at proving the absence of this tripeptide in cells.

Adenylsuccinase deficiency is rare. It involves damaged purine nucleotide metabolism resulting in severe psychomotor delay and autism. The metabolites of diagnostic value in urine are succinylpurines, adenylosuccinate and succinylamino-imidazole-carboxamid ribotide. These compounds can be detected by HPLC, but not by GC–MS. Urinary HPCE analysis typically gives results as presented in Fig. 16. The separation was done in 0.05 mol/l sodium phosphate buffer (pH 2.5) at 20 kV with pressure injection for 3.8 s (Beckman PACE instrument).

Another example also reported by Jellum *et al.* [78] is the determination of aminoethanesulphonic acid (taurine) in heart biopsies. Taurine has attracted considerable interest because of its possible role in the development of cardiomyopathy. The procedure is as follows: to homogenized biopsies [0.05 mol/l phosphate buffer (pH 7.5)] are added 20 μl of 1 mol/l perchloric acid solution to precipitate the proteins; the precipitate is spun off, the supernatant neutralized with a few microlitres of 1 mol/l trisodium phosphate, mixed with a 5–10-fold excess (on a molar basis) of FMOC reagent (5 mmol/l in acetonitrile) and after 20 min of

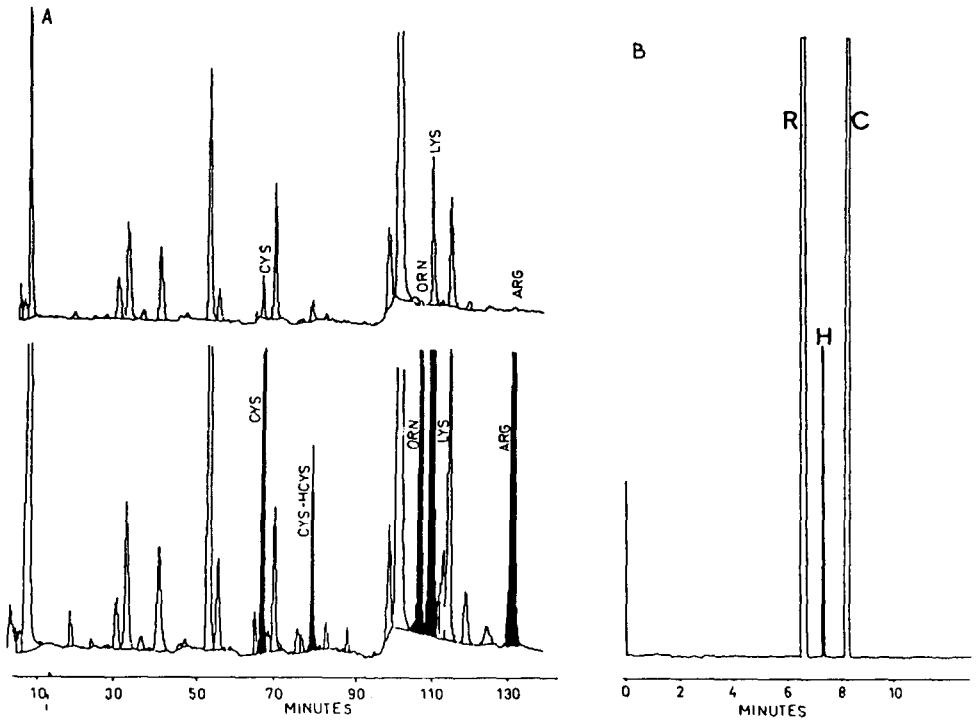


Fig. 13. (A) Amino acid analysis by classical ion-exchange chromatography of urine from a control (top) and a patient with cystinuria (bottom). (B) High-performance electrophoretic analysis of urine from a patient. The sample was reduced with dithiothreitol and derivatized with monobromobimane before analysis. Peaks: as in Fig. 12. From ref. 78, with permission.

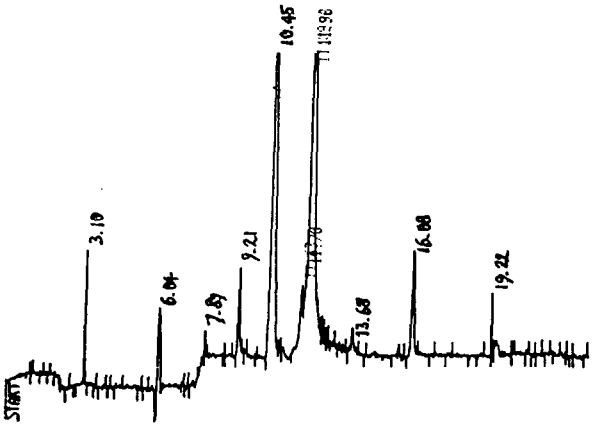


Fig. 14. Typical electropherogram of an extract of serum from a phenylketonuria-affected patient, containing Phe at a level of 250 μg/ml as measured with an amino acid analyser. Separation at 20 kV in a 65 cm (45 cm to the detector) × 50 μm I.D. capillary. Buffer, 25 m*M* borate (pH 10.0). Peaks: phenylalanine represents the second dominant peak in the electropherogram. From ref. 57, with permission.

reaction at room temperature the derivatized amino acids are separated by CZE with fluorescence detection.

8. Monitoring of drugs in body fluids

Electrokinetic separation technology is capable of exploiting a number of separation principles that makes it well suited for monitoring drugs and their metabolites in body fluids such as serum, saliva and urine. The possibilities can go further and trace amounts of drugs or bound metabolites can be detected in tissues. Glycation of structural proteins or deposition of drugs in hair may serve as demonstrative examples. Regarding the separation technology itself, most attention has been focused on micellar electrokinetic capillary chromatography because it permits the separation and determination of

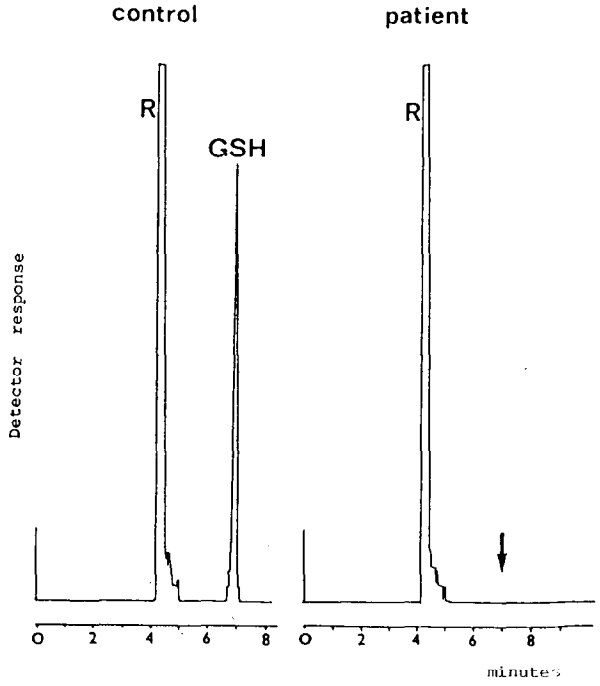


Fig. 15. Capillary electrophoretic analysis of reduced glutathione in red blood cells from controls (left) and a patient with glutathione synthetase deficiency right). Experimental conditions similar to those in Fig. 13. GSH = reduced glutathione. From ref. 78, with permission.

drugs with discrimination based largely on the hydrophobic properties of the molecules.

Selectivity in micellar electrokinetic chromatography depends on the nature of the micelle-forming compound, the pH of the background buffer and the use of additives, including organic modifiers and salts. For monitoring of drugs and their metabolities the selection of the buffer is extremely important. This topic was discussed in detail by Thormann *et al.* [79]. The data summarized in Table 2 illustrate the difficulty in determining optimized conditions for micellar electrokinetic chromatography of a number of drugs having reasonable elution time intervals. Employing small amounts of methanol, 2-propanol or acetonitrile as organic modifiers, the impact of solubilization changes is strongest with 2-propanol on the hydrophobic side. However, the effect with compounds (drugs) having a more hydrophilic character, such as benzoylecgonine or morphine glucuronide, is different: these two compounds, as shown by Thormann *et al.* [79], are best separated in the absence of organic modifiers in the running electrolyte, they can be separated in the presence of methanol but are impossible to separate in the presence of 2-propanol. Thormann *et al.* [79] concluded that optimized micellar electrokinetic separation conditions can be typically established for only a few compounds of interest. This means that micellar electrokinetic separations will need selective drug extraction prior to analysis or, alternatively for screening purposes, stepwise extraction followed by sequential analysis of each eluate.

On arrival, the samples have to be prepared for analysis. This preparation steps involve centrifugation, dilution, filtration, release of the analyte from the sample matrix (hydrolysis, sonication), removal of endogenous compounds and procedures to increase the sensitivity and selectivity of the assay. A general review covering all these steps was published recently by McDowall [80]. Although not devoted directly to micellar electrokinetic separations, the procedures described in that review may well be applied to samples intended to be subjected to capillary electrophoretic techniques.

As stressed by Thormann *et al.* [79], micellar electrokinetic chromatography with sodium dodecyl sulphate micelles was shown to allow the direct injection of protein-containing fluids (serum). The situation here is similar to that in HPLC working with micellar mobile phases. The proteins are solubilized in the presence of SDS micelles and elute as a broad zone after uric acid. Ultrafiltration prior to sample application removes the proteins and results in a considerably simplified profile. It is worth mentioning that ultrafiltration changes the electrophoretic pattern after the uric acid peak, but not before it. There are essentially two interference-free windows in the electropherogram bracketed by creatinine and uric acid. Serum levels of drugs that elute within these windows can be determined by direct sample injection without any pretreatment. Cefpiramide, aspoxicillin, phenobarbital, caffeine and theophylline belong to this category.

Naturally, for drugs that elute after uric acid

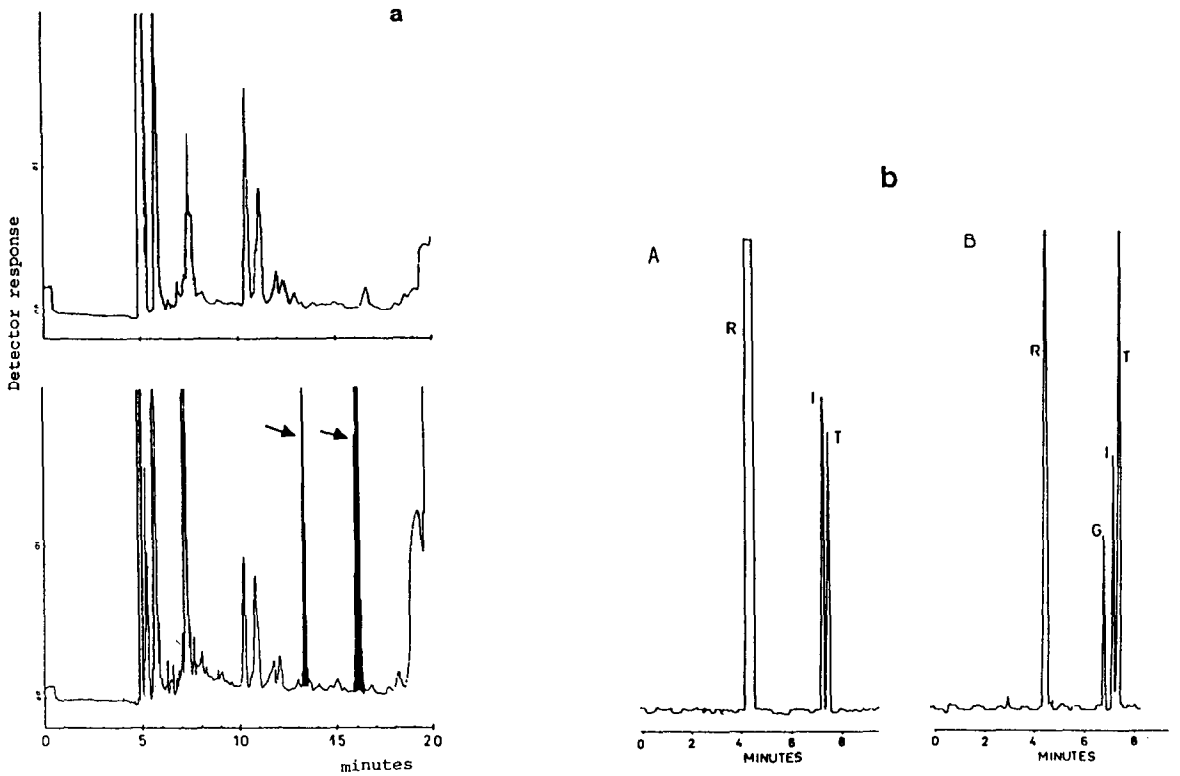


Fig. 16. (a) Capillary electrophoretic profiles from a control (top) and from a patient with adenylsuccinase deficiency (bottom). Detection by absorbance at 214 nm; the succinylpurines are marked by arrows. (b) Capillary electrophoretic analysis of a submiligram sample of heart biopsy. (A) separation of taurine and the internal standard; (B) analysis of the biopsy to which internal standard was added before derivatization with FMOC (9-fluorenylmethyl chloroformate) and electrophoresis. Peaks: R = reagent peak; I = internal standard; T = taurine; G = tentatively identified as reduced glutathione. From ref. 78, with permission.

pretreatment of the sample is necessary. Ultrafiltration followed by micellar electrokinetic capillary separation gave acceptable results and no interferences relating to protein removal were found with commercial control serum samples spiked with more than 30 drugs covering antiarrhytmics, anticonvulsants, antiasthmatics, antineoplastics, antibiotics, antidepressants, antipsychotics, analgesics and an immunosuppressant (cyclosporin).

Several examples of micellar electrokinetic chromatography of drugs present in ultrafiltered sera have been published. Fig. 17 shows the difference between direct serum injection and the sample purified by ultrafiltration. Fig. 18 shows three-dimensional plots for a serum containing phenobarbital, ethosuximide, primidine and phenytoin [81]. Another example is shown in Fig. 19, where an example of the separation and determination of phenobarbital and carbamatepine in ultrafiltered saliva is presented [79]. Direct injection of urine was shown to provide complex electropherograms within the first half of the elution range, resulting in difficulties in the unambiguous identification of zones. With proper buffer selection, *e.g.*, caffeine metabolites can be identified. However, for fast-moving drugs and drugs or metabolites present at low concentrations, appropriate sample pretreatment is necessary. Fig. 20 shows the identification of morphine and benzoylecgonine in a solid-phase-extracted urine specimen. Another example has been published by Lee *et al.* [82], who demonstrated the possibility of

Table 2
Impact of buffer modifications on MECC elution range and capacity factors of selected drugs

System	Additive	Current (μA)	t_0 (min)	t_{mc} (min)	Capacity factors of eluting compounds[a]				
					15	1	6	4	12
1	None	78	6.60	25.77	0.567	1.51	50.3	73.3	115
2	5% methanol	68	7.78	34.26	0.546	0.960	30.4	43.2	67.1
3	5% 2-propanol	70	7.84	38.16	0.517	0.570	12.8	18.1	40.8
4	5% methanol, 5 m*M* Brij	74	7.71	28.90	0.597	0.540	17.1	22.4	42.2
5	5 m*M* Brij	88	6.50	22.36	0.566	0.656	25.1	34.7	76.8
6	10 m*M* Brij	90	6.38	17.32	0.699	0.472	13.4	17.2	64.8
7	None	77	6.81	23.24	–	1.68	63.3	81.5	133
8	5% acetonitrile	72	7.69	33.70	–	0.854	27.0	36.8	58.9
9	10% acetonitrile	72	7.94	45.85	–	0.468	10.1	14.2	24.5

Methanol and methadone served as markers for the electroosmotic flux (t_0) and micelle elution (t_{mc}), respectively. A borate–phosphate buffer (pH 9.2) with 75 m*M* SDS was employed. Buffers containing organic solvents were diluted by the content of the solvent (5 to 10%). From ref. 79, with permission.

[a] Capacity factors were calculated by $k' = (t - t_0)/t_0(1 - t/t_{mc})$, where t is the elution time of the compound. Compound identification: 1 = benzoylecgonine; 4-methamphetamine; 6 = amphetamine; 12 = diazepam; 15 = morphine-3-glucuronide.

assaying antiepileptics [ethosuccimide, primidone, valproic acid, phenobarbital, hexobarbital (internal standard), phenytoin and carbamazepine] in extracted serum samples. The reproducibility of the retention times in such assays can be judged from Table 3.

Further information about drugs and endogenous compounds that have been analysed by electrokinetic techniques can be obtained from refs. 82 and 85–97.

A specific topic is the analysis of urine, blood or hair samples for abused drugs and their

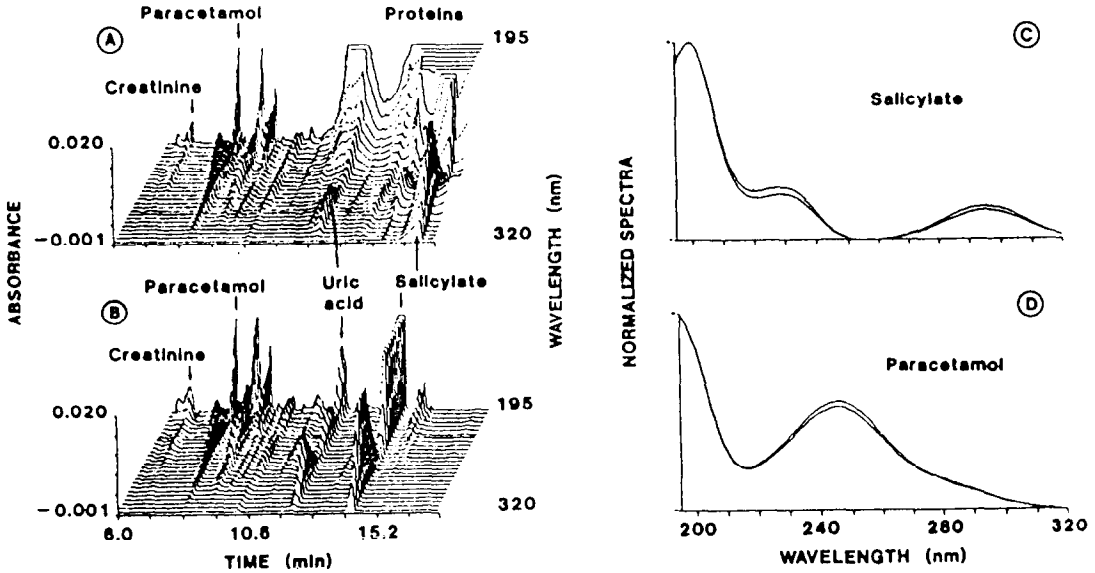


Fig. 17. Three-dimensional micellar electrokinetic separation data for (A) directly injected and (B) ultrafiltered patient serum containing salicylate and paracetamol. (C) and (D) represent spectral identity proofs for salicylate and paracetamol. From ref. 81, with permission.

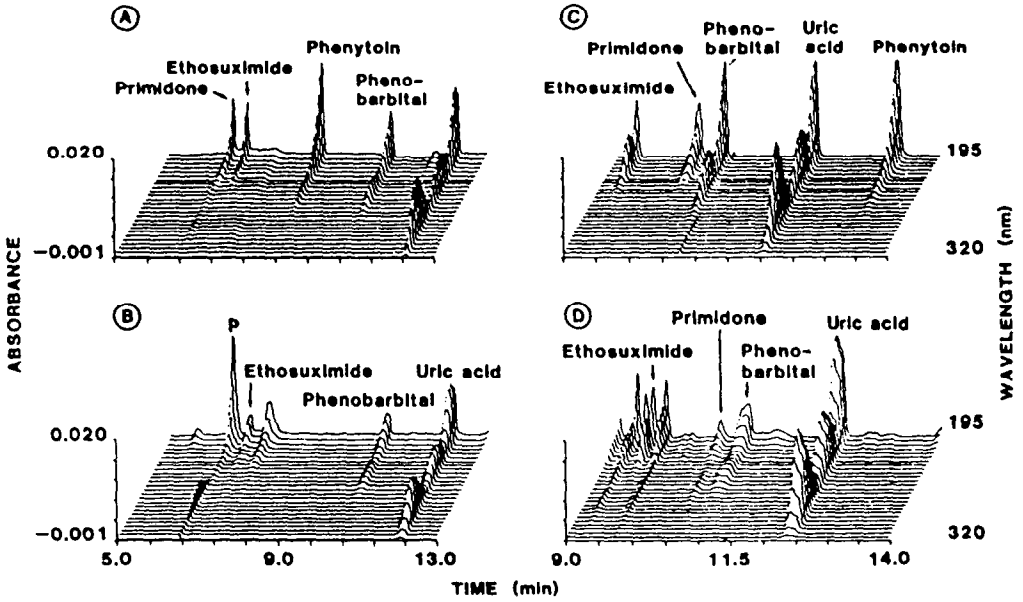


Fig. 18. Three-dimensional capillary electrophoresis data representing separation in the presence of a surface-active agent (A and B) and in its absence (C and D). Separation of four antiepileptics and uric acid. Standards in (A) and (C), ultrafiltered serum of a patient under multiple anticonvulsant drug therapy in (B) and (D). Peak P in (B) is the peak in which primidone is co-migrating with endogenous compounds. Sample injection, electrophoretic, 5 s, 20 kV, 75 m*M* SDS–6 m*M* borax–10 m*M* Na_2HPO_4 (pH 9.1). The same buffer was used for runs in the capillary zone electrophoresis mode except without SDS. from ref. 81, with permission.

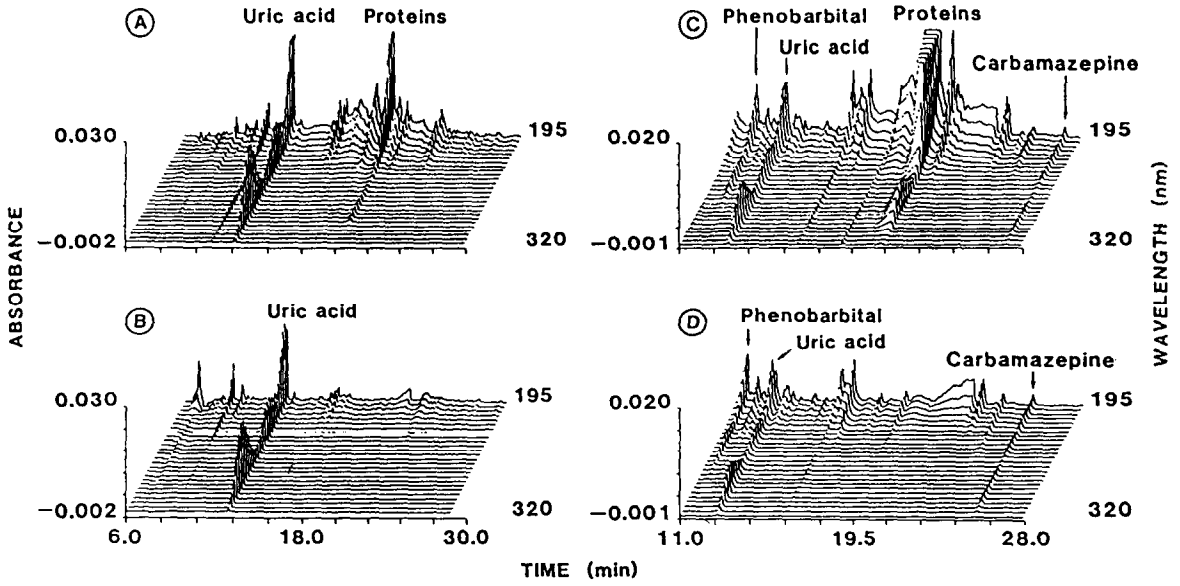


Fig. 19. Three-dimensional plot of a micellar electrokinetic separation of saliva blank, (A) directly injected and (B) ultrafiltered. (C) and (D) are corresponding saliva data after phenobarbital and carbamazepine administration. From ref. 79, with permission.

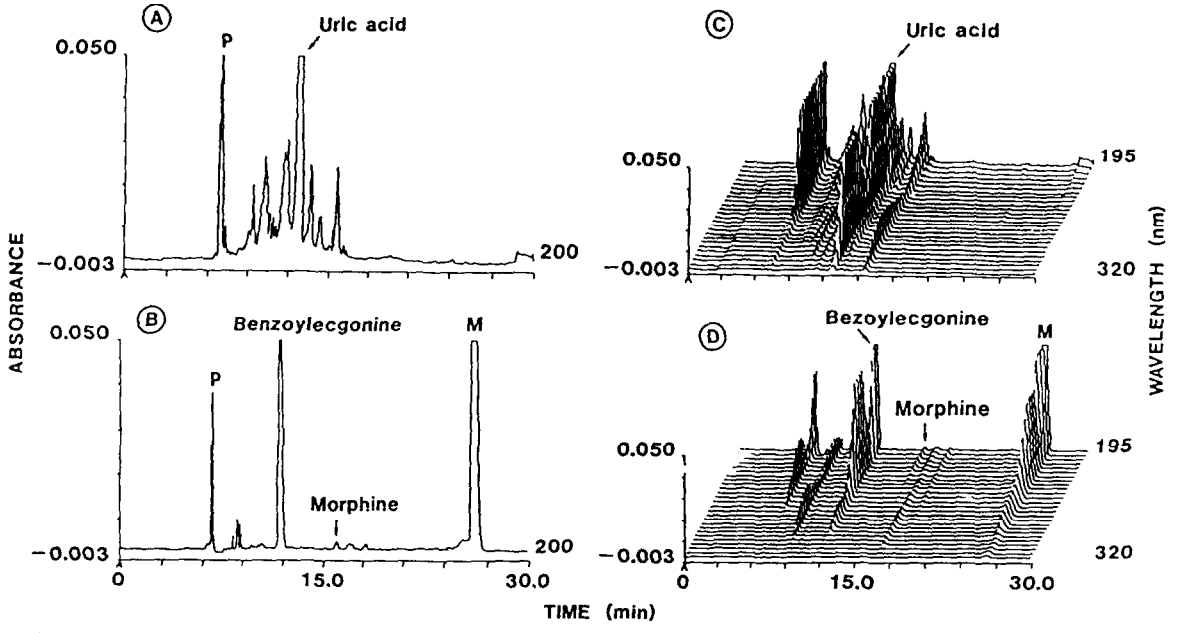


Fig. 20. Micellar electrokinetic separation of an opiate- and cocaine-positive urine, (A, C) after direct injection and (B, D) the same samples after solid-phase extraction. P and M refer to initial and micelle peak, respectively. From ref. 79, with permission.

Table 3
Reproducibility of retention times and peak areas for antiepileptics

Compound	Concentration (μg/ml)	Retention time (min)	Relative peak area
Ethosuccimide	25	4.45 ± 0.01 (0.10)	0.25 ± 0.01 (4.71)
	75	4.41 ± 0.14 (0.33)	0.58 ± 0.04 (8.27)
	100	4.44 ± 0.01 (0.32)	1.03 ± 0.06 (6.30)
	200	4.41 ± 0.01 (0.08)	1.84 ± 0.08 (2.54)
Primidone	5	5.47 ± 0.01 (0.16)	0.53 ± 0.03 (5.81)
	20	5.39 ± 0.01 (0.10)	1.46 ± 0.03 (2.41)
	30	5.45 ± 0.02 (0.38)	2.21 ± 0.03 (1.59)
	40	5.42 ± 0.01 (0.35)	2.80 ± 0.03 (1.31)
Phenobarbital	5	6.03 ± 0.01 (0.20)	0.54 ± 0.01 (2.57)
	20	5.87 ± 0.01 (0.16)	1.79 ± 0.03 (1.93)
	40	5.82 ± 0.01 (0.29)	3.57 ± 0.10 (2.94)
	60	5.98 ± 0.03 (0.52)	5.15 ± 0.09 (1.86)
Phenytoin	5	10.58 ± 0.03 (0.28)	1.04 ± 0.01 (1.52)
	20	10.40 ± 0.01 (0.16)	3.51 ± 0.14 (4.12)
	30	10.39 ± 0.05 (0.50)	3.13 ± 0.07 (2.46)
	40	10.55 ± 0.05 (0.55)	2.96 ± 0.06 (2.11)
Carbamazepine	5	12.09 ± 0.03 (0.32)	1.77 ± 0.01 (0.39)
	20	11.88 ± 0.02 (0.19)	6.13 ± 0.23 (3.82)
	30	11.81 ± 0.06 (0.53)	9.90 ± 0.39 (4.01)
	40	12.10 ± 0.06 (0.53)	13.23 ± 0.32 (1.32)

Values are means ± S.D. ($n = 5$); values in parentheses are coefficients of variation (%). From ref. 82, with permission.

metabolites. Although Thormann *et al.* [79] used micellar electrokinetic separations for this purpose, capillary zone electrophoresis can serve a similar purpose as shown by Tagliaro *et al.* [83] and Marigo *et al.* [84]. Wernly and Thormann [85] assayed the main urinary metabolite of the most commonly abused drug 9-tetrahydrocannabinol, 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid, using basic hydrolysis, extraction and concentration of a 5 ml-urine specimen. The separation was carried out in a 70 cm × 75 μm I.D. capillary at 20 kV. Runs were performed with a grounded cathode and the sample was introduced by gravity. Drug concentrations down to 10 ng/ml can be monitored unambiguously. Peak assignment was achieved through comparison of the retention time and absorption spectrum with the eluting metabolite peak with those in computer-stored model runs.

9. Conclusions

In addition of the wealth of data now available about the conditions for the capillary electrophoretic separation of a large number of both endogenous and exogenous compounds, this technique appears well suited for specific tasks, such as the analysis of complex biological matrices, monitoring of drugs, assaying their binding capacity to proteins or miniaturization of enzymic activity determination. The advantages are obvious: minimum sample consumption, minimum demands on the solvents used, in most instances worked out separation techniques that can be directly applied with minimum alterations, the possibility of determination and the possibility of coupling the electrophoretic system with, *e.g.*, MS. Although UV detection and, perhaps, fluorescence detection predominate today, indirect detection by using suitable UV-absorbing compounds in the background electrolyte makes this method applicable to mixtures consisting exclusively or in part of solutes that are impossible to detect in UV light. This is particularly welcome in situations where solutes of different chemical natures are to be assayed side-by-side, a very frequent situation in biomedical analysis. Although it is generally believed that the separation efficiency of the technique can go up to 10^6 theoretical plates per capillary (about 60 cm long), with samples of biological material this rarely reaches half of this value, being mostly in the range 200 000–300 000.

Introduction of the micellar electrokinetic separations by Terabe *et al.* [98] extended the original limited applications of the technique into the area of hydrophobic compounds, allowing separations of, *e.g.*, drugs, drug forms and decomposition products. Of particular interest is the observation that in the presence of a micelle-forming surfactant in the background electrolyte, charged compounds are separated according to their charge, while solutes with a distinct hydrophobic molecular domain are separated on the basis of their affinity to the micelle. Hence mixtures that differ considerably in their polarity can be assayed in a single run. This is important, *e.g.*, in the separation of hydrophobic drugs and their decomposition products, as the latter are in many instances highly hydrophobic (bearing carboxylic or other charged groups).

The versatility and in a way the robustness of capillary electrophoretic separations make them applicable to all fields of biomedicine and biological research and practice. With the advancing automation and computerization of capillary electrophoresis apparatus, electrokinetic methods will certainly in the near future become suitable tools in clinical and biomedical laboratories where they will not only be complementary to current separation methodologies such as HPLC, but will in part replace them or will represent an alternative choice for the chemist.

10. References

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