ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
16 (1997) 377–393

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

HPCE methods for the identification and quantitation of antibodies, their conjugates and complexes

I.S. Krull *, X. Liu, J. Dai, C. Gendreau, G. Li

Department of Chemistry, 102 Hurtig Building, Northeastern University, 360 Huntington Avenue, Boston, MA 02115, USA

Received 1 December 1996; received in revised form 15 February 1997; accepted 18 February 1997

Abstract

We review here much of the existing literature that deals with analysis, resolution, characterization, and (at times) quantitation of antibodies in capillary electrophoresis modes. Each major mode of CE shown applicable to antibody analysis is described, along with the major applications of that mode for antibodies. Discussions are presented as to the mechanisms of antibody resolution in CE, interactions of various buffer components with the proteins leading to resolution, and methods of quantitation for antibodies. The literature is critically reviewed with regard to true application of CE for antibody analysis, limitations, information possible, information implied, and which samples have actually been assayed by CE modes. The literature is critically reviewed up to and including 1996, both for the scientific and commercial literature, especially vendor applications and real world applications possible. © 1997 Elsevier Science B.V.

Keywords: Antibodies; Capillary electrophoresis; Analysis of proteins; Immunorecognition in CE; CIEF; CGE; CZE; MECC; Affinity CE

1. Introduction and background to importance and need for HPCE methods for identification and quantitation of antibodies (Abs), their conjugates and complexes.

Quite aside from the analytical interest and opportunities, antibodies (Abs) have become of commercial interest over the past few years for several reasons. They have their own inherent pharmaceutical properties in attacking undesired foreign chemicals, natural products, and viruses that enter the body. They can be administered as therapeutics, at times to replace the body's natural Abs that may not be produced in sufficient quantities by an individual. They can be used in a wide variety of immunoassays and in immunochemistry studies, and they have become an important commercial entity, with many firms making and selling Abs for analytical, clinical assays, and immunoaffinity chromatography applications. There are several biotechnology firms now producing a variety of Abs for therapeutic purposes (Centocor, Merck, Genentech, and others). Abs have also been chemically attached to drugs to be used as drug delivery systems with a high specificity for a target bioreceptor (e.g. tu-

* Corresponding author.

PII S0731-7085(97)00071-X

mors). They are also used for many environmental applications, as immunoassay components and as instrumentation devices (e.g. ImmunoSystems, Pharmacia's BiaCor systems). There is significant interest at present in generating relatively large amounts of specific Abs, which can then be utilized as pharmaceuticals in their own right or as drug delivery systems [1–7].

Abs are basically very large proteins with certain sections containing carbohydrates of varying compositional arrangements and sequences. Most antibodies have molecular weights (MWs) in the neighborhood of 150 000 Daltons, though some can be quite higher, few are lower, other than for fragments. Abs, Fig. 1, consist of specific regions that can be chemically and/or enzymatically separated from each other, such as Fab, Fab', Fc, heavy and light chains, carbohydrates, and so forth [1,8–11]. In antibody terminology, the Fab is a smaller fragment of the original, intact Ab, derived by suitable enzymatic digestion, to release a protein derivative (without carbohydrate attachments) that contains the antigen recognition site (epitope) with free thiol groups in place of the original disulfide bonds in the intact Ab. The Fab' fragment is a dual Fab species, which comes about by a different enzymatic digestion of the intact Ab, but now contains two Fab fragments linked by one or more disulfide bonds, without any carbohydrate regions, containing two antigen recognition sites (epitopes). Finally, the Fc species is derived by deglycosylation of the original Ab, and contains no antigen recognition sites, but only carbohydrates from the intact Ab. The Fab and Fab' species serve to recognize the antigen, are free of carbohydrates, contain only protein, and have lower molecular weights than the original, intact Ab. They are less heterogeneous than the intact Ab. The Fc species or region of the intact Ab contains the carbohydrate portions and is used for membrane/cellular transport of the antibody, without which the Ab could not function correctly in vivo. Basically, all Abs consist of protein and carbohydrate regions, with the carbohydrates limited to only certain portions of the overall Ab, usually the Fc or crystalline region. Abs differ with regard to the nature of their proteins, location and number of disulfide linkages between chains, location and structure (sequence) of their carbohydrate regions, total MW and conformation or shape, as well as their epitope or recognition sites.

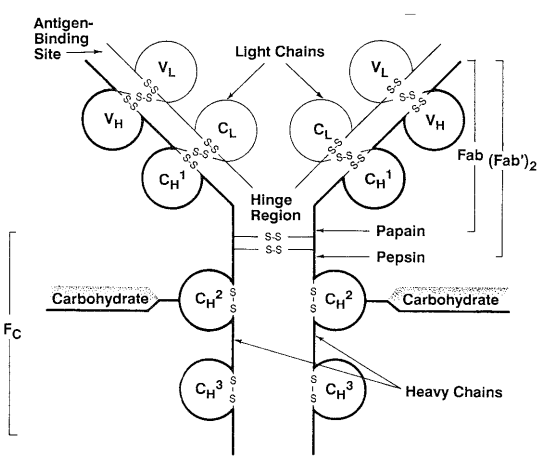

Fig. 1. Schematic illustration of antibody structure, showing Fab, Fab′, and Fc regions, bonds holding heavy and light chains together, disulfide bridges, and so forth. Reproduced with permission of the copyright holder and Pierce Chemical Company (Rockford, IL).

Of all protein containing species, perhaps Abs are the most complex in terms of protein sequence and length (MW), as well as the nature, size, complexity, sequence, and sites of attachments of the carbohydrate regions. In the past, several analytical methods have evolved that permit for the analytical determination and/or structure assignment for Abs [12–40]. Of these, perhaps HPLC and MS have evolved to become the paramount analytical methods, with conventional, flat-bed electrophoresis still retaining popularity and importance [41–48]. Over the past decade or more, HPCE has clearly begun to overtake conventional electrophoresis in terms of its advantages and opportunities for Ab analysis [49–59].

The commercial production, purification, and formulation of Abs presents more challenges than the production of single, individual proteins, which are usually more homogeneous after recombinant formation/synthesis. Most Abs, whether mono- or polyclonal derived, are heterogeneous, often containing microheterogeneity in the Fc (crystalline portion) or carbohydrate re-

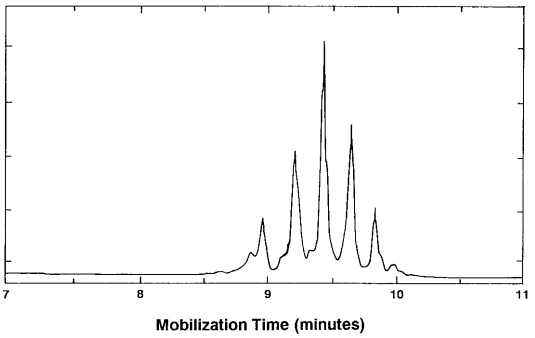

Fig. 2. CIEF separation of monoclonal Ab [75]. Reproduced with permission of the copyright holder and J. Liquid Chromatogr., Marcel Dekker, Inc., Publishers.

gions, and several protein species, which may prove difficult to resolve [60–62]. The proteins derived after immunizing an animal, in the form of the Abs against the antigen or hapten, almost always are more than a single, pure species. This heterogeneity is usually due to the carbohydrate regions, but it can also extend to the Fab or recognition site regions. Though HPLC has shown abilities, especially in the ion-exchange mode, to resolve similar Ab structures, this is not always the case [60–62]. Good techniques for active Ab species present (fingerprints) are essential in process stream manufacturing, quality control, research and development, in vivo metabolism, and for regulatory purposes in any pharmaceutical firm manufacturing and hoping to commercialize recombinant or natural Abs (animal derived).

Various major pharmaceutical firms have recently begun to investigate the potentials of HPCE for Ab analysis, purity determination, micropreparative isolation, interfacing with MS, and interfacing with Western/Southern Blotting after separation in order to improve separation, identification, and eventual quantitation of individual Abs or their conjugates that might be present in crude samples (fermentation broths, formulations, biofluids, and so forth). When first developing newer CIEF methods for proteins, several pharmaceutical firms approached us with their desire to apply our approaches for recombinant proteins and Abs [49–51]. There have been several commercial publications demonstrating the utility and applicability of various HPCE modes for Abs [12–25,49–52,55,57–59,63,64].

Most of the literature dealing with HPLC and HPCE of antibody-conjugates has dealt with Ab-enzyme or Ab-FL probes, species that have been utilized for many years for immunoassays and immunodetection; there is little in the literature related to Ab-drug conjugates. Much has been written about HPLC of Abs and their conjugates. However, it is now apparent that HPCE offers much more resolving ability when compared with HPLC separations. For these reasons, perhaps the ideal analytical approach would use HPCE-ESI-MS, utilizing electrospray ionization (ESI) for the interface. An alternative approach might be HPCE-ESI-TOFMS, but the successful interfacing of HPCE-MALDI-TOFMS has yet to be demonstrated with any routine success. This final approach would still require optimized HPCE conditions, whether these become CZE, CIEF, CGE, or others. The final optimized HPCE conditions must also be fully compatible with the ESI-MS requirements, in terms of buffer modifiers, pH, organic content, and so forth.

2. Capillary isoelectric focusing of antibodies, their conjugates and complexes.

CIEF is a high resolution technique that can be used to separate proteins, peptides and Abs based on their pIs. In CIEF, a coated capillary or an uncoated capillary with polymer additive in the buffer is generally employed. Single point, on-column UV detection at 280 nm is most commonly used. Most work on Ab separations by HPCE has been used to separate the monoclonal Ab. Monoclonal Abs produced by hybridoma or genetic engineering techniques have often displayed a certain degree of microheterogeneity when analyzed by HPCE [57,65–73]. This microheterogeneity may be caused by differences in glycosylation, variations in protein sequences, post-translational modifications, improper folding and other factors [74].

IEF is one of the assays suggested by FDA for characterizing monoclonal Ab products. Com-

pared with conventional IEF, CIEF is much less labor intensive or time consuming, it is easy to quantitate and automate, and less sample is required. Several papers have been published on CIEF separations of antibodies [58,75–78], and the resolution was compared with other modes of separation techniques. Silverman et al. reported the separation of a recombinant Ab [75], maB b72.3. Five major peaks were observed for the Ab in both IEF and CIEF, as shown in Fig. 2, which offered better resolution than that of ion exchange chromatography. Costello et al. described the CIEF separation of another Ab [57], humanized anti-TAC monoclonal Ab. The same resolution was achieved in IEF and CIEF, which was much better than SDS-PAGE, free zone capillary electrophoresis and gel IEF. In both papers, salt mobilization was used after focusing [79].

A more detailed study was reported by Tom Pritchett [80]. In this work, six peaks were observed for the monoclonal Ab, called anti-CEA. The isoelectric point of each peak was determined by running the Ab together with internal pI markers. Percent area of each peak was determined by peak area integrations. The RSD of migration times was around 0.5%, and %RSD of peak areas was in the range of 0.6–4.7%. A very good linearity between Ab concentration and peak area was achieved in the Ab concentration range of 25–250 μg ml^{-1}. Another application reported by Kundu et al. showed slightly different results [76]. They found that the pIs of mAbs determined by a fitted, linear calibration curve showed appreciable deviation from the pIs determined by slab gel methods. Therefore, they calculated pIs from a fitted non-linear calibration curve and used pI standards with pIs below and above the pI ranges of mAbs. The differences between calculated and literature pI values of the tested proteins were less than 0.04 unit when fitted non-linear calibration curves were used. This could be compared to 0.3 pI unit when fitted linear curves were employed. This result was consistent with other observations [77].

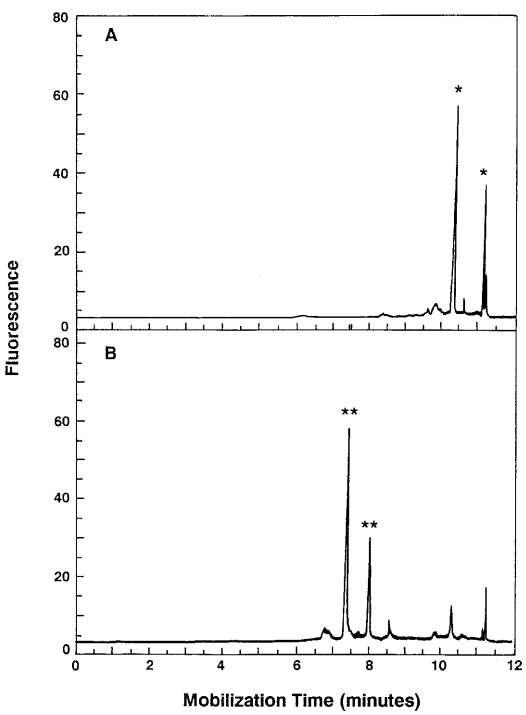

Fig. 3. Example of an approach to immunoaffinity recognition in CIEF using a FL probe Fab' fragment of the Ab [78]. Reproduced with permission of the copyright holder, Anal. Chem. J. and Am. Chem. Soc.

Chen et al. reported the CIEF separation of a monoclonal antibody named HER2 [7]. Both IEF and CIEF methods showed five charged isoforms with estimated pI values ranging from 8.6–9.1. The CIEF assay demonstrated good precision with percent relative standard deviations of 0.7–3.7% and 0.4–4.2% for intra- and interassay analyses, respectively. The limit of detection for the main peak was determined to be 2 ppm.

In addition to the characterization of mAbs, CIEF has been used to perform immunoassays of proteins. In the work of Shimura and Karger that dealt with the immunoassays of hGH [78], they used CIEF to separate the Ab/antigen complexes from the antigen and Ab. To avoid any microheterogeneity of the Ab that might complicate their immunoassays, a Fab' fragment from the Ab was used to interact with the Ag. The Ab was mixed with the antigen sample of interest, and incubated before they were introduced into the capillary. Since the Ab and Ag/Ab complex had different pIs, the excess Ab fragment was separated from the complex in the capillary, as shown in Fig. 3. The Ab sample was quantitated either

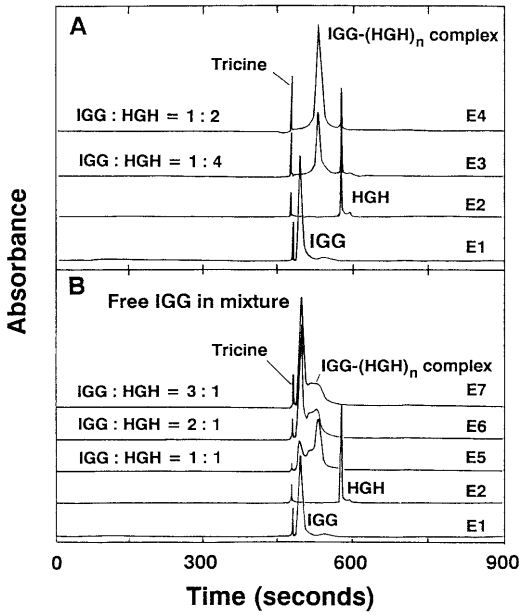

Fig. 4. Separation of IgG, hGH, and IgG-(hGH)$_n$ complexes by CZE. (A) Electropherograms of IgG, hGH and mixtures containing an excess of hGH. (B) Electropherograms of IgG, hGH and mixtures containing an excess of IgG. Experimental conditions are described elsewhere [84]. Reproduced with permission of the copyright holder, J. Chromatogr. and Elsevier Science Publishers, Inc.

by the Ab or complex peak. Due to the focusing effect and the great sensitivity of LIF, a detection limit of 0.1 ng ml^{-1} of hGH was achieved.

In summary, CIEF is a very good method for the characterization of mAbs. Its high resolving power makes it possible to discriminate the subtle change of mAb isoforms. With the development of improved capillary coating techniques, both quantitative and qualitative analyses may be achieved. The focusing effect of CIEF is a great advantage when performing immunoaffinity HPCE.

3. Free solution capillary electrophoresis of antibodies, their conjugates and complexes.

Analyses of Abs, their conjugates and complexes with FSCE have often found their applications in immunoassays [79–90]. In 1989, Grossman et al. [84] were able to separate anti-hGH, hGH and their complex by using an uncoated capillary with 100 mM tricine, pH 8.0, and 200 nm ultraviolet detection (Fig. 4). The detection limit was not evaluated in this case. Chu et al. [86] reported the determination of the binding stoichiometry of human serum albumin (HSA) to

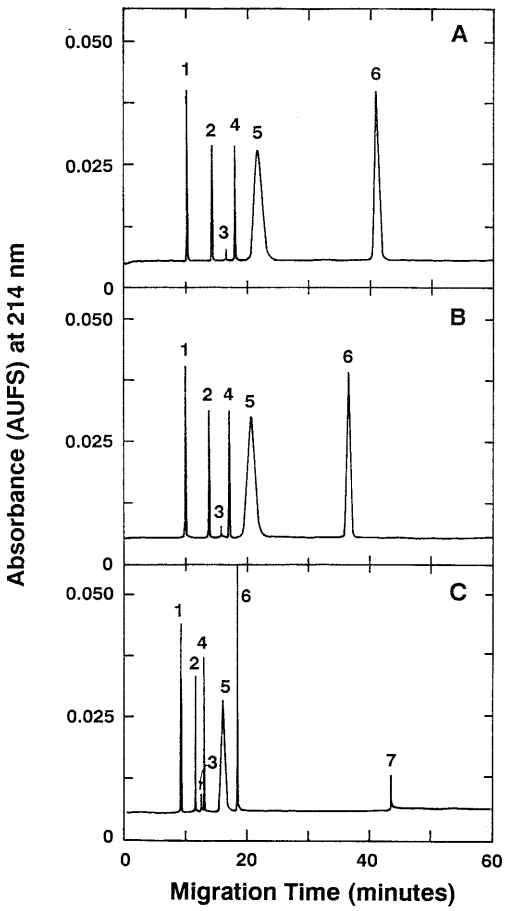

Fig. 5. Capillary electrophoresis profile of fluorescamine-derivatized anti-TAC separated under three different buffer conditions: (A) 0.05 M sodium tetraborate buffer, pH 8.3; (B) 0.05 M sodium tetraborate buffer, pH 8.3, containing 0.025 M TMAPS; and (C) 0.05 M sodium tetraborate buffer, pH 8.3, containing 0.5 M TMAPS. The humanized anti-TAC MAb used here was present in a simple liquid formulation dosage form at a concentration of 40 μl (240 μg or 1.60 pmol) 100 μl^{-1} of reaction mixture, containing 0.2 mg ml^{-1} of Tween-80 [72]. Reproduced with permission of the copyright holder, J. Chromatogr. and Elsevier Science Publishers, Inc.

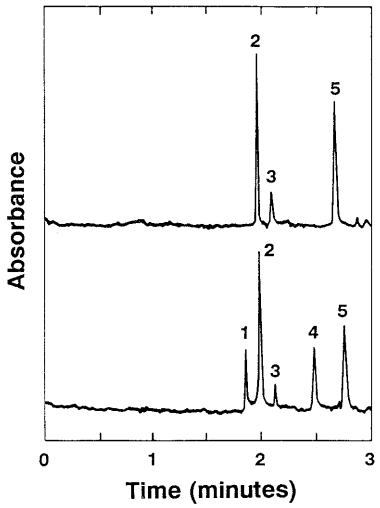

Fig. 6. Electropherogram (top) of 100 nM FITC-insulin under conditions described elsewhere [87]. Electropherogram (bottom) of 100 nM FITC-insulin and 50 nM Fab. Peaks 2, 3, and 5 are FITC-insulin. Peaks 1 and 4 are due to the formation of complex of Fab with FITC-insulin in peaks 2 and 5, respectively. A He-Cd laser was the excitation source [87]. Reproduced with permission of the copyright holder, Anal. Chem. and Am. Chem. Soc.

its mouse monoclonal IgG Ab (anti-HSA), now using affinity HPCE in 192 mM glycine + 74 mM Tris buffer at pH 8.7. The detection wavelength was set at 200 nm. They were also able to detect concentrations as low as 0.16 μM. Zou et al. [91] also made use of CZE for the analysis of hGH and its Ab.

In 1992, Guzman et al. [72] separated and quantitated a humanized monoclonal Ab anti-TAC in an uncoated capillary with 50 mM sodium borate buffer (pH 8.3) and different concentrations of TMAPS (Fig. 5). The Abs were first derivatized with fluorescamine before analysis and the absorbance was monitored at 214 nm.

Detection limits for Abs were greatly optimized with FL derivatized products. In 1991, Guzman et al. [66] came up with multiple parallel capillaries for the analysis of purified monoclonal Ab. They claimed that by using multiple parallel capillaries, the detection limit for Ab using ultraviolet detection was in the range of 1/10s of μg ml^{-1}, and in the range of 1/10s of ng ml^{-1} for FL detection. With LIF, the detection limit could be further lowered. Schultz and Kennedy [87] showed successful separation of Fab, FITC-insulin, and complex of Fab and FITC-insulin in an uncoated capillary with LIF detection (Fig. 6). The detection limit was about 2 nM. The detection limit for the complex of digoxigenin-labeled β-phycoerythrin and its Ab using LIF detection was in the low 10^{-11} M range in Chen and Pentoney's work [88]. Chen and Evangelista also reported the application of cyanine dye for the analysis of antigen-antibody complex [89,90].

In some cases, ultraviolet detection is still used and this usually applies to analyses with coated capillaries. David Mao and colleagues from J. and

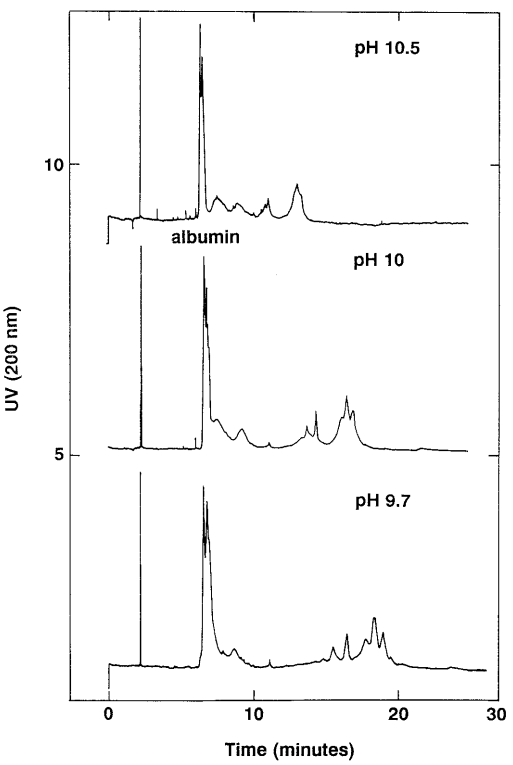

Fig. 7. CZE separation of monoclonal antibody in mouse ascites fluid at various pH conditions. CZE conditions: FC coated capillary; effective length: 15 cm; applied voltage: 230 V cm^{-1}; buffer: 30 mM phosphate with 0.01% FC surfactant; sample: Mab (Sigma) [92]. Reproduced with permission of the copyright holder, J. and W. Scientific, Inc., Folsom, CA.

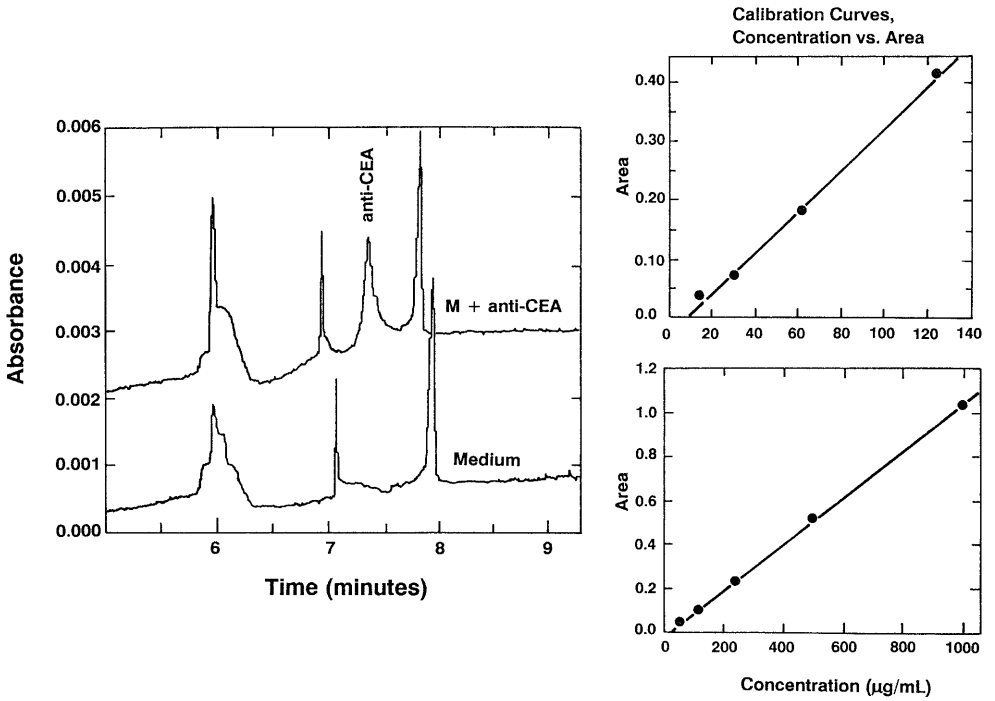

Fig. 8. CZE analysis of anti-CEA MAb in tissue culture medium and calibration curves, concentration versus area. Reproduced with permission of the copyright holder and Beckman Instruments, Inc. [56,58].

W. Scientific [92] have separated different isoforms of Abs using fluorocarbon-coated (FC) capillaries with fluorine containing surfactants (Fig. 7). Pritchett [95] from Beckman used an eCAP neutral coated capillary, 20 mM citrate at pH 3.0 with UV detection at 214 nm to quantitate an anti-carcinoembyonic antigen (CEA) murine MAb in serum-free hybridoma culture medium. The detection limit was 16 μg ml^{-1} and the limit of quantitation was 25 μg ml^{-1} (Fig. 8).

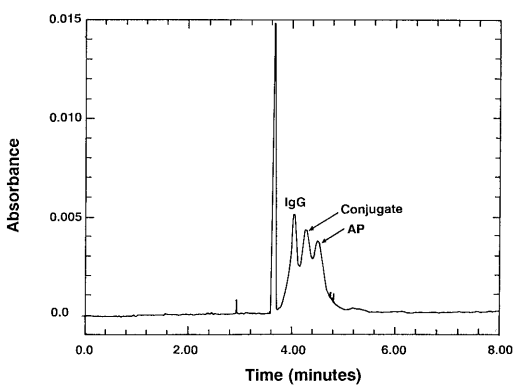

Fig. 9. Electropherogram of the MEKC separation of IgG, IgG-AP complex and AP [71]. Reproduced with permission of the copyright holder, J. Chromatogr. and Elsevier Science Publishers, Inc.

In one case, Harrington et al. [71] used HPCE as a fast in-process control for enzyme labeled monoclonal Ab conjugates. Methyl cellulose (0.5%) was added to 100 mM borax and 0.5 mM SDS in a pH 10 running buffer for the separation of alkaline phosphatase (AP), immunoglobulin G (IgG), and the AP-IgG conjugate in an uncoated capillary. The absorbance was detected at 280 nm. When AP and IgG were mixed together, three unresolved peaks were observed. IgG migrated first, followed by the conjugate, and last was AP (Fig. 9). These conditions could not separate the three components without sample pretreatment. With simplicity and improved FL or LIF detection, CZE will find more applications in the analyses of therapeutic drugs in the future.

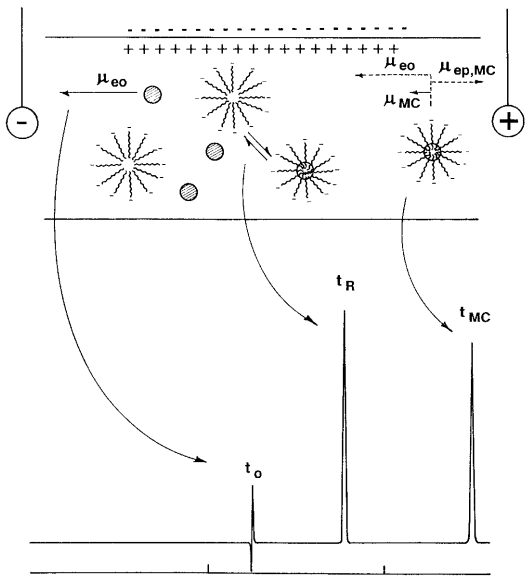

Fig. 10. Schematic illustration of MEKC, showing the times of elution for an analyte not interacting at all with the micelle (t_0), one that does partition between the micelle and mobile phase (t_R) and one that spends all of its time in the micelle (t_{MC}) [96]. Reproduced with permission of the copyright holder, Beckman Instruments, Inc.

4. Micellar electrokinetic capillary chromatography of antibodies, their conjugates and complexes

Micellar electrokinetic chromatography (MEKC), also sometimes called micellar electrokinetic capillary chromatography (MECC), is a technique that was developed to aid in the separation of neutral compounds. It can basically be described as CZE with the addition of a charged surfactant at a concentration above the critical micellar concentration (CMC) to the background electrolyte. Above the CMC, the surfactant forms micelles, which are species that are hydrophobic on the inside and hydrophilic on the outside. Thus, neutral analytes will partition between the hydrophobic inside of the micelle and the hydrophilic aqueous buffer. Although developed initially for neutral compounds, MEKC works equally well for ionic compounds and even mixtures containing both charged and uncharged compounds. As can be seen in Fig. 10, very polar compounds will spend all of their time in the aqueous phase and will elute first at t_o, while very nonpolar compounds will spend all of their time in the interior of the micelle and will elute last, with the micelle, at t_{MC}. All of the analytes will elute somewhere in between these two extremes. This partitioning between the micellar and aqueous phases increases the selectivity and aids in the separation of compounds with very similar electrophoretic mobilities [96].

Not much work has been reported using MEKC to separate Abs. Indeed, as the mass of the most commonly used sodium dodecyl sulfate (SDS) micelle is similar to that of the immunoglobulin G (IgG) Ab, the exact mechanism of the separation remains unknown. Possibly it could be due to ion-pairing between the SDS micelle and hydrophobic protein regions on the antibody. Alternatively, it could be due to simple differential migration of negatively charged antibodies through negatively charged micelles [49]. At this time, there is no known published work to support either case, nor any to present another.

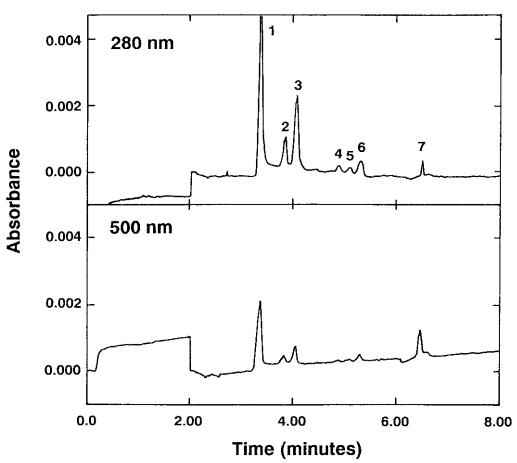

Fig. 11. Electropherograms of the MEKC separation of Ab-drug complex (peaks 1–3), Fab fragment (peak 4), heavy and light chains (peaks 5–6) and the unconjugated drug (doxorubicin) (peak 7). The top represents detection at 280 nm, showing the proteinaceous species, with the bottom representing detection at 500 nm, showing the species conjugated to the drug [49]. Reproduced with permission of the copyright holder, J. Chromatogr. and Elsevier Science Publishers, Inc.

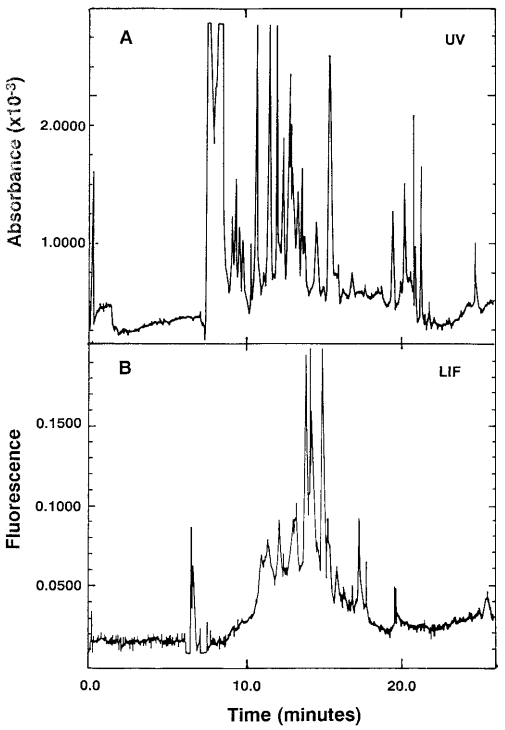

Fig. 12. Electropherograms of the MEKC separation of a tryptic digest of Ab-drug conjugate using 214 nm ultraviolet detection (top) and LIF detection (bottom) [97]. Reproduced with permission of the copyright holder, J. Chromatogr. and Elsevier Science Publishers, Inc.

The first published work on using MEKC for the analysis of Abs was that of Hughes and Richberg, in 1993 [49]. They described a method of using MEKC and multiwavelength chromatographic characterization to separate and identify parts of an immunotoxin (an IgG Ab with a specificity towards cancerous tumor cells conjugated with a cytotoxic drug) [49]. Throughout, the proteinaceous species were identified at 280 nm, while the drug-containing ones were simultaneously monitored at 500 nm. The concentration of SDS was varied above the CMC to see its effects on the separation. The effects of buffer concentration and pH were also analyzed. As is shown in Fig. 11, the authors were successful in separating and identifying the conjugated antibody, antibody light chains, antibody heavy chains, antibody fragments and the unconjugated drug (doxorubicin, an anthracycline, in this case) [49].

Two years later, in 1995, another paper involving MEKC of Abs was published by the same group [50]. They used MEKC and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) to study the thermal stability of polyclonal or monoclonal IgG Abs. Unfortunately, the peaks collected by MEKC could not be directly analyzed by MALDI-MS due to the fact that SDS, at levels as low as 0.01%, has been shown to cause spectral degradation. In this case the MALDI-MS was used separately to give enhanced detection specificity to aid in species identification. Electropherograms and mass spectra of the Ab, after up to 166 h of thermal stress (60°C), were shown and discussed. It was determined that the stress caused the loss of light chains and Fab arms, and the formation of separated heavy chain and light chain moieties [50].

In 1996, Liu et al. published a method to do comparative peptide mapping of tryptic digests of monoclonal Ab and corresponding doxorubicin immunoconjugate using MEKC and capillary liquid chromatography (CLC) [97]. Both ultraviolet and LIF detection were employed (doxorubicin is naturally fluorescent). This results in a unique 'fingerprint' which can be used for structural analysis. The method was developed using a model cystine containing peptide and then applied to the monoclonal Ab. Fig. 12 shows the 'fingerprint' electropherograms for 214 nm ultraviolet detection (top) and LIF detection (bottom) using MEKC conditions of the tryptic digest of an Ab-doxorubicin conjugate [97].

In conclusion, MEKC seems to offer an enhancement in resolution over CZE when used to analyze antibodies. It has been shown to be a valuable tool in the separation of antibodies, their fragments and various antibody conjugates.

5. CGE methods for antibodies, their conjugates and complexes.

Capillary gel electrophoresis (CGE) takes advantage of HPCE and slab gel electrophoresis. In the presence of excess SDS and a reducing

reagent, such as 2-mercaptoethanol or mercaptoethylamine, most proteins are fully denatured and hydrophobically bind a constant amount of SDS per unit weight (1.4 g of SDS g^{-1} of protein). This results in a detergent-protein complex of approximately constant mass-to-charge ratio, and practically identical electrophoretic mobilities in a free solution or FSCE. However, due to the presence of a sieving medium (e.g. agarose or polyacrylamide) that exists in CGE, the electrophoretic mobility of each complex is proportional to the log effective molecular radius and thus, to the log molecular weight of the protein.

Because of the correlation with slab gel techniques, CGE with its automated and quantitative features demonstrates great potential for replacing its more lengthy and labor intensive slab gel counterparts. In the capillary format, the basic 'sieving' effect for protein separation can be achieved in various gel matrices, such as crosslinked or non-crosslinked polyacrylamide, methyl cellulose and derivatives, 'liquefied' agarose, dextran and polyethylene glycol (PEG).

Amongst commercial suppliers, Beckman Instruments has developed a SDS-CGE kit which is used for quantitative analysis of anti-CEA in serum-containing medium [98]. An eCAP™ neutral capillary and SDS 14-200 gel buffer were used in this method. The sample buffer consisted of 0.12 M Tris/HCl/1% SDS, pH 6.6 [99]. The sample was denatured by mixing with the sample buffer and incubating at 100°C for 10 min. A field strength of 250 V cm^{-1} was applied for the separation and the detection wavelength was 200 nm. A typical calibration curve resulting from injection of increasing concentrations of anti-CEA (4–62 μg ml^{-1}) exhibited good linearity (r^2 = 0.9977). Spiking anti-CEA into serum-containing medium at 25 μg ml^{-1} level provided 98% recovery, and the relative S.D. was ±3%, providing good accuracy and precision.

Another application of the Beckman kit was reported by Liu et al. in 1995 [100]. They utilized the kit for the separation and analysis of a monoclonal Ab, chimeric BR96 and the corresponding immunoconjugate prepared between BR96 and the anticancer drug, doxorubicin. In the protein denaturation and reduction steps, the run buffer and 2-mercaptoethanol were mixed with sample. The mixture was placed into a heating block at 100°C for 5 min, removed, and then placed in an ice bath to cool prior to injection for analysis. For samples undergoing denaturation without reduction, the Ab and conjugate were incubated with sample buffer at 100°C for 5 min. The separation was performed with a coated capillary column (100 μm × 47.1 cm), using a field strength of 300 V cm^{-1}. Under these conditions, the Ab was well separated from its fragments (Fig. 13) and the separation of the conjugates was demonstrated by LIF detection.

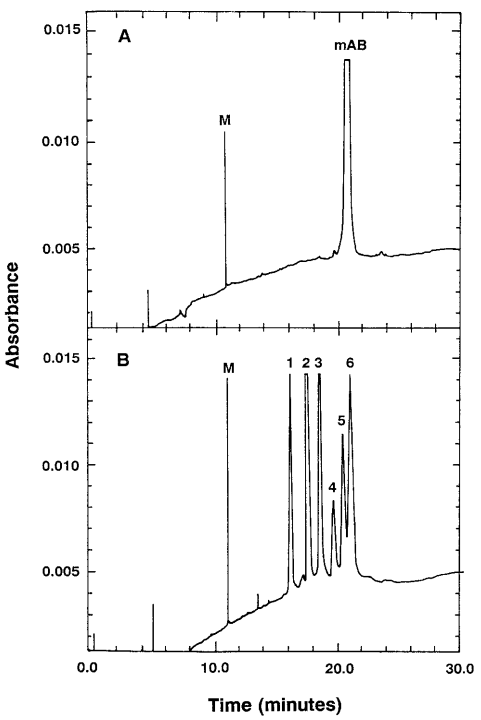

Fig. 13. SDS-CGE of MAb BR96 (A) and its fragments (B) under denaturation conditions with ultraviolet absorbance detection. M, reference marker, MAb, native, nonreduced monoclonal Ab BR96; peak numbers correspond to Ab fragments or combinations of fragment chains. Sample buffer consists of 0.12 M Tris/HCl/1% SDS, pH 6.6. Coated capillary, 100 μm i.d. × 47.1 cm (40.6 cm effective separation length) filled with Beckman gel buffer. Applied electric field, 300 V cm^{-1}. Pressure injection, 5–10 s. Reproduced with permission of the copyright holder, Anal. Biochem. J. and Academic Press, Inc. [100].

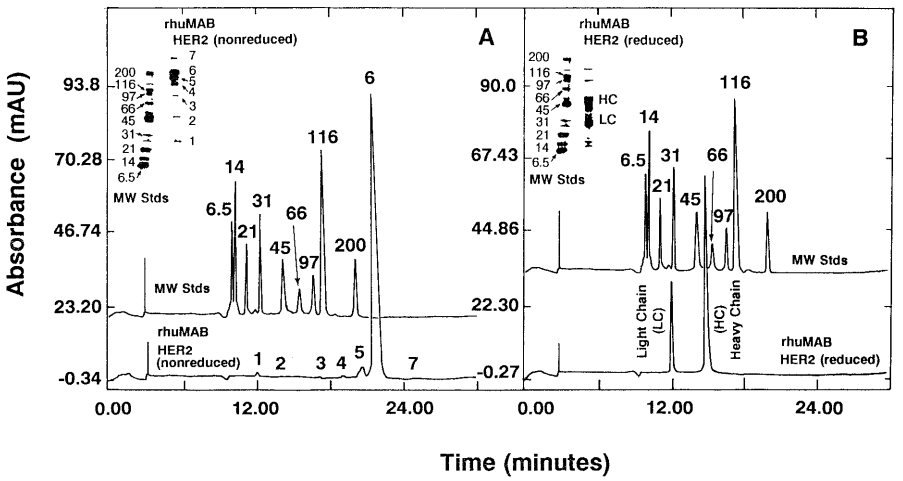

Fig. 14. (A) SDS-PAGE (insert) and SDS-CGE of rhuMAbHER2 under nonreduced conditions (bottom) and molecular mass markers (top). Analytical conditions are described elsewhere [6]. (b) SDS-PAGE (insert) and SDS-CGE of rhuMAbHER2 under reduced conditions (bottom) and molecular mass markers (top). Analytical conditions are described elsewhere [6]. Reproduced with permission of the copyright holder, J. Chromatogr. and Elsevier Science Publishers, Inc. [6].

Hunt et al. reported a CGE method for recombinant humanized monoclonal Ab, rhuMAbHER2 [6]. They used an uncoated silica capillary of 24 cm × 75 μm i.d, 19.5 cm effective length as column. The SDS sample buffer and SDS running buffer were purchased from Bio-Rad. The experiment was performed on a Bio-Rad BioFocus 3000 CE system. The sample was diluted to approximately 1 mg ml^{-1} and 15 mM ionic strength with purified water. This was then analyzed in both nonreduced and reduced forms. The sample was injected using electrophoretic methods. Separation was performed at 333 V cm^{-1}, constant voltage, and the column temperature was maintained at 20°C. For the nonreduced sample, seven peaks were observed (Fig. 14). This correlated well with the seven bands observed in SDS-PAGE. In addition, the peak area percents attributed to high-molecular mass aggregates seen using SDS-CGE of the nonreduced samples were consistent with those found with size-exclusion chromatography using a SDS containing mobile phase. Also, the SDS-CGE method provided excellent resolution of the light and heavy chains of the Ab. The limit of detection (LOD) for the intact rhuMAbHER2 under nonreduced conditions was determined to be 0.5 ppm (μg ml^{-1}). The Pearson correlation coefficient for the peak area of intact rhuMAbHER2 was >0.99, thus demonstrating linearity for the concentration range of 0.5–500 μg ml^{-1}. The RSD values of migration time ranged from 0.8 to 1.7% in three replicate injections of a single sample.

It is noteworthy that the intact rhuMAbHER2 migrated at a position which resulted in a higher than expected molecular weight determination under nonreduced conditions for SDS-CGE (Fig. 14). A possible explanation for this anomalous migration is that Abs are glycoproteins. The lack of SDS binding by carbohydrate moieties of the proteins may lead to lower charge-to-mass ratios for SDS-glycoprotein complexes, resulting in decreased migrations and an over-estimation of molecular weights. A proposed solution for obtaining a better estimation of molecular weight for this class of proteins is to use the conditions for a Ferguson plot [7].

6. Summary and conclusions. Prospects for the future.

Although there are numerous publications that describe the application of HPCE, in various

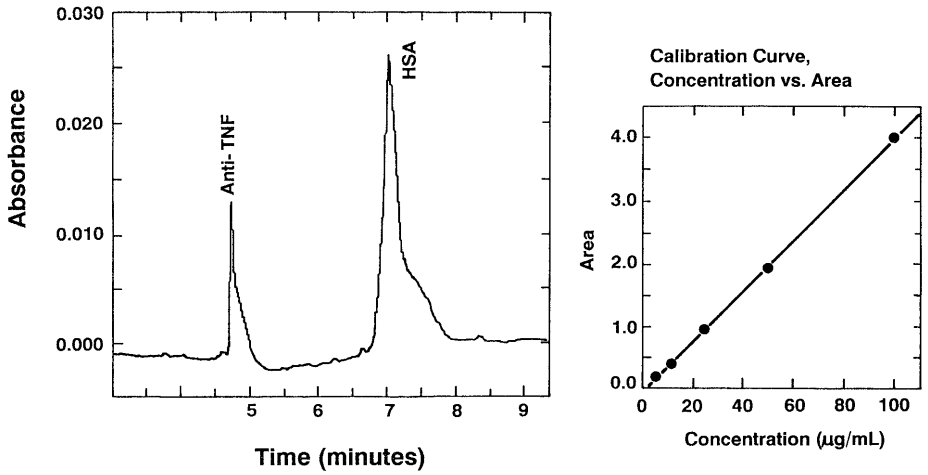

Fig. 15. Quantitative CIEF analysis of anti-TNF dosage form and calibration curve, concentration versus area. Reproduced with permission of the copyright holder, Beckman Instruments, Inc. [56,58].

modes, for the separation and detection of antibodies, actual applications for real world samples appear somewhat lacking at the present time. That is, analysis of Abs in cell growth medium, fermentation broths, tissue culture medium, degraded samples, stability studies, formulations, biofluids, and so forth, are missing in large numbers from the available literature. The excellent work of Pritchett at Beckman is perhaps an exception in this regard, since he has clearly demonstrated the ability to utilize CIEF, CZE, and SDS-CGE for the separation of various Abs, such as anti-TNF dosage forms (Fig. 15), anti-CEA in tissue culture medium (Fig. 8), and anti-CEA in serum containing medium (Fig. 16). These approaches are clearly able to separate the main Ab peak from the other sample components, and to obtain quantitative determinations of recovery and the levels present. The specific operating conditions for these Ab assays are available from Beckman [58]. Calibration plots were derived in each HPCE mode, with excellent linearities, and utilized for the quantitation of Abs in the above described samples. Peak shapes for the Abs were quite broad, again a result of the extreme microheterogeneity of most Abs. However, despite such broadness, it is very clear that accurate and precise quantitations for Abs in real samples is very feasible at the present time, perhaps instrument and capillary gel dependent.

In the application of HPCE methods for the qualitative or quantitative determination of Abs for production or quality control purposes, such assays must have certain capabilities. As Pritchett has indicated: (1) they should be rapid, so that process control decisions can be made rapidly; (2) they should be adaptable for quantitation of the Ab in a variety of analytical matrices of varying complexity; (3) methods development time should be short; (4) assays should provide high resolution to achieve the required specificity and selectivity; and (5) the assays should exhibit good accuracy, precision, and linearity [58].

All HPCE methods must preserve the integrity of the original Ab sample, so that sample preparation steps do not change the nature of the species present prior to the actual HPCE separations (MECC may be problematic here). Because Abs are so heterogeneous, most HPCE modes of separation result in broad peaks, which usually contain numerous, unresolved species. The purity of Abs is a difficult question to assess by HPCE methods alone, since impurity peaks or even inactive Abs may comigrate along with the active, desired Ab species. What does a single Ab peak in any HPCE mode really signify? Can we actually

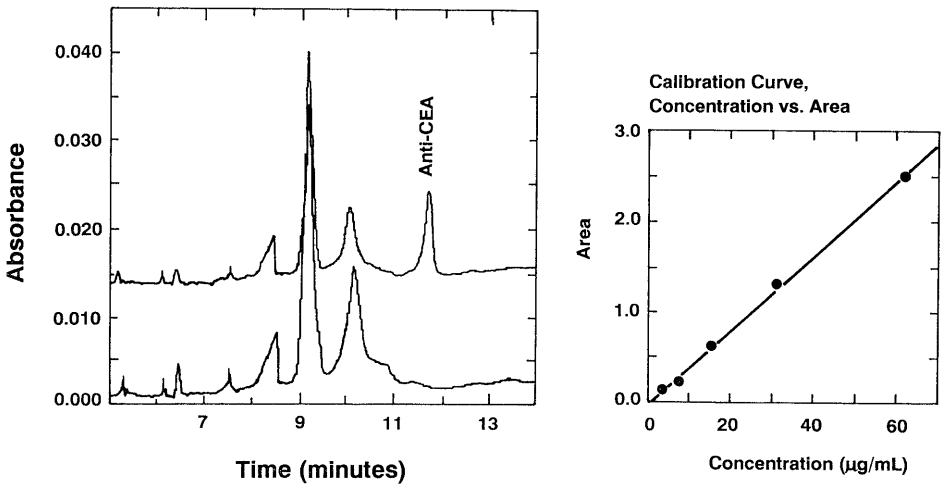

Fig. 16. Quantitative analysis of anti-CEA in serum containing medium: SDS-capillary gel electrophoresis and calibration curve, concentration versus area [56,58].

identify the nature of the species present under that single, perhaps broad Ab peak, without further identification by perhaps MS or MS/MS methods of detection? Does that single peak signify all active Ab species or are both active and inactive peaks comigrating? Several well resolved peaks in CIEF may be more advantageous to indicate the qualitative (perhaps quantitative) nature of a particular Ab sample, rather than a single peak in MECC, CZE, or CGE modes. What is it that one really wants from the final HPCE electropherograms, a single, sharp peak well resolved from other sample components? Or, do we want a series of well resolved peaks all coming from the same Ab sample, again resolved from non-Ab components, but now more illustrative of the Ab species present? There are very real differences in these analytical goals, perhaps sample dependent, and one must first define what is needed from the HPCE assay, perhaps Ab and sample dependent. One must also decide in advance if qualitative and/or quantitative data are the goals.

Specific Ab samples may require different combinations of ampholyte buffers in CIEF to provide really high resolutions. At times, different Abs may require different gels in CGE to provide useful resolutions from other sample components, without resolution of the Ab species themselves, usually very difficult to accomplish with any CGE gel. The future developments in HPCE for Ab analysis will need to demonstrate: (1) a simplicity of sample preparation without loss of sample integrity; (2) a high degree of accuracy, precision, and reproducibility; (3) a simplicity of sample introduction, separation and detection; (4) improved qualitative identification of the groups of Ab species present (as now possible in CIEF modes); and (5) resolution of active from inactive Ab species perhaps by the use of affinity or immunoaffinity HPCE methods.

Glossary (alphabetical)

Ab	antibody
Ab-drug	antibody-drug conjugates
Abs	antibodies
Ag	antigen
Ab-FL	antibody-fluorescent tagged probe in HPCE
Ab-En	antibody-enzyme conjugate
Ab-Ag	antibody-antigen complex
anti-hGH	antibody to hGH (antigen)
anti-IgG	antibody to IgG, an anti-antibody species
anti-Ab	anti-antibody

anti-TNF	antibody to tumor necrosis factor
anti-CEA	antibody to carcinoembryonic antigenic proteins
ampholytes	mixture of amphoteric species for IEF and CIEF
AP	alkaline phosphatase
CGE	capillary gel electrophoresis
CIEF	capillary isoelectric focusing
CMC	critical micellar concentration
CZE	capillary zone electrophoresis (aka FSCE)
DL(s)	detection limit(s)
[En]	enzyme concentration
EOF	electroosmotic flow in HPCE
Fab	antigen recognition regions of antibody
Fab'	combination of two Fab species thru disulfide bridge
Fc	crystalline region of antibody (carbohydrate containing)
FC	fluorocarbon
FL	fluorescence detection
FSCE	free solution capillary electrophoresis (aka CZE)
hGH	human growth hormone
HPLC	high performance liquid chromatography
HPCE/CE	high performance capillary electrophoresis
HPIAC	high performance immunoaffinity chromatography
HSA	human serum albumin
IEF	isoelectric focusing
IgG	immunogammaglobulin (antibody)
LIF	laser induced fluorescence detection
LOD	limit of detection
MALDI-TOFMS	matrix-assisted laser desorption/ionization-time of flight mass spectrometry
mAb	monoclonal antibody
mAbs	monoclonal antibodies
MECC	micellar enhanced capillary chromatography
MEKC	micellar electrokinetic chromatography
MS	mass spectrometry
MC	methyl cellulose
MW	molecular weight
PEG	polyethylene glycol
pI	isoelectric point
RI	refractive index
SDS	sodium dodecyl sulfate
t_o	elution time for unretained analyte in MEKC that does not partition into the micelle
t_{MC}	elution time of the micelle in MEKC
TMAPS	trimethylammoniumpropyl sulfonate
UV/VIS	ultraviolet-visible spectroscopy

Acknowledgements

We are very grateful to many individuals and outside industrial firms who have collaborated with us over several years in areas of improved derivatization and/or detection of proteins and antibodies by both HPLC and HPCE. These include the following firms: The Pharmacia and Upjohn Company, Troy Chemical Company, Teva Pharmaceutical Industries, Ltd., SmithKline Beecham Pharmaceuticals, Thermo Separation Products, Thermo Bioanalysis Corporation, Waters Corporation, Zeneca Pharmaceuticals, Wyeth-Ayerst Research Corporation, Pfizer and Company, Gilson Biomedical Electronics, Genentech Corporation, PerSeptive Biosystems, Biogen Corporation, Viscotek, ISCO, and others. Professor Hanfa Zou is acknowledged for his early collaborations in the areas of HPIAC, ID, and immuno-CE. Dr. David Mao of J and W Scientific was extremely helpful in providing us with technical support, FC coated capillaries, and FC buffer additives for the MECC/CZE analysis of various antibodies. Dr Ming Huang of Supelco Corporation was similarly helpful in providing us with guidance and guidelines for the selection of suitable HPCE conditions for various antibodies using certain coated capillaries and buffer additives.

References

[1] Principles and Practice of Immunoassay, Edited by C.P. Price and D.J. Newman, Stockton Press, New York, 1991.

[2] J. Clausen. Immunochemical Techniques for the Identification and Estimation of Macromolecules, Laboratory Techniques in Biochemistry and Molecular Biology, Volume 1, Part 3, Elsevier Science Publishers, Amsterdam, 1988.

[3] T.M. Phillips. Analytical Techniques in Immunochemistry, Marcel Dekker, NY, 1992.

[4] Immunochemical Methods for Environmental Analysis, Ed. by J.M. Van Emon and R.O. Mumma, ACS Symposium Series No. 442, American Chemical Society, Washington, DC, 1990.

[5] I. Weeks. Chemiluminescence Immunoassay, Elsevier Science Publishers, Amsterdam, 1992.

[6] G. Hunt, K.G. Moorhouse, A.B. Chen, J. Chromatogr. 744 (1996) 295.

[7] W.E. Werner, D.M. Demorest, J.E. Wiktorowicz, Electrophoresis 14 (1993) 759.

[8] H. Zola. Monoclonal Antibodies: A Manual of Techniques, CRC Press, Boca Raton, FL, 1987.

[9] J.W. Goding. Monoclonal Antibodies: Principles and Practice, Academic Press, New York, NY, 1983.

[10] Pierce Catalog and Handbook of Life Science and Analytical Research Products, Pierce Chemical Company, Rockford, IL, 1995–96.

[11] E. Harlow and D. Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, LI, NY, 1988.

[12] New Directions in Electrophoretic Methods, J.W. Jorgenson and M. Phillips, Editors, ACS Symposium Series, Volume 335, American Chemical Society, Washington, D.C., 1987.

[13] Analytical Biotechnology: Capillary Electrophoresis and Chromatography, Cs. Horvath and J. G. Nikelly, Editors, ACS Symposium Series, Volume 434, American Chemical Society, Washington, D.C., 1990.

[14] S.F.Y. Li, Capillary Electrophoresis: Principles, Practice and Applications, Elsevier Science Publishers, Amsterdam, 1992.

[15] Capillary Electrophoresis—Theory and Practice, Edited by P.D. Grossman and J.C. Colburn, Academic Press, Incorporated, San Diego, CA, 1992.

[16] Capillary Electrophoresis-Technology, N. Guzman, Editor, Marcel Dekker, New York, 1993.

[17] R. Weinberger, Practical Capillary Electrophoresis, Academic Press, San Diego, CA, 1993.

[18] CRC Handbook of Capillary Electrophoresis: Principles, Methods, and Applications, Edited by J.P. Landers, CRC Press, Boca Raton, FL, 1994.

[19] D.N. Heiger, High Performance Capillary Electrophoresis—An Introduction, A Primer, Second Edition, Hewlett-Packard Corporation, Waldbronn, Germany, 1992.

[20] D.R. Baker, Capillary Electrophoresis, Techniques in Analytical Chemistry Series, J. Wiley and Sons, Incorporated, New York, 1995.

[21] Capillary Electrophoresis in Analytical Biotechnology, Edited by P.G. Righetti, CRC Series in Analytical Biotechnology, CRC Press, Incorporated, Boca Raton, FL, 1996.

[22] Capillary Electrophoresis, Theory and Practice, Edited by P. Camillari, CRC Press, Boca Raton, 1993.

[23] R.A. Mosher and W. Thormann, The Dynamics of Electrophoresis, VCH Publishers, Weinheim, FRG, 1992, Chapter 7.

[24] K.D. Altria and M.M. Rogan, Introduction to Quantitative Applications of Capillary Electrophoresis in Pharmaceutical Analysis, A Primer, Beckman Instruments, Inc., Fullerton, CA, 1995.

[25] Capillary Electrophoresis Guidebook, Principles, Operation, and Applications, Edited by K.D. Altria, Methods in Molecular Biology/52, Humana Press, Totowa, NJ, 1996.

[26] C.A. Poole and S.K. Poole, Chromatography Today, Elsevier Science Publ. Co., Amsterdam and New York, 1991.

[27] Chromatography: Fundamentals and Applications of Chromatographic and Electrophoretic Methods, Part A: Fundamentals and Techniques, Edited by E. Heftmann, Elsevier Science Publ. Co., Amsterdam and New York, 1983.

[28] Chromatography, Fifth Edition, Edited by E. Heftmann, Elsevier Science Publ. Co., Amsterdam and New York, 1992, Parts A and B.

[29] Liquid Chromatography in Pharmaceutical Development, Edited by I.W. Wainer, Aster Publ. Co., Springfield, Oregon, 1985.

[30] High Performance Liquid Chromatography in Biotechnology, Edited by Wm. S. Hancock, Wiley and Sons, NY, 1990.

[31] Pharmaceutical and Biomedical Applications of Liquid Chromatography, Edited by C.M. Riley, W.J. Lough, and I.W. Wainer, Elsevier Science, Inc., Amsterdam, 1994.

[32] W.S. Hancock and J.T. Sparrow, HPLC Analysis of Biological Compounds, A Laboratory Guide, Marcel Dekker, New York, 1984.

[33] High Resolution Separation of Biological Macromolecules, Methods in Enzymology Series, Edited by B.L. Karger and Wm. Hancock, Academic Press, New York, 1996.

[34] O. Mikes, High Performance Liquid Chromatography of Biopolymers and Biooligomers. Part A, Materials and Techniques, Part B, Applications, Elsevier Science, Amsterdam, 1988.

[35] High-Performance Liquid Chromatography of Peptides and Proteins: Separation, Analysis, and Conformation, Edited by C.T. Mant and R.S. Hodges, CRC Press, Boca Raton, FL, 1991.

[36] HPLC of Biological Macromolecules, Methods and Applications, Edited by K.M. Gooding and F.E. Regnier, Marcel Dekker, New York, 1990.
[37] HPLC of Proteins, Peptides, and Polynucleotides, Edited by M.T.W. Hearn, VCH Publishers, New York, 1991.
[38] S.I. Sivakoff. Antibodies. In HPLC of Biological Macromolecules, Methods and Applications, K.M. Gooding and F.E. Regnier, Editors., Marcel Dekker, New York, 1990, Chapter 19.
[39] High Performance Liquid Chromatography: Principles and Methods in Biotechnology, Edited by E.D. Katz, J. Wiley and Sons, New York, 1996.
[40] Methods in Enzymology, Volume 270, High Resolution Separations and Analysis of Biological Macromolecules, Part A, Fundamentals, Edited by B.L. Karger and Wm.S. Hancock, Academic Press, San Diego, 1996.
[41] G. Siuzdak, Mass Spectrometry for Biotechnology, Academic Press, San Diego, CA, 1996.
[42] G.D. Roberts, W.P. Johnson, S. Burman, K.R. Anumula, S.R. Carr, Anal. Chem. 67 (1995) 3613.
[43] D.A. Lewis, A.W. Guzzetta, Wm.S. Hancock, and M. Costello. Anal. Chem. 66 (1994) 585.
[44] L. Tarditi, M. Camagna, A. Parisi, C. Vassarotto, L.B. DeMonte, M. Letarte, F. Malavasi, and M. Mariani. J. Chromatogr. 599 (1992) 13.
[45] S.I. Sivakoff, BioChromatography 1 (1) (1986) 42.
[46] P. Carty, R. O'Kennedy, J. Chromatogr. 442 (1988) 279.
[47] R. Vola, A. Lombardi, M. Mariani, BioTechniques 14 (4) (1993) 65.
[48] H. Graf, J.N. Rabaud, J.M. Egly, Bioseparation 4 (1994) 7.
[49] D.E. Hughes, P. Richberg, J. Chromatogr. 635 (1993) 313.
[50] A.J. Alexander, D.E. Hughes, Anal. Chem. 67 (1995) 3626.
[51] J.R., Mazzeo and I.S., Krull, In Capillary Electrophoresis-Technology, Edited by N. Guzman, Marcel Dekker, NY, 1993, Chapter 29.
[52] X. Liu, Z. Sosic, I.S Krull, J. Chromatogr., Biomed. Applics. 735 (1996) 165.
[53] J.R. Mazzeo, J. Martineau, and I.S. Krull. In CRC Handbook of Capillary Electrophoresis: Principles, Methods, and Applications, Edited by J.P. Landers, CRC Press, Inc., Boca Raton, FL, 1994, Chapter 18.
[54] B.L. Karger and F. Foret. In Capillary Electrophoresis Technology, Edited by N.A. Guzman, Marcel Dekker, NY, 1993, Chapter 1.
[55] R. Palmieri and J.A. Nolan, In Handbook of Capillary Electrophoresis, Edited by J.P. Landers, CRC Press, Boca Raton, FL, 1994, Chapter 13.
[56] M.A. Costello, C. Woititz, J. de Feo, D. Stremlo, L-F.L. Wen, D.J. Palling, K. Iqbal, B.A. Guzman, J. Liquid Chrom. 15 (6/7) (1992) 1081.
[57] M.A. Costello, C. Woititz, J. De Feo, J. Liq. Chromatogr. 15 (1992) 1081.
[58] (a) T. Pritchett, Beckman Newsletter, Issue 1, 9 (1994), Beckman Instruments, Fullerton, CA; (b) T. Pritchett, Application Information, Protein (A-1662A), Beckman Instruments, Fullerton, CA, 1994.
[59] O.W. Reif, R. Lausch, and R. Freitag. In Advances in Chromatography, Volume 34, Edited by P.R. Brown and E. Grushka, Marcel Dekker, New York, 1993, Chapter 1.
[60] J. Cacia, N. Bjork, R. Keck, J. Frenz, Paper 0803-WA presented at HPLC '94, Minneapolis, MN, May, 1994; (b) J. Cacia, R. Keck, J. Frenz, Paper 0807-WA-L presented at HPLC '94, Minneapolis, MN, May, 1994; (c) J. Frenz, C.P. Quan, C. Democko, R. Bridenbaugh, T. McNerney, Anal. Chem. 66 (1994) 335.
[61] L. Varady, H. Hodjiat, P. Bureau, R. Hussoin, and R. Mhatre. Paper 0820-WB presented at HPLC '94, Minneapolis, MN, May, 1994.
[62] (a) R. Mhatre, W. Nashabeh, D. Schmalzing, X. Yao, M. Fuchs, D. Whitney, F. Regnier, J. Chromatogr. 707 (1995) 225; (b) E. McCarthy, G. Vella, R. Mhatre, Y.-P. Lim, J. Chromatogr., 743 (1996) 163; (c) E. McCarthy, G. Vella, R. Mhatre, Y.-P. Lim, J. Chromatogr. 743 (1996) 171.
[63] L. Gadowski, A. Abdul-Wajid, J. Chromatogr. A715 (1995) 241.
[64] E.A. Charter, J. Fichtali, K.V. Lo, Intl. J. Bio-Chromatogr. 1 (1995) 199.
[65] N.A. Guzman, L. Hernandez, B.G. Hoebel, BioPharm 2 (1989) 29.
[66] N.A. Guzman, M.A. Trebilcock, J.P. Advis, Anal. Chim. Acta 249 (1991) 247.
[67] S.K. Hjerten, F. Kilar, J.-L. Liao, A. Chen, C. Siebert, M. Zhu, J. Chromatogr. 403 (1987) 47.
[68] T. Wehr, M. Zhu, R. Rodriguez, D. Burke, K. Duncan, Amer. Biotech. Lab. 8 (1990) 22.
[69] M. Zhu, R. Rodriguez, T. Wehr, J. Chromatogr. 559 (1991) 479.
[70] B.J. Compton, J. Chromatogr. 559 (1991) 357.
[71] S.J. Harrington, R. Varro, T.M. Li, J. Chromatogr. 559 (1991) 385.
[72] N.A. Guzman, J. Moschera, K. Iqbal, A.W. Malick, J. Chromatogr. 608 (1992) 197.
[73] T.-L. Huang, P.C.H. Shieh, N. Cooke, Chromatographia 39 (1994) 543.
[74] D. Schmalzing, W. Nashabeh, X.-W. Yao, R. Mhatre, R.E. Regnier, N.B. Afeyan, N. Fuchs, Anal. Chem. 67 (1995) 606.
[75] C. Silverman, M. Komar, K. Shields, G. Diegnan, J. Adamovics, J. Liquid Chrom. 15 (1992) 207.
[76] S. Kundu and C. Fenters. Presented at The 6th Frederick Conference on Capillary Electrophoresis, Hood College, Frederick, Maryland, October 23–25, 1995.
[77] M. Conti, C. Gelfi, P.G. Righetti, Electrophoresis 16 (1995) 1485.
[78] K. Shimura, B.L. Karger, Anal. Chem. 66 (1994) 9.
[79] F.-T.A. Chen, J. Chromatog. A680 (1994) 41.
[80] T. Pritchett, R.A. Evangelista, F-T.A. Chen, Bio/Tech. 13 (1995) 1449.
[81] N.H.H. Heegaard, J. Chromatogr. A680 (1994) 405.

[82] O.-W. Reif, R. Lausch, T. Scheper, R. Freitag, Anal. Chem. 66 (1994) 4027.
[83] L.J. Cole, R.T. Kennedy, Electrophoresis 16 (1995) 549.
[84] P.D. Grossman, J.C. Colburn, H.H. Lauer, R.G. Nielsen, R.M. Riggin, G.S. Sittampalam, E.C. Rickard, Anal. Chem. 61 (1989) 1186.
[85] R.G. Nielsen, E.C. Richard, P.F. Santa, D.A. Sharknas, G.S. Sittampalam, J. Chromatogr. 539 (1991) 177.
[86] Y.-H. Chu, W.J. Lees, A. Stassinopoulos, C.T. Walsh, Biochemistry 33 (1994) 10616.
[87] N.M. Schultz, R.T. Kennedy, Anal. Chem. 65 (1993) 3161.
[88] F.-T.A. Chen, S.L. Pentoney Jr., J. Chromatogr. A680 (1994) 425.
[89] F.-T.A. Chen, R.A. Evangelista, Clin. Chem. 40 (9) (1994) 1819.
[90] R.A. Evangelista, F.-T.A. Chen, J. Chromatogr. A680 (1994) 587.
[91] H. Zou, Y. Zhang, P. Lu, I.S. Krull, Biomed. Chromatogr. 10 (1996) 78.
[92] (a) D.T. Mao, G.M. Satow, and R.M.A. Lautamo. Application Notes from J and W Scientific, Inc., Folsom, CA, 1995; (b) J. and W. Scientific Publication, High Voltage, 1 (2), 1996; (c) D.T. Mao, L. Yu, and R. Lautamo. Paper presented at The Seventh Frederick Conference on HPCE, Hood College, Frederick, MD, October, 1996.
[95] T. Pritchett. Beckman Application Information Bulletin A-1772A, 1994.
[96] J. Vindevogel and P. Sandra, Chromatographic Methods: Introduction To Micellar Electrokinetic Chromatography, Huthig Buch Verlag, Heidelberg, 1992.
[97] J. Liu, H. Zhao, K. Volk, S. Klohr, E. Kerns, M. Lee, J. Chromatogr. A 735 (1996) 357.
[98] The eCAP 14-200 kit, Beckman Instruments, part No.477420. Application Information A-1772A. Beckman Instruments, Inc., Fullerton, CA, 1994.
[99] Beckman Instruments, Instruction Manual, Number 015-726434-B. Beckman Instruments, Inc., Fullerton, CA, 1994.
[100] J. Liu, S. Abid, M.S. Lee, Anal. Biochem. 229 (1995) 221.