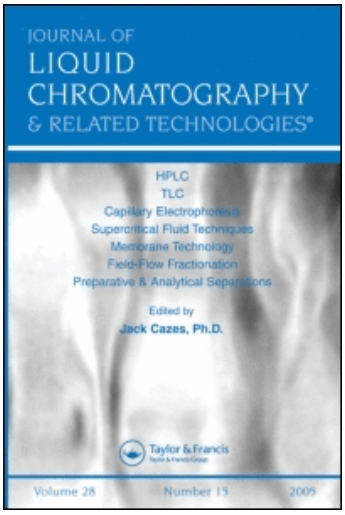

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REVIEW OF APPLICATIONS OF CAPILLARY ELECTROPHORESIS FOR CLINICAL ANALYSIS

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REVIEW OF APPLICATIONS OF CAPILLARY ELECTROPHORESIS FOR CLINICAL ANALYSIS

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ABSTRACT

As an analytical tool, CE offers several advantages in the clinical laboratories. In addition to high resolution of the CE, the ease of modifying the selectivity by simple additives to the buffer allows complex mixtures of analytes in biological samples to be resolved easily. The driving forces for the widespread use of CE at the present time are simplicty, speed, high resolution, and low operating costs. Four general application areas were identified which are expected to have a great impact on the routine clinical analysis by CE. These areas are proteins (serum, CSF, urine), ions, drug screening, and DNA analysis. The analysis of these compounds is rapidly expanding and it is mature enough for bringing them into the routine work. The DNA applications for sequencing and PCR analysis have progressed to the extent that few specialized instruments dedicated to these analyses are on the market. Proteases as well as other enzymes can be analyzed conveniently by CE. The CE can offer also basic physical and structural information for the peptides and proteins such as charge (pI), size, and isoforms. The application of capillary electrophoresis in clinical analysis is expected to become more widespread in the near future with the introduction of more commercial instruments designed for specific applications.

INTRODCTION

Capillary electrophoresis (CE) is a general analytical technique for separation and quantification of a wide variety of molecules based not only on charge, but also on size, hydrophobicity, and stereo specificity. Numerous clinical tests can be adapted to CE; however, some are better suited than others for analysis by this method. In this work we will examine the future of this technique for clinical work and more important what type of applications are most suited for CE and point out those assays most suitable for day–to-day in routine work.

The CE offers specific advantages for clinical analyses: a high plate number, rapid analysis time, low cost per test, and full automation. However, the CE has several major challenges such as poor sensitivity of detection, problems with sample matrix, adsorption to the capillary, a need for better precision, and high instrument cost. Great progress has been achieved towards solving these problems. Because of its high resolution and because of the ability to add vast types of compounds to the buffer which affect the separation, CE is well suited for resolving complex samples, which contain large numbers of related compounds such as: screening for drugs of abuse in urine, or DNA sequencing.

Clinical assays encompass a wide variety of tests; some are uncommon basic science research, while others are common tests used routinely. In this review we will not try to cite all the different clinical applications. Instead we will focus on four types of clinical assays which, in our opinion, are well suited for CE and which show the greatest promise to become common routine tests: proteins (serum, CSF, urine), ion, drug screening, and DNA analysis. Furthermore, we expect, in the near future, dedicated instruments to be designed and commercially sold specifically for these applications.

PROTEINS

Serum Proteins and Immunofixation

Serum proteins are comprised of more than a hundred different proteins at varying concentrations. They are separated into 5-12 bands by agarose gel electrophoresis (AG), which is a time consuming method. They are analyzed routinely on a daily basis in most large hospitals to detect several disorders such as renal failure, infections, and monoclonal gammapathies.

Based on our experience, serum protein analysis by capillary zone electrophoresis (CZE) has definite advantages over AG. Because of the strong absorption of the peptide bond, protein can be detected directly and easily at 214 nm. This detection avoids the need for staining and simplifies instrument

automation. Proteins, especially those with a high pI, tend to adsorb strongly to the capillary walls thus distorting the separation and affecting the precision of the migration time. Several approaches are used to decrease protein binding and to obtain a high plate number in CE: the use of high ionic strength buffers,[1] addition of zwitterions,[2] use of coated capillaries,[1,3-5] or operating at a pH far away from the isoelectric point of the protein.[7,8]

In CE, serum proteins can be separated into 5-12 zones using different buffers such as Tris,[6] borate,[9,10-13,14] Tricine,[13] and glycine,[13] with a pH of 8-11 with the separation being better at pH >8. Chen[12] has described a serum protein separation in narrow capillaries which can be completed in about 90 seconds. The speed of the CE system can be contrasted to 1-2 hrs for AG.

Kline and Joliff [15,16] and Bossuyt[17] described serum protein separation on a commercial instrument performing 6 samples simultaneously in 6 capillaries of narrow diameter (25 μm). The narrow capillaries produce much better resolution than the wider ones with a shorter migration time. Serum electropherograms from patients with different disorders were described to illustrate the clinical significance of this system and how it gives the same clinical information similar to that of the AG.[15,16,17] The correlation coefficient between CE and AG was good[18,19] (0.872, 0.93 and 0.96) for albumin, α2, and gamma, respectively while it was low (0.535, 0.434) for α1 and β fractions respectively.[18] The CVs for peak area were 5-16% and for migration times 1-3%. These results are comparable to those obtained with AG.

Immunoglobulins are composed of heavy and light chains and classified based on their reaction with specific antibodies into IgG, IgA, IgM, IgD, and IgE. Kline and Jollif[15,16] described a method suitable for CE for the immunofixation based on reacting the serum proteins with specific antibodies bound to a solid matrix. The sample is assayed before and after binding. The difference between the two "immunosubtraction" represents the specific type of the m-spike. This method has been shown to be reliable and more suitable than the AG method.[20-22] Serum protein separation by CE has been reviewed previously.[23-26]

Protein analysis by CE has matured enough to be practical and reliable for routine analysis. Furthermore, commercial instruments are now on the market for this specific analysis.

Cryoglobulins

Cryoglobulins are immunoglobulins which reversibly precipitate from serum at cold temperatures. This is a difficult specialized test performed in selected centers.

Cryoglobulins can be classified as monoclonal, a mix of polyclonal-monoclonal or a mix of polyclonal-polyclonal immunoglobulins.[27] Cryoglobulins can precipitate in different tissues of the body such as the kidney and the extremities. They are associated with several immune-type disorders.

Cryoglobulins are detected by precipitating an aliquot of the serum at 4°C, centrifuging and dissolving the precipitate in a buffer followed by electrophoresis using the same conditions as those for serum proteins. The main advantages of CE for cryoglobulins analysis are the higher sensitivity, speed (20 min vs. 6 hrs), and improved quantification.

Urinary Proteins

Because of the low concentrations and the presence of numerous interfering ultraviolet-absorbing compounds, urinary proteins are more difficult to measure by CE when compared to serum. However, the urine contains several proteins of clinical interest, especially Bence-Jones proteins. A few, urine samples from pathological specimens can be analyzed directly without any preparation.[28] However, the majority require concentration and clean-up steps before the analysis by CE. The use of the commercial membrane concentrators for these steps is quite convenient.[29] The same buffers and conditions for serum proteins are basically used for analysis of urine protein. Alcohol precipitation can also be used to prepare the samples for analysis by CE and tends to give very clean electropherograms.[29]

Cerebrospinal Fluid (CSF) Proteins

The main clinical significance of CSF protein electrophoresis is for the detection of the oligoclonal bands which are present in multiple sclerosis in the gamma region. Because proteins in CSF occur at much lower concentrations than in serum (100 times less) a 10-20-fold sample concentration is needed.[30] CSF protein separation can be accomplished in less than 10 min with CE versus 2 hours for AG with the ability to detect oligoclonal banding by this technique.

Serum Lipoproteins

Serum lipoproteins are divided into 5 main groups: Chylomicrons, VLDL, IDL, LDL, and HDL. They are considered mainly as markers of atherosclerosis and coronary heart disease. Schmitz et al.[31, 32] separated lipoproteins into 9-14 fractions based on staining with a fluorescent lipophilic dye followed by isotachophoresis. The HDL was separated into 3 fractions by this method. Tady and Purdy[33] have described the separation of LDL and HDL by CE after ultracentrifugation in borax buffer containing 0.1% SDS. Lehman et al.[14] separated Apo I and Apo II by CE. Goux et al.[34] separated apo I and II in a coated capillary by

MEKC after sample ultra-centrifugation. Apo I gave a single peak while apo II was heterogeneous. Mcfarlane group[35,36] found that both SDS and acetonitrile in the buffer modify the separation of HDL and LDL. They also separated by CE the different lipoprotein fractions after ultracentrifugation with delipidation in the capillary tube.[36]

Enzymes

Analysis of enzymes by CE can be achieved by direct absorbency and also by catalytic activity. Enzymes which are present in high concentration can be measured as mass directly by their light absorbency. Each approach has advantages as well as disadvantages and in some instances does not yield the same answer. In CE, the enzyme, the substrate, and the products all can be measured. Proteolytic enzymes are well suited for analysis by CE because the peptides have strong absorbency in addition to possessing a charge. Furthermore, two related enzymes such as alpha-amylase and glucoamylase can be measured simultaneously as the Terabe group[37] has demonstrated.

Catalytic activity is more convenient and offers better sensitivity in enzymatic assays because the reaction product can be amplified easily several folds. In CE catalytic activity can be measured in several ways:

Incubation in the capillary: Several methods have been described for enzymatic activity based on adding the enzyme and the substrate directly and the capillary is used as a microreactor.[38,39,40,41]

On-line post-capillary reactor: Emmer and Roeraade[42] separated glucose-6-phosphate and 6-phospho gluconic dehydrogenase by CE then added the substrate after the separation using a post capillary reactor.

Incubation outside the capillary: If a long incubation step is needed then it is more convenient to perform that outside the instrument. Proteolytic enzymes with low activity are quite suited for analysis by CE in this manner. Landers et al.[43] have measured the enzyme chloramphenicol acetyl transferase activity by CE. Glutathione peroxidase was measured in cell-free preparations by incubating the enzyme and the substrate outside the capillary then separating by CE the reduced and oxidized glutathione.[44] Ornithine transcarbamylase activity was measured by MEKC based on the incubation of the enzyme with ornithine and carbamyl phosphate and monitoring the formed citruline.[45]

Proteolytic Enzymes

For the proteolytic enzymes, CE can separate the products of the reaction from the substrates and give basic information on the physical properties of the peptides such as ionization, charge to mass, etc. Angiotension converting

enzyme[46] and carboxypeptidase Y[47] were assayed by CE. A tripeptidase from lactococcus lactis was measured rapidly (10 min) by CE without derivatization.[48] The enzyme was reacted outside the capillary with the substrate gly-gly-phe and the products were separated using a citrate buffer. Protease from Pseudomonas aeruginosa was also measured by CE-MS.[49] Elastase activity has been measured by micellar electrokinetic capillary chromatography (MEKC).[50]

Cathepsin D is a proteolytic lysosomal enzyme with an optimum pH of 3.5. Tissue enzyme levels have been found to be a good predictor of tumor malignancy in general and of breast carcinoma in particular.[51] Initially, the enzyme was assayed by its catalytic activity on several proteins and more recently by immunoassays.[51] The action of Cathepsin D on releasing different peptides from hemoglobin and its velocity has been studied by MEKC.[52] We measured this enzyme by its catalytic activity outside the capillary on buffered hemoglobin. Acetonitrile was added to stop the reaction and precipitate the hemoglobin. A specific cleaved peptide was separated by CZE in borate buffer and detected at 214 nm in less than 5 min.[53] A sensitive and general approach for analyzing proteases has been demonstrated by Craig et al.[54] in which the a synthetic biotynylated peptide labeled with fluorescein is hydrolyzed by the enzyme and detected by CE-LIF.

HEMOGLOBIN VARIANTS, A1C, AND GLOBIN CHAINS

The most encountered variants of hemoglobin (Hb) are A,F,S,C; while the common globin chains of the hemoglobin molecule are α, β, γ, δ, and ε. Chen et al.[11] have shown that the common variants can be separated in untreated capillaries by CZE. Because of the small charge difference (pI), the hemoglobin variants do not separate well by CZE especially between HbA and HbF.[55] For good separation of hemoglobin variants by CZE, a high buffer concentration, a narrow capillary 20-30 μm (i.d.), minimum volume of sample, and low voltages are required. Either a 0.8 M Tris or 1.1 M Tricine buffer at pH 8-8.4 gives a good separation.[55] The separation between HBA and F is better at pH 7.9.[55] The separation by CZE resembles very closely that of the alkaline separation by AG. Mario et al.[56] used a commercial reagent where the charge on the capillary is altered to give better separation and reproducibility.

Although the CE instruments are not well designed for capillary isoelectric focusing (CIEF) several workers have successfully separated these variants much better by CEIF than by CZE. Zhu et al.[57,58] using coated capillaries were able to separate, in addition to the common variants, HBA_{1c}, G Philadelphia, and Bart's. Molteni et al.[59] used methylcellulose in untreated capillaries to suppress the electroosmotic flow during electrophoresis. They were able also to separate A_{1c}, A, F, C, S, E, and A2. The separation compared well to HPLC and gel isoelectric focusing. Hempe and Carver[60] separated the major hemoglobin variants including A_{1c} on a coated capillary. Yao et al.[61] and Yao and Regnier[62] described coatings for capillaries which were suited for CIEF of the hemoglobin including

HbA_{1c}. Conti et al.[63] described the problems involved in using coated capillaries in the CEIF of the hemoglobin F separation in cord blood samples. Because of the popularity of the analysis of HbA_{1c} and the low cost of the operating CE, it is expected in the future a dedicated CE instrument for this test to be commercially produced. The variants have been also analyzed by CIEF with special absorption imaging detectors. These types of detection devices do not require pushing or moving the peaks to the detector and can simultaneously detect several capillaries with better precision than the CE instruments.[21]

The globin chains, which are useful for investigating the thalassemias, have been determined by CZE in phosphate buffer either at pH 11.8,[64] or at pH 2.5 - 4.5[65] after acetone precipitation. Furthermore, the tryptic digests of the globin chains were analyzed by CZE using 50 mM phosphate buffer pH 2.5 with detection at 200 nm for the study of the variants.[59,66] The analysis was found to be better than that by HPLC.

ION ANALYSIS

CE, with its ease in measuring both organic and inorganic acids, opens a new door for introduction of these specialized tests into the routine clinical lab. Serum total and ionized Ca were measured by indirect detection in CE with good comparison to an ion specific electrode method and atomic absorption.[67] The addition of crown ethers to the buffers changed the selectivity of these cations.[68] Several inorganic cations: $NH4^+$, Na^+, K^+, Li^+, Ca^{++}, Mg^{++} were measured by indirect UV detection using a buffer composed of cupric sulfate, formic acid, and crown-6 in less than 6 min.[69] Nitric oxide is a mediator of many cellular functions such as vascular tone, signal transmission, and phagocytosis. It is rapidly oxidized to nitrite and nitrate in the blood. Nitrite and nitrate were measured by CE on a coated capillary in urine at 214 nm after 40 fold dilution with water.[70] Plasma NO2 and NO3 were analyzed after sample dilution using absorbance at 214 nm[71] and after serum deproteinization with acetonitrile.[72] We found that CE is much faster and less expensive for nitrate analysis when compared to the enzymatic methods.

Many of the organic acids were measured by both indirect UV detection and by direct UV absorbency, 185-214 nm. For example, oxalate and citrate, which are important in stone formation, have been measured after urine dilution by both indirect detection[73,74] and also by direct UV detection.[75] The CE offers speed, precision, and specificity over other methods.[73] Lactate, pyruvate, ascorbate, and oxalate were measured by CE in the CSF of patients in borate buffers. The analysis was complete in 10 min.[76] Patients with bacterial meningitis and with cerebral infarction showed an elevated pyruvate to lactate ratio. Ascorbate was decreased in inflammatory disorders. Several organic acids in serum such as pyruvate, citrate, malate, acetoacetate, and lactate were determined with direct sample injection with indirect UV detection using ε-aminocaproic and mandelic acids at pH 3.8. The detection limit for citrate was 8 μM.[77] Shirao et al.[75] determined sev-

eral organic acids such as oxalate, citrate, hippurate, lactate, and acetate in urine by absorbency at 185nm in about 12 min. Prochazkova et al.[78] have used a combination of isotachophoresis and CZE to determine ascorbic acid in serum at low sensitivity.

Methylmalonic acid is a sensitive measure of vitamin B_{12} deficiency preceding any clinical symptoms or changes in the serum level of this vitamin. It is also elevated in patients with methylmalonic aciduria. Methylmalonic acid has been determined in serum by CE after reaction with pyrenyldiazomethane and detection with laser-induced fluorescence (LIF).[79] Marsh and Nutall[80] analyzed methylmalonic acid in urine after sample extraction with ethyl acetate and injection onto the CE. Few other acids such as citric, aconitic, ketoglutaric, and succinic acids were detected also in the same run with analysis in less than 6 min. The separation of the organic acids was affected by the addition of divalent ions to the electrophoresis buffer.

DRUG SCREENING

Because of the low concentration and the continuous introduction of new substances, drug screening is a very difficult subject. Furthermore, law regulation and the need for fast results for patient treatment complicates the screening further. Immunoassays which screen for a few general classes of drugs and the GC-MS which is used mainly for confirmation are used in most labs. Both of these methods have certain limitations especially the cost. Both chromatographic and electrophoretic methods identify drugs based on retention/elution/migration time. Unfortunately the migration time in CE is not reproducible enough for identification of the numerous drugs. On the other hand the mobility data are much more reproducible and can be used for this purpose. CE has the potential of being a simple, economical, and powerful method of separation.

The CE offers two powerful modes of separation which can be complementary to each other, the CZE and MEKC. Early work of several groups Thormann, McCord, and Husdson have shown the promise of the CE for drug analysis. Drug Screening by CE is a very enormous task which requires and awaits the resources of a strong commercial company in order to build up a computerized data base for all the controlled substances with their mobility and spectral data. Thus the future growth of this area will depend to a large extent on the entry of a large commercial company.

Northrop et al.[81,82] have described the analysis of 34 compounds (mostly drugs of abuse acidic, basic, and neutral) by MEKC using a short capillary of 25 cm x 50 μm in a borate–phosphate buffer which can be completed in 40 min. Chee and Wan[83] described screening for 17 basic drugs such as amphetamines, codeine, diazepam, and methaqualone in urine and serum by CE in phosphate buffer 0.05 M, pH 2.35 and detection at 214 nm. Thormann and his group[84-89]

have described in several publications the screening of drugs of abuse in serum and urine. Several benzodiazipenes (e.g. diazepam, lorazepam, oxazepam) were separated from human urine by MEKC after hydrolysis and solvent extraction.[85] The methods were shown to be superior to the immunoassays in terms of sensitivity and specificity. They compared the three variations of CE (MEKC, CZE, capillary isotachophoresis) for screening serum and urine in patients with drug overdoses such as barbiturates, acetaminophen, and salicylates. Serum or urine can be injected directly without extraction provided the drug concentration is high with the analysis being completed in 30 min.[86]

Wernly and Thormann[87] described analysis of several drugs of abuse in urine such as benzoylecogonine, amphetamines, and methaqualone. After solid phase extraction of urine, the drugs were analyzed by MEKC using a borate-phosphate buffer. Levels down to 100 mg/L can be detected with a multi-wavelength detector. They also[88] have shown that tetrahydrocannabinol carboxylic acid can be determined in urine by MEKC under similar conditions down to 10 mg/L. Thormann et al.[89] have reviewed the forensic application of CE and tabulated many drugs which have been analyzed by MEKC and CZE. Hudson et al.[90] have accumulated data on several hundred drugs and their metabolites for the analysis by CE and shown that the mobility data for these drugs are very reproducible. Watzig, Degenhardt and Kunkle[91] have reviewed drug analysis in general by CE, including methods for better precision. Coderc et al.[92] reviewed drug analysis by CE-LIF to improve the detection limits. Kok et al.[93] reviewed the different hyphenated methods suitable for identification of drugs by CE.

Hair analysis is used occasionally in forensic drug analysis because it can indicate the chronic use of drugs.[94-96] Tagliaro et al.[94] have shown that cocaine and morphine can be detected in hair by hydrolysis and extraction. As little as 0.15 ng/mg of Cocaine or morphine in 100 mg of hair can be detected. Tagliaro et al.[95] showed the separation for 20 illicit drugs and pointed out the advantages of capillary electrophoresis (both MEKC and CZE) for hair analysis, e.g. as no derivatization is needed and that it can be used also for confirmation.

DNA ANALYSIS

The PCR, the Genome project, viral detection, blood transfusion safety, and forensic identification, all are pushing the widespread DNA analysis further than most investigators have expected. Many agencies are resorting to DNA for collecting information on law offenders. Gel electrophoresis, which includes several types of analysis such as PCR, restriction fragment length polymorphism, hybridization, etc, remains the major method for DNA analysis.

The CE offers several advantages over the gel, mainly speed and automation. However, it has some limitations e.g. the need for LIF detection, the need for coated capillaries, and only one sample at a time can be run in the capillary.

However, since few- manufacturers such as Beckman and Perkin-Elmer have introduced specialized instruments for DNA analysis using multi-capillaries this field will no doubt see a great explosion in the near future.

The dsDNA fragment separation in CE is accomplished as in the slab gels based on molecular sieving. The ssDNA requires also a denaturing substance as urea or formamide. Cross-filled polyacrylamide gel capillaries have been used in the early studies.[97,98] These gels gave excellent separations. A single bp resolution up to 450 bp[97,98] can be obtained; however, they had several problems mainly filling the capillary without introducing air bubbles and short lifetime. Later on, solutions of uncrossed polymer network have been used more frequently. These include, uncrossed polyacrylamide,[99,100] different derivatives of cellulose,[101,102] poly-(vinylpyrrolidone),[103] poly(ethyleneoxide),[104] dextran.[105] These polymers are replaceable gels and separate fragments of DNA < 100bp to >2 kpb, and in some instances up to 12 kpb [98,106] with resolution of ~10 bp. Very dilute solutions of polymers can also separate DNA indicating that the entangled polymer network is not necessary. Sunada and Blanch[107] have concluded that cellulose polymers are more suited for large fragments while the linear polyacrylamide is more suited for the smaller fragments. However, the optimum concentration and the molecular weight are more important than the type of the polymer.[102] Heller[105] used 10% dextran for DNA separation because of its low viscosity.

For DNA sequencing a single bp separation is important and this can be achieved with some polymers after addition of ethidium br or other intercalator dyes. Karger group[108] reported on a special high molecular weight linear polyacrylamide which can be used for sequencing up to 1000 bases. While Kim and Yeung[109] used a mixture of poly(ethyleneoxide) to perform sequencing of also up to 1000 bases.

The addition of different dyes in the buffer stiffen the structure of the DNA and improves the separation in addition to that they are also important for the LIF detection. They also enable the use of LIF detection which improves the sensitivity over the uv detection by a factor of at least 100 and eliminates the interferences from the nucleotides in the reaction mixture which absorb light in the uv detection. Also, because the sample is diluted they overcome the matrix effects of the sample too and enable electrokinetic injection. The challenge is to sequence large fragments ~ 1000 bases with minimum errors. The search for a polymer with low viscosity which can resolve 1 base separation with large fragments and can be used in an untreated capillary[103, 104] will keep going on for some time. The use of HEPES buffer in place of Tris allows untreated capillaries to be used for dsDNA separation.[110] Chan et al. [111] described fast separation of DNA based on using short capillaries. Muller et al.[112] described ultra-fast separations of DNA based on short capillaries and moderate to high voltages. DNA separation based on microchips and capillary arrays to speed up the analysis further is a growing area of research.[103,113,114] Several specific reviews have discussed DNA analysis by CE in detail.[115-119]

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