Capillary Electrophoresis in the Development of Recombinant Protein Biopharmaceuticals

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1.1 Summary
The understanding of the physicochemical properties of proteins in solution is required to understand the separations taking place in the capillary. For example, during isoelectric focusing, it is important to ensure the lack of protein precipitation during focusing and mobilization otherwise artifacts occur and the true resolution of charge variants is not achieved. Similarly, buffer conditions, including additives, composition of buffer, and pH can influence the resolution of product variants. With diligent efforts one can achieve desired separations. As knowledge of buffer conditions and use of these techniques increase, the methods will be improved.

MECC can be useful for the characterization of recombinant proteins and monoclonal antibodies. However, the relative long analyte migration times may be a deterrent for routine use in quality control situations. Pharmaceutical companies will employ new techniques which provide advantages over current technology. CE has advantages in certain applications. Applications which use slab gel techniques, IEF and SDS-PAGE can be replaced by the CE methods for formulation development, characterization of charge isoforms, and for purity determinations. CZE and CEHF can be used for rapid identity tests which do not require peptide mapping. CZE has a tremendous advantage in analyzing basic proteins, and CZE and CE-SDS can be used to quantify protein in the presence of interfering excipients. It is the responsibility of the analytical chemist to determine applications which are appropriate for CE. It is apparent that these are currently being defined for the biotechnology industry, and the usage of CE should continue to increase.

1.2 Introduction
Since the cloning and expression of insulin, the biotechnology industry has experienced a boom in the number of products that have been developed, tested in clinical trials, and approved for unmet medical needs for many indications. The development of proteins as pharmaceuticals is somewhat different than the development of traditional small molecule pharmaceuticals. These differences are mainly due to the size and complexity of proteins. With the experience gained after the approval of recombinant proteins such as insulin, growth hormone (rhGH), recombinant human erythropoietin (rhEPO), recombinant tissue plasminogen activator (rt-PA), and a number of monoclonal antibodies, the required characterization and process controls for the approval of a protein product are becoming more established. After the protein has been cloned and expressed in eukaryotic or prokaryotic cells, there are usually several rounds of purification, product characterization, evaluation of the production process, and preclinical and toxicology studies leading to the filing of an IND to begin clinical trials. Assays are required for quantitation of the protein during various stages of production including the crude cell paste or cell culture fluid to determine the yield of the production process. During production, various post-translational modifications can occur such as deamidation, oxidation, isoaspartate isomerization, and proteolytic cleavages. Hence, it may be necessary to analyze for size, charged, or hydrophobic variants, especially if these variants have different biological potencies than the desired product. According to the ICH guideline, Q6B ‘Guidance on Specifications: Test Procedures and Acceptance Criteria for Biotechnological Biological Products’ [1], a product variant which does not have the same safety and efficacy profile as the desired product is termed a product-related impurity. For example, isomerization of a heavy chain aspartate residue of a therapeutic monoclonal antibody to isoaspartate resulted in a variant with low potency [2]. The manufacturer may choose to remove these impurities during the purification process or monitor their concentration during lot release.

Product characterization is required throughout the development of a recombinant product. In early preclinical stages, it is necessary to confirm if the product manufactured is the desired product prior to beginning pharmacokinetic and toxicological studies. In the case of glycoproteins, there could be heterogeneity due to varying degrees of glycosylation and occupancy of glycosylation sites. These variations could affect the intrinsic biological activity of the protein or the pharmacokinetic profiles of these proteins. For exam-
ple, it has been shown that glycosylation is necessary for the antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxic activities of monoclonal antibodies [3]. Carbohydrate moieties play a role in the clearance of rt-PA [4] and biological activity; e.g., type II rt-PA with an unoccupied glycosylation site displays more in-vitro activity than type I rt-PA which has all three glycosylation sites occupied [5]. In these instances, it may be necessary to monitor glycosylation to demonstrate consistent manufacture of a quality product. When the results of clinical trials are promising, more extensive characterization is usually performed for regulatory filings to demonstrate that the manufacturing process is well understood in order to avoid surprises when full production occurs. Some of the methods that are used in characterization are validated for final product lot release testing. Analytical characterization is also required to support methods for use in the quality control system. Formulation studies are performed to optimize the excipients and conditions necessary for the stability of the recombinant protein over a proposed shelf-life. Formulation studies are also performed to determine the modes of degradation of the protein molecule. Thus, formulation studies normally include accelerated stability studies of the protein molecule under various conditions. Following successful clinical trials and the finalizing of the manufacturing process, validation of the methods to be used in the quality control of the product is initiated. The control system for lot release is determined based upon product characterization and manufacturing history. Tests for identity, strength, purity, and potency are among those required for final product lot release. The primary identity test used requires peptide mapping to confirm the amino acid sequence of the protein. Identity tests are also used to confirm the product's identity, for example during packaging, or transfer of product to collaborators and contract labs. Stability studies are required on bulk and final product to confirm the shelf life of the recombinant product. The traditional chromatographic methods such as reversed-phase high performance liquid chromatography (RP-HPLC), size-exclusion chromatography (SEC), ion-exchange chromatography (both cation and anion), hydrophobic interaction chromatography, and gel electrophoretic methods such as sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF) methods have been used extensively for the analysis of recombinant proteins. CE analyses add to the repertoire of available analytical methods and will be discussed as they support the requirements for product development.

### 1.3 Capillary Electrophoresis Reviews

Several excellent reviews have been written in the use of capillary electrophoresis for the analysis of proteins including biotechnology-derived proteins. These reviews have provided information based upon the individual capillary electrophoresis (CE) techniques such as capillary zone electrophoresis (CZE) ([6–8]), capillary isoelectric focusing (cIEF, [9, 10]), capillary electrophoresis using sodium dodecyl sulfate (CE-SDS, [11]) and micellar electrokinetic chromatography (MEKC, also called micellar electrokinetic capillary chromatography [MECC, [12]]. Reviews have also been written for the analysis of recombinant proteins in general [13–15], antibodies [16], and glycoproteins [17, 18].

#### 1.3.1 CZE

The simplest mode of capillary electrophoresis is that of free solution capillary electrophoresis or CZE. In this mode, the intrinsic properties of the molecule, its charge and size are used to characterize its electrophoretic behavior, determine its purity, and as a means of quantification. In Dolnik's review [6] of the CZE of proteins, he addressed modeling the migration behavior of proteins, sample pretreatments such as preconcentration and derivatization to increase sensitivity, methods to reduce interactions with the capillary wall, and ways to improve the selectivity of the method. He also briefly reviewed CE-SDS using cross-linked and non-cross-linked gels. When more sensitive methods than UV detection are required, laser-induced fluorescence (LIF) or mass spectrometry methods have been used. The detection limit for bovine serum albumin (BSA) decreases from 0.5 μg mL⁻¹ to 25 ng mL⁻¹ when UV absorption is replaced with argon-laser-induced fluorescence [19]. In his subsequent review, Dolnik [7] updated the information on these same topics. Preconcentration can be performed by three on-line methods. In the first, sample self-stacking was used to determine trace concentrations of recombinant interleukins [20]. The second procedure utilizes solid-phase extraction on a cartridge containing Sepharose CL, with the concentrated peptides or proteins being released with acetonitrile. The latter method was used to analyze a tryptic digest of bovine serum albumin. The third method for preconcentration used a semi-permeable hollow fiber between the sample vial and the capillary inlet. An injection current is applied which concentrates the proteins into the hollow fiber. Subsequently, an electric field is applied through the hollow fiber to begin the electrophoretic separation. This method can lower the detection limit by a factor of 1000 [21]. Because the walls of fused silica capillaries carry negative charges due to ionized silanol groups, some proteins may adsorb to the walls during electrophoresis. Either dynamic (buffer additives) or static (permanently coated) wall coatings are used to reduce adsorption to capillary walls. For improving the selectivity of a CZE method, optimizing the composition of the background electrolyte (BGE) is usually a more effective procedure with adjusting the pH of the BGE as the first choice. A considerable amount of research has been done in describing buffer additives for CZE which minimize protein-capillary interactions, improve selectivity and resolution, and control electro-osmotic flow (EOF). Corradini [22] has summarized these additives which include neutral polymers, ionic salts and zwitterions, amine modifiers, surfactants, and ion-pairing agents used in protein CZE methods. In some cases cyclodextrins have been successfully used to resolve protein mixtures. Thus, carboxymethylated β-cyclodextrin was used to improve the separation of a model mixture of proteins (2-chymotrypsinogen A, cytochrome c, lysozyme, and ribonuclease A) in the cationic mode at pH 2.5.

A fundamental requirement in the analysis of a recombinant protein pharmaceutical is the need to demonstrate identity with respect to its primary amino acid sequence by peptide mapping. The method may also be used to show, by comparison with an appropriate reference material that changes in the primary amino acid sequence have not occurred, confirming product consistency and/or genetic stability [23]. The method requires considerable expertise for performing peptide