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Evaluation of the iCE280 Analyzer as a potential high-throughput tool for formulation development

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Abstract

The iCE280 Analyzer (iCE280) was evaluated for its potential application as a high-throughput tool to determine p*I* and separate charge related species using glycosylated, non-glycosylated and pegylated protein therapeutics as models. Resolution was achieved for glycosylated and non-glycosylated molecules, but remained a challenge for pegylated proteins. The sources of charge variants were determined to be the presence of C-terminal lysine residues, sialic acid content, and deamidation. Limited assay performance evaluation demonstrated that the method was linear in the concentration range of 2–333 μg/ml of IgG with linear regression coefficients of 0.984, 0.998, and 0.990 for acidic, main and basic species, respectively. Limit of detection and limit of quantitation were determined to be 3 and 11 μg/ml. The R.S.D. for intra- and inter-day precision as well as reproducibility was determined to be 0.2% or less for all p*I* values and 1.4% or less for acidic and main peak area distribution; the R.S.D. for basic peak area distribution was 5.7% or less. Robustness testing was performed by deliberately deviating ±50% of pharmalyte concentration away from the desired condition. This deviation revealed a p*I* shift of only 0.06 units and resulted in no significant impact on area percent distribution. Utilization of iCE280 Analyzer eliminated the mobilization step associated with traditional capillary isoelectric focusing analysis and increased analytical throughput at least 2-fold.

Keywords: Imaged capillary isoelectric focusing; iCE280 Analyzer; Deamidation; Protein; Pegylation

1. Introduction

Slab gel isoelectric focusing (IEF) technique has been routinely used to determine the isoelectric point (p*I*) of proteins and to monitor their purity, stability and microheterogeneity [1–7]. Nevertheless, the technique is labor-intensive and semi-quantitative [1,8]. Recently, isoelectric focusing performed in a capillary format (cIEF) has demonstrated and offers many advantages over conventional slab gel IEF. These include improved resolution [1], better quantitation, and automation capability [1]. Several years ago, an *imaged* cIEF instrument (iCE280 Analyzer) was introduced to the market by Convergent Bioscience. Prior to the introduction of this instrument, most cIEF analyses were carried out using traditional CE instruments with a two step approach: (1) protein focusing after being introduced into the capillary and (2) post-focusing mobilization of protein passing through a capillary detector window located at one end of the capillary [7,9,10–12]. There were problems associated with the mobilization step. The pH gradient established during the focusing step could be distorted [10–12]. This often resulted in band broadening, reduced resolution, and poor reproducibility [10–12]. Imaged cIEF completely eliminated the mobilization step by taking the whole capillary absorption image using a charge-coupled device camera. The detection system consists of a whole column optical absorption imaging detector operated at 280 nm. The light source of the absorption detector is a deuterium (D2) lamp. During the focusing, the light beam from the lamp is focused onto the separation capillary by a bundle of optical fibers and a cylindrical lens. The final whole capillary UV absorption image is captured by a camera with an imaging lens and a charge-coupled device sensor [11].

This work was intended to evaluate the iCE280 Analyzer as a potential high-throughput tool for formulation development.

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The scope of this work includes: (1) use of iCE280 Analyzer to attain resolution of charge related species with minimum development time; (2) identification of the source of charge heterogeneity using this instrument in conjunction with other techniques, such as HPLC, mass spectrometry, and enzymatic digestion; (3) investigation of parameters affecting the performance of the iCE280 Analyzer. Three diverse protein molecules were used as models, a glycosylated immunoglobulin (IgG) molecule, a non-glycosylated protein (Genotropin®) molecule, and a pegylated protein (SOMAVERT®) molecule.

2. Experimental

2.1. Materials

Pharmalytes pH 3–10 and 8–10.5 were obtained from Sigma. The Testing Kit for the iCE280 Analyzer, including 80 mM phosphoric acid, 50% sodium hydroxide, 1% methylcellulose and hemoglobin were purchased from Convergent Bioscience. 5.3 and 6.5 p*I* markers were obtained from BioRad. 3.78 and 9.5 p*I* markers were from Convergent Bioscience. Modified, sequence grade trypsin isolated from porcine was from Promega. N-glycanase, recombinant from Chryseobacterium [Flavobacterium] meningosepticum expressed in *E. coli* and Sialidase A, recombinant from Arthrobacter ureafaciens expressed in *E. coli* were purchased from Prozyme. Carboxypeptidase B, from human pancreas was purchased from Sigma–Aldrich and urea, U.S.P. grade was from Mallinckrodt. Deionized water $\geq$18 MΩ cm was generated using Milli-Q water purification system from Millipore. IgG, Genotropin® and SOMAVERT® were supplied as active pharmaceutical ingredients by Pfizer Bioprocess Research and Development Department with concentration of 11 mg/ml in acetate buffer pH 5.5, 37 mg/ml in phosphate buffer pH 6.8, and 7.5 mg/ml in histidine buffer pH 6.5, respectively. They were diluted to 1–2 mg/ml using deionized water prior to use.

2.2. Imaged capillary isoelectric focusing

Imaged capillary isoelectric focusing was performed using an iCE280 Analyzer (Convergent Bioscience, Toronto, Canada). The separation cartridge used in the study is commercially available and was purchased from Convergent Bioscience. The purchased separation cartridge contains a 5 cm long, 100 μm i.d. × 200 μm o.d. separation capillary that has its inner surface pre-coated with fluorocarbon compound. This capillary was fixed on to a glass substrate and separated from the catholyte and anolyte by two pieces of hollow fiber membrane. The anolyte was 80 mM phosphoric acid and the catholyte 100 mM NaOH. A sample of IgG was prepared by mixing 30 μl of 2 mg/ml IgG, 1 μl of 5.3 p*I* marker, 1 μl of 9.5 p*I* marker, 70 μl of 1% methylcellulose, and 8 μl of pharmalytes (pH 3–10 or a mixture of pH 3–10 and 8–10.5 at ratio of 20:80 or 15:85). The resulting solution was diluted to 200 μl with deionized water and centrifuged for 2 min. Genotropin® was less soluble in the sample matrix; consequently, it was diluted from 37 mg/ml down to 1 mg/ml first in 4 M urea. Then the sample was prepared by mixing 30 μl of 1 mg/ml Genotropin®, 1 μl of 3.78 p*I* marker, 1 μl of 6.5 p*I* marker, 70 μl of 1% methylcellulose, and 8 μl of pharmalytes pH range 3–10. The resulting solution was diluted to 200 μl with deionized water and centrifuged for 2 min. SOMAVERT® was prepared similarly to Genotropin® except that it was diluted in deionized water first prior to mixing with sample matrix. Additionally, the high p*I* marker used in this sample preparation has p*I* of 7.2 instead of 6.5. Samples were introduced to the capillary through an autosampler (Prince) and were focused for varied lengths of time to achieve the optimum resolution. The final image of the IEF trace was captured by the 280 nm deuterium lamp detector.

2.3. Enzymatic reaction

2.3.1. Carboxypeptidase B (CPB) enzymatic reaction

2.2 mg of IgG in 200 μl of 20 mM acetate buffer pH 5.5 was mixed with 1.8 μg of CPB in 50 μl of 50 mM acetate pH 5.0, and 1 M NaCl. Additional 50 μl of 500 mM phosphate buffer pH 7 was added. The resulting solution was incubated at 37 °C for 3 h and quenched with 1% TFA. A control reaction was performed simultaneously without addition of enzyme.

2.3.2. Sialidase A enzymatic reaction

2.2 mg of IgG in 200 μl of acetate buffer pH 5.5 was mixed with 12.5 μg of sialidase A in 100 μl of 20 mM Tris–HCl pH 7.5, and 25 mM NaCl. The resulting solution was incubated at 37 °C overnight and quenched with 1% TFA. A control reaction was performed simultaneously without addition of enzyme.

2.3.3. N-glycanase enzymatic reaction

2.2 mg of IgG in 200 μl of 20 mM acetate buffer pH 5.5 was mixed with 2.5 μg of *N*-glycanase in 20 μl of 20 mM Tris–HCl pH 7.5, 50 mM NaCl, and 1 mM EDTA. The resulting solution was incubated at 37 °C for 7 h. Additional 2.5 μg of enzyme in 20 μl of 20 mM Tris–HCl pH 7.5, 50 mM NaCl, and 1 mM EDTA was added and incubation was continued at 37 °C overnight. The reaction was quenched with 1% TFA. A control reaction was performed simultaneously without addition of enzyme.

2.3.4. Trypsin digestion of Genotropin®

0.2 mg of stressed Genotropin® in 12.5 μl of Tris–HCl buffer pH 9.0 was mixed with 8 μg of trypsin in 16 μl of 50 mM Tris–HCl pH 7.5. The resulting solution was incubated at 25 °C overnight and quenched with 1% TFA. A control reaction was performed simultaneously.

2.3.5. Trypsin digestion of IgG

0.4 mg of thermally stressed IgG in 100 μl of acetate buffer pH 5.5 was mixed with 20 μg of trypsin in 20 μl of 50 mM Tris–HCl pH 7.5. Additional 10 μl of 125 mM Tris–HCl buffer pH 8.5 was added to the reaction mixture. The resulting solution was incubated at 37 °C for 24 h and then quenched with 1 M HCl.

2.4. HPLC and mass spectrometry condition

2.4.1. Digested Genotropin®

HPLC analysis was performed using Agilent 1100 series HPLC system (Agilent Technologies Inc., California, USA). Ten microliter of digested Genotropin® was injected to a Phenomenex Jupiter C18 reversed-phase column (2.1 mm × 250 mm, 5 μm). The tryptic peptides were eluted at a flow rate 0.2 ml/min with mobile phase A of 0.1% TFA in water and mobile phase B of 0.085% TFA in acetonitrile. A multi-step gradient was applied as the following: 2–16% B in 27 min, 16–28% B in 60 min, 28–34.5% B in 20 min, 34.5–45% B in 25 min, 45–95% B in 5 min, and then hold at 95% B for 5 min. The tryptic peptides were monitored by UV at absorbance of 216 nm. The HPLC was also coupled with a Q-Tof micro™ mass spectrometer from Waters Micromass, previously calibrated with myoglobin. The mass spectra were acquired and analyzed using MassLynx 4.0 software.

2.4.2. Digested immunoglobulin

The HPLC system used in this analysis was the same as for the digested Genotropin®. One hundred microliter of digested IgG was injected to a Zorbax 300SB C18 (4.6 mm × 150 mm, 3.5 μm) reversed-phase column. The tryptic peptides were eluted at flow rate 0.5 ml/min with mobile phase A of 0.1% TFA in water and mobile phase B of 0.085% TFA in acetonitrile. A multi-step gradient was used for the analysis as the following: 3–15% B in 22 min, 15–16% B in 5 min, 16–21% B in 3 min, 21–27% B in 30 min, 27–42% B in 25 min, 42–90% B in 2 min, and hold at 90% B for 10 min. The tryptic peptides were monitored using the same detection system as for Genotropin®.

2.4.3. Intact Genotropin®

The HPLC system used in this analysis was the same as for the digested Genotropin®. Fourty-five microliter of intact Genotropin® was injected to two Tosoh Bioscience Super Q-5PW (7.5 × 75) Anion exchange (AEX) columns. The column temperature was kept at 45 °C throughout the analysis. The sample was eluted with mobile phase A of deionized water and mobile phase B of 200 mM potassium dihydrogen phosphate buffer, pH 6.75. An isocratic condition followed by a multi-step gradient was used as the following: Isocratic with 23% B in 20 min at flow rate 0.4 ml/min, 23–91.2% B in 18 min at flow rate 0.4 ml/min, hold at 91.2% B in 5 min at flow rate 0.4 ml/min, 91.2–100% B in 2 min at flow rate 0.8 ml/min, and then go back to 23% B in a step gradient and hold for 10 min at flow rate 0.4 ml/min. Genotropin® peaks were monitored by UV at absorbance of 220 nm.

3. Results and discussion

3.1. Glycosylated protein

3.1.1. Optimization of resolution

For the iCE280 Analyzer, the capillary is adhered to a glass substrate. The length and coating are fixed. Additionally, no mobilization step is required. Therefore, the parameters that need to be modified to improve the resolution of charge related species are focusing time and the ratio of different pH range pharmalytes if solubility is not an issue. As a result, less time is needed for method development. In this study, the optimum condition was established through the following two steps: (1) 0.3 mg/ml IgG in 4% pharmalytes, pH range of 3–10, and 0.35% methylcellulose was focused from 5 to 10 min to obtain profiles of resolution changes with focusing time and (2) narrow pH range of pharmalytes was added in different ratios according to the first step's screening to further improve the resolution by varying the focusing time. Initial screening with broad pH range pharmalytes indicated the presence of three peaks centered at p*I* of approximately 8. The maximum resolution between main and acidic peaks using broad range pharmalytes alone was 1.2 at 5 min focusing. The maximum resolution between main and basic peaks was 1.7 at 8 and 10 min focusing. With the addition of narrow pH range pharmalytes, the maximum resolution between main and acidic peaks was 1.4 at 12 min focusing at broad and narrow pH range pharmalyte ratio of 20:80, but the resolution between main and basic peaks at this condition decreased slightly compared to the condition using broad pH range pharmalytes alone. Further increasing the ratio of narrow pH range pharmalytes showed little impact. The majority of the acidic species were stability related, whereas the basic species were not. These remained unchanged over the course of the stability study. Therefore, the resolution between main and acidic peaks was used as a benchmark. As a result, broad and narrow pharmalyte ratio of 20:80 and 12 min focusing time was considered the optimum condition for further study. The resolution obtained in this study was similar to the one reported by Hunt et al. [13] who validated the mouse/human chimeric monoclonal antibody following ICH guideline using a traditional CE instrument. The profiles of resolution vs. ratios of different pH range of pharmalytes and focusing time are shown in Fig. 1. At optimized condition, the main peak had p*I* of 8.28, the acidic peak adjacent to the main peak had p*I* of 8.11, and the basic peak had p*I* of 8.51. The percent area distribution was 69.2, 26.2, and 4.6% for main, acidic and basic peaks, respectively.

The profiles of slab gel IEF and Imaged cIEF analyses were compared as shown in Fig. 2. They were analogous to each other in terms of number of species observed; however, the p*I* values were slightly varied. This was attributed to the fact that the slab gel IEF and Imaged cIEF were run under different ratios of broad and narrow pH range pharmalytes. For slab gel IEF, the pharmalytes used was a mixture of pH 3–10, 7–9 and 8–10.5 at ratio of 16:42:42. Whereas for Imaged cIEF, the pharmalytes used was a mixture of pH 3–10 and 8–10.5 at ratio of 20:80. Additionally, the p*I* markers used in the two techniques were different. For slab gel IEF, the p*I* marker used was a high p*I* marker kit from Amersham Biosciences, but p*I* markers from Bio-Rad (5.3) and Convergent Bioscience (9.5) were used for Imaged cIEF analysis.

3.1.2. Identification of IgG charge variants using enzymatic reaction

IgG is an *N*-glycosylated protein. Sources of charge related heterogeneity have been well established. These include the

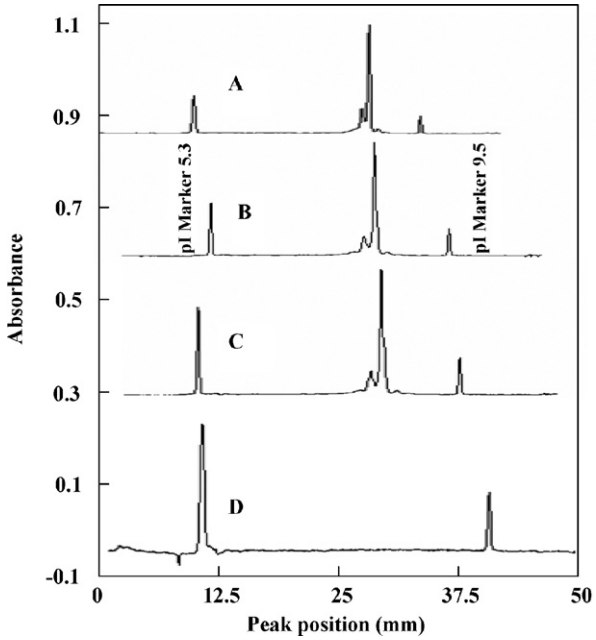

Fig. 1. Impact of pharmalyte pH range on resolution: (A) IgG in broad pH range pharmalytes (pH 3–10) focused for 5 min; (B) IgG in broad and narrow pH range pharmalytes (pH 3–10 and 8–10.5) at ratio of 20:80 and focused for 12 min; (C) IgG in broad and narrow pH range pharmalytes (pH 3–10 and 8–10.5) at ratio of 15:85 and focused for 12 min; (D) blank in broad and narrow pH range pharmalytes at ratio of 20:80 and focused for 12 min.

presence of terminal sialic acids, incomplete post-translational cleavage of C-terminal lysines, deamidation, and succinimide formation [14–16]. Grant and co-workers [14] have demonstrated that these charge variants can be detected and quantified by ion exchange HPLC and cIEF with traditional CE instruments. They also demonstrated that the qualitative and quantitative profiles obtained from these two techniques agreed with each other very well [14]. Additionally, these charge variants could be characterized using a traditional cIEF instrument in conjunction with other instrumentation and methods [14]. To demonstrate that the iCE280 Analyzer has similar functions as the traditional CE instrument, and can be used not only as a quantitation tool but also as characterization tool in early candidate selection, pre-formulation, and formulation development, IgG was treated with several enzymes, including carboxypeptidase B, *N*-glycanase, sialidase A, and trypsin to identify the source of charge heterogeneity.

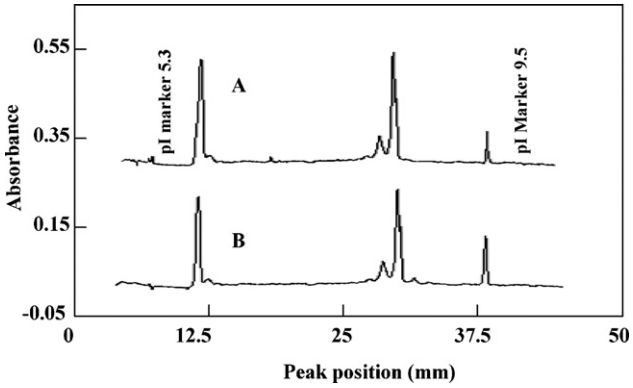

Fig. 3. Electropherograms of carboxypeptidase B (CPB) digest: (A) IgG treated with CPB; (B) IgG as control without CPB. It demonstrated that high p*I* species was C-terminal Lys variant. Enzyme digestion and analysis conditions are described in Section 2.

Carboxypeptidase B (CPB) is an enzyme that selectively cleaves Lys and Arg residues at the C-terminal end of proteins and peptides. Therefore, if any of the isoforms are C-terminal Lys variants, CPB treatment will cleave the terminal Lys from the end of the protein and the associate peaks should shift toward the acidic region. Grant et al. [14] identified basic charge variants present in D2E7, a fully human IgG by treating the molecule with CPB and analyzing it by traditional cIEF. This same procedure was followed here. When model IgG was treated with CPB, the basic peak disappeared but it remained unchanged in the control sample. This illustrated that the ~4% basic peak observed in the iCE280 analysis was originated from incomplete post-translational cleavage of C-terminal Lys. This result was also consistent with the preliminary mass spectrometry analysis of heavy chain that showed approximately 5% of Lys variant was present in the molecule (data not shown). Representative electropherograms of CPB reaction analysis by iCE280 Analyzer are shown in Fig. 3.

N-glycanase is an enzyme that selectively removes N-linked carbohydrates from glycosylated proteins and peptides. Neutral oligosaccharides do not introduce charge variants on a protein unless sialic acid is present. Deglycosylation of the IgG

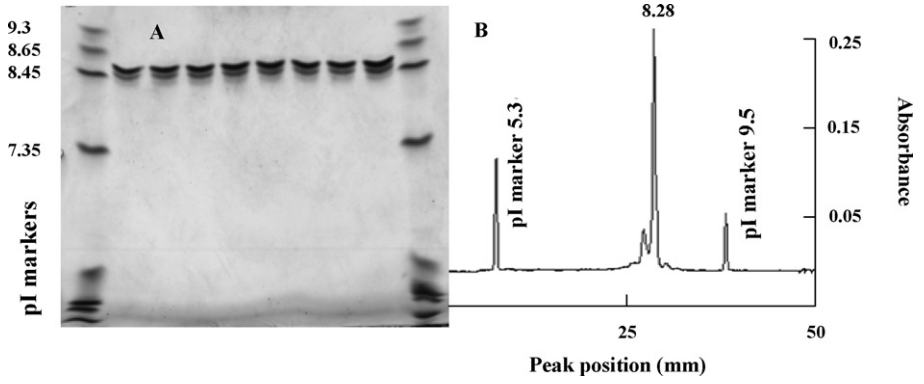

Fig. 2. Profile comparison of slab gel IEF and Imaged cIEF: (A) slab gel IEF run with mixture of broad and narrow pH range pharmalytes 3–10:7–9:8–10.5 = 16:42:42; (B) imaged cIEF ran with mixture of broad and narrow pH range pharmalytes 3–10:8–10.5 = 20:80 and focused for 12 min.

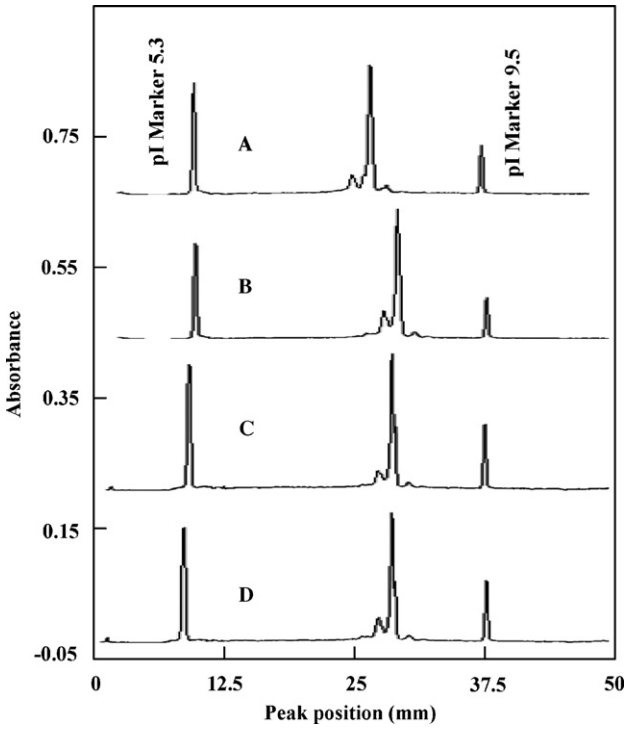

Fig. 4. Electropherograms of enzyme digests: (A) IgG treated with *N*-glycanase; (B) IgG control without *N*-glycanase; (C) IgG treated with Sialidase A; (D) IgG control without Sialidase A. Enzyme digestion and analysis conditions are described in Section 2.

with *N*-glycanase showed approximately 7% reduction of acidic species but did not remove it entirely. This indicated ~7% sialic acid may be present in the molecule. Representative electropherograms of the *N*-glycanase reaction analysis by iCE280 Analyzer are shown in Fig. 4A and B. All the peaks shifted toward acidic region after treatment with *N*-glycanase because asparagine linked with carbohydrate was hydrolyzed into aspartic acid after enzymatic reaction. Thus, IgG was treated with sialidase A to further investigate the source of charge heterogeneity.

Sialidase A is an enzyme that specifically cleaves terminal sialic acid residues from carbohydrates. The analysis of sialidase A treated sample using iCE280 Analyzer showed a 6% reduction of acidic species in comparison to control sample. This data combined with the *N*-glycanase reaction analysis data indicated that approximately 6–7% of sialic acid was present in the molecule and contributed to only a fraction of the charge heterogeneity of acidic species. The remaining ~20% acidic species were, therefore, likely to be the deamidation species. Representative electropherograms of sialidase A reaction analysis by iCE280 Analyzer are shown in Fig. 4C and D. IgG was subjected to tryptic map to confirm the presence of deamidation in the molecule.

Reversed-phase HPLC coupled with mass spectrometry analysis of tryptic digest of stressed IgG indicated the presence of 2 deamidated tryptic peptides in the heavy chain, one in T4 fragment and the other in T32 fragment. Both of these peptides contain an Asn-Gly residue which has been shown to readily undergo deamidation [17–19]. The mass for each pair of the intact and deamidated peptides was 2757 and 2758 Da for T4 and 2544 and 2545 Da for T32, respectively. The mass difference for each pair is 1 Da. This supports the results from iCE280 analysis that one major and one minor acidic species were observed in electropherograms in Fig. 1. As demonstrated earlier, there was approximately 6–7% acidic species contributed by sialic acid, the major acidic species, therefore, could be the combination of mono-deamidation and intact IgG with sialic acid, the minor species could be the combination of mono-deamidation with sialic acid and di-deamidation species. Due to the stage of the project, direct assignment of degradation products was not pursued.

3.2. Non-glycosylated protein

3.2.1. Optimization of resolution

Genotropin® was chosen as a model of non-glycosylated protein. According to slab gel IEF analysis, this molecule has a p*I* of about 5.2. Therefore, p*I* markers of 3.78 and 6.5 were used in the Imaged cIEF study. Initial attempts using 0.3 mg/ml Genotropin®, 4% pharmalytes, and focusing for different lengths of time did not yield reproducible profiles but smears of broad bands and spikes as shown in Fig. 5A. This is a typical phenomenon of protein precipitation in the capillary during the isoelectric focusing. Mao and Pawliszyn [20] observed a similar phenomenon in their work and they resolved the problem through addition of 20% glycerol in the sample matrix. Righetti and co-workers [21] also encountered a similar problem. They explored a series of mild additives capable of completely preventing or largely alleviating protein precipitation in the capillary during the isoelectric focusing. These

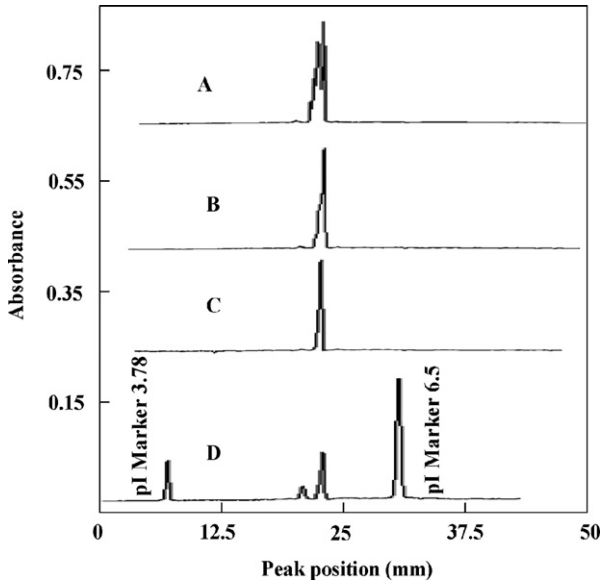

Fig. 5. Optimization of Imaged cIEF for Genotropin®: (A) Genotropin® was diluted in deionized water to 2 mg/ml, then added to pH 3–10 pharmalytes sample matrix and focused for 5 min; (B) Genotropin® was diluted in 4 M urea to 2 mg/ml and ran using the same condition as A; (C) Genotropin® diluted in 4 M urea to 1 mg/ml and ran using the same condition as A; (D) stressed Genotropin® diluted in 4 M urea to 1 mg/ml and ran using the same condition as A.

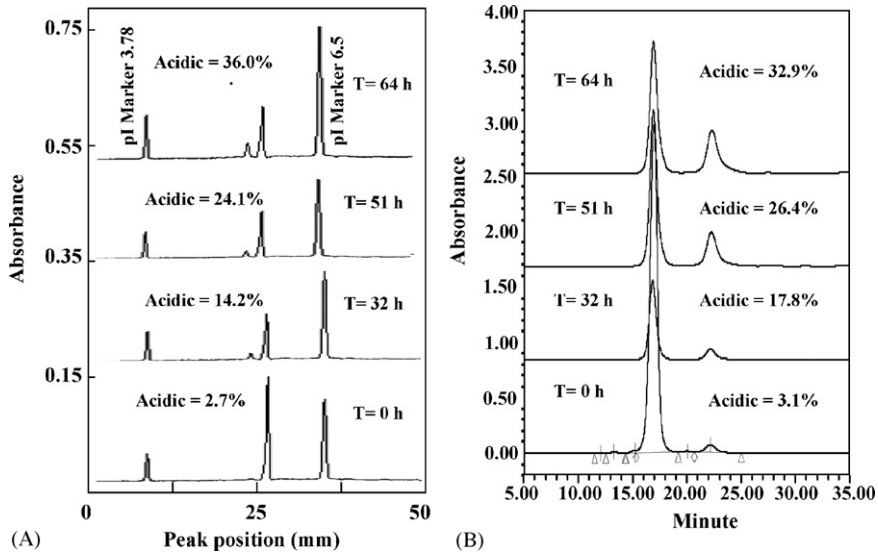

Fig. 6. Imaged cIEF and ion exchange HPLC profile comparison: (A) electropherograms of Genotropin® stressed in pH 9 tris buffer at 40 °C over different times; (B) ion exchange HPLC chromatograms of the Genotropin® stressed at the same condition as A. Electrophoresis and ion exchange HPLC conditions are described in Section 2.

additives included ethylene glycol, propylene glycol, sucrose, sulphobetaines and others [21]. Here, Genotropin® was diluted in 4 M urea first and then mixed with sample matrix. Urea is a non-ionic detergent and has been widely used to enhance protein solubility in a variety of studies, including cIEF [22]. The final protein concentration in sample matrix was also reduced from 0.3 to 0.15 mg/ml. Through these modifications, the focusing became possible as demonstrated in Fig. 5C and D. A control sample gave a single peak after 5 min focusing. When a stressed sample was analyzed, a low p*I* species was observed in addition to the parent peak. The resolution between the two species was >3.

3.2.2. Imaged cIEF and anion exchange HPLC profile comparison

Stability profiles of Imaged cIEF and anion exchange HPLC were compared. Genotropin® was buffer exchanged to a 20 mM tris buffer, pH 9 and placed in a 40 °C incubator for up to 64 h. Samples were withdrawn at different time points for analysis. As shown in Fig. 6, the number of peaks and relative abundance were consistent with each other, except the peak order was reversed. The reason is that the deamidation products of asparagine are iso-aspartic and aspartic acid and, an extra negative charge was introduced into the molecule. The more acidic the molecule, the stronger the interaction of the molecule with column resins is in anion exchange chromatography. Therefore, stronger elution solvent is required to elute deamidated species off the column, and thus longer retention times are observed.

3.2.3. Identification of Genotropin® charge variants using enzymatic reaction

The stressed sample was subjected to tryptic digestion and analyzed by HPLC/MS. A new species appeared next to T15 fragment in HPLC chromatogram. This species was not observed in the control sample. The mass of the new species was 1490 Da, a difference of 1 Da from T15 (1489 Da). T15 has two sites susceptible to deamidation, one at Asp-149 and the other at Asp-152. Therefore, the shoulder peak from tryptic map was likely to be a mono-deamidated T15. The sequencing analysis was not performed. Thus, the precise site of deamidation was not identified. These data support that the low p*I* species observed in the eletropherogram is deamidated Genotropin®.

3.3. Pegylated protein

Pegylation of biologically active proteins is one of the strategies in drug development and life cycle management for the pharmaceutical industry [23]. A number of existing drugs were conjugated with different sizes of polyethylene glycol (PEG) to achieve prolonged biological half-life in less frequent dosing regimens. This was exemplified by PegIntron, a pegylated form of IFN-α2b (Intron-A) for treatment of hepatitis C [23]. Generally, analysis of pegylated molecules are more challenging than analysis of the protein alone. In this study, SOMAVERT®, a pegylated protein for treatment of patients with acromegaly, was selected as model to evaluate the application of iCE280 Analyzer for analysis of pegylated proteins. SOMAVERT® is a complicated molecule. The protein portion of the molecule has a similar primary structure to Genotropin® except for nine amino acid residues which were altered to yield the antagonistic action on the somatropin receptor. Pegylation yields multi-pegylated species which was demonstrated by SDS-PAGE and capillary zone electrophoresis analysis [24]. Averages of four to six 5000 MW polyethylene glycol molecules are covalently bound to each protein molecule and generate heterogeneous mixtures of molecules in term of sites and extent of pegylation. Attempts to resolve SOMAVERT® and its charge related variant were less successful. General observations for this molecule were that the peak was relatively broad and distorted. Modification of pharmalyte contents, methylcellulose concentration,

protein concentration, focusing time, and addition of other additives could not yield reasonable peak shapes. The hydrophilic nature of polyethylene oxide unit and the mobility of PEG in solution might force the charged surface of protein to be buried. Thus, the separation based on surface charge of the molecule was difficult.

3.4. Limited assay performance evaluation

Limited assay performance was conducted using IgG to evaluate the instrument performance. These include: linear response, limit of detection (LOD), limit of quantitation (LOQ), repeatability, specificity, and robustness. Spike recovery for accuracy was not performed due to unavailability of other techniques to isolate charge variants.

3.4.1. Linearity

Linearity was assessed by varying the protein concentration in sample matrix from 333 μg/ml down to 2 μg/ml. The absolute areas of acidic, main, and basic peaks were plotted against total protein concentration. The equations of linear regression for acidic, main and basic species were $y = 191.3x - 1583.1$, $y = 565.39x - 1310.5$, and $y = 30.26x + 416.87$, respectively. The correlation coefficients for acidic, main, and basic species were 0.984, 0.998, and 0.990. Statistical analysis showed that 95% confidence intervals for all three intercepts included zero. Therefore, there is no statistically significant bias. Additionally, all three slopes were significantly different from 0 at 95% confidence intervals. These demonstrate capacity of the iCE280 method for quantitative analysis.

3.4.2. Limit of detection and quantitation

The limit of detection was established as the minimum concentration at which the main species can be detected with signal-to-noise ratio of ≥3. The limit of quantitation was established as the minimum concentration at which the main species can be reliably measured with signal-to-noise ratio of ≥10. The LOD and LOQ for major species at the above concentrations were determined to be 3 μg and 11 μg/ml, respectively. The signal-to-noise ratios at these concentrations were 4 and 10, respectively. These values are similar to the ones reported in the literature for a mouse/human chimeric monoclonal antibody [13].

3.4.3. Repeatability

Repeatability was assessed using a single sample preparation and six replicate injections within the same day. As shown in Table 1, the repeatability for p*I* values and area distribution was demonstrated by R.S.D. of 0.1% for all p*I* measurement and less than 0.5% for all peak area percent distribution measurements. The repeatability for the cIEF analysis using iCE280 Analyzer was equivalent to the literature report values for two other antibodies using traditional CE instrument with two step approach [2,13].

3.4.4. Intermediate precision

Intermediate precision was evaluated using a single sample preparation and nine injections over 3 days. Again, as shown in Table 1, good intermediate precision for p*I* values and area percent distribution was demonstrated with R.S.D. of 0.2% or less for all p*I* measurements; and R.S.D.s of 1.4, 0.6, and 5.7% for acidic, main and basic peak area percent distribution measurements. The intermediate precision for p*I* measurement using iCE280 Analyzer was slightly better than the literature reported value using traditional CE instrument with two step approach for an antibody, but R.S.D. for area percent distribution was slightly higher [2]. However, the differences were not significant.

Table 1
Precision measurement for IgG using iCE280 Analyzer

	Acidic	Main	Basic
p*I* measurement			
Repeatability[a]			
Mean	8.11	8.28	8.51
R.S.D. (%)	0.1	0.1	0.1
Intermediate[b]			
Mean	8.11	8.27	8.50
R.S.D. (%)	0.1	0.1	0.2
Reproducibility[c]			
Mean	8.10	8.27	8.50
R.S.D. (%)	0.1	0.1	0.2
Area measurement (%)			
Repeatability[a]			
Mean	26.2	69.2	4.6
R.S.D. (%)	0.3	0.5	0.3
Intermediate[b]			
Mean	26.1	69.3	4.6
R.S.D. (%)	1.4	0.6	5.7
Reproducibility[c]			
Mean	26.3	69.2	4.6
R.S.D. (%)	1.3	0.5	5.7

[a] Single sample preparation and 6 injections in 1 day.
[b] Single sample preparation, 3 injections per day for 3 consecutive days.
[c] Two sample preparations and analyzed in two different laboratories. Six injections were done in laboratory 1 and 6 injections in laboratory 2.

3.4.5. Reproducibility

Reproducibility was assessed by two independent sample preparations and separate analysts in two separate laboratories with 6 injections in each laboratory. The data were pooled for calculations. As shown in Table 1, good reproducibility was demonstrated with R.S.D. for p*I* values of 0.2% or less for all peaks, and R.S.D. for acidic, main, and basic peak area percent distributions of 1.3, 0.5, and 5.7%, respectively.

3.4.6. Specificity

Specificity is defined as the ability to discriminate between components of closely related structures as well as other species which are likely to be present in the sample, e.g. degradation products, excipients, and buffer species that may overlap with the peaks of interested. Thus, it is necessary to demonstrate the absence of these interferences. As shown in Fig. 1, specificity was demonstrated by near baseline resolution of main peak from other charge related species and by the absence of any UV

absorbance in the presence of excipients in the blank solution analysis.

3.4.7. Robustness

Robustness is an indication of analytical method reliability. It can be determined by deliberately varying key method parameters and assessing the impact on the method performance and final results. For this method, sample preparation is one of the key aspects which could potentially introduce variation and affect performance. For instance, pharmalytes, a key component for the sepration, are highly viscous solutions and how an analyst dispenses them may directly affect the quantity in the sample matrix. The other factor is the quality of the capillary cartridge. These two parameters were evaluated.

3.4.7.1. Variation of pharmalyte concentration. While keeping all other method parameters constant, pharmalyte concentration was intentionally altered by ±50% of desired concentration. A minimum of three injections was performed at each condition. The variation of p*I* values was 0.06 units or less. The variation of area percent distribution was 0.4% or less. This demonstrates that the cIEF method using iCE280 Analyzer can be operated under a broad range of pharmalyte concentrations without affecting the quantitative output of the analyte. Therefore, the method was deemed robust. A summary of the data is in Table 2.

3.4.7.2. Capillary lot to lot variability. Three capillary cartridges from separate lots were evaluated using a single sample preparation and triplicate injections for each cartridge. The variations of p*I* values were 0.07 or less, and there was no significant impact on area percent distributions. Detailed data are summarized in Table 3.

Table 2
Robustness test by deliberately varying the pharmalyte concentration

	Acidic	Main	Basic
p*I* measurement			
−50% Pharmalytes[a]			
Mean	8.05	8.29	8.48
R.S.D. (%)	0.1	0.1	0.1
Desired condition[b]			
Mean	8.11	8.28	8.51
R.S.D. (%)	0.1	0.1	0.1
+50% Pharmalytes[c]			
Mean	8.12	8.31	8.54
R.S.D. (%)	0.1	0.1	0.2
Area measurement (%)			
−50% Pharmalytes[a]			
Mean	26.4	69.0	4.6
R.S.D. (%)	1.6	0.6	3.9
Desired condition[b]			
Mean	26.5	69.0	4.5
R.S.D. (%)	1.5	0.4	3.4
+50 Pharmalytes[c]			
Mean	26.8	68.7	4.9
R.S.D. (%)	2.1	0.2	9.4

Triplicate injections.
[a] 2% of pharmalytes (broad:narrow = 20:80) and focused for 12 min.
[b] 4% of pharmalytes (broad:narrow = 20:80) and focused for 12 min.
[c] 8% of pharmalytes (broad:narrow = 20:80) and focused for 12 min.

Table 3
Lot to lot cartridge comparison

	Acidic	Main	Basic
p*I* measurement			
Lot 99574			
Mean	8.11	8.28	8.51
R.S.D. (%)	0.1	0.1	0.1
Lot 99417			
Mean	8.09	8.26	8.49
R.S.D. (%)	0	0	0.
Lot 99653			
Mean	8.02	8.20	8.43
R.S.D. (%)	0.2	0.2	0.2
Area measurement (%)			
Lot 99574			
Mean	26.4	69.1	4.5
R.S.D. (%)	1.4	0.6	5.6
Lot 99417			
Mean	26.2	69.2	4.6
R.S.D. (%)	1.1	0.5	6.1
Lot 99653			
Mean	26.1	69.1	4.8
R.S.D. (%)	1.0	0.2	4.5

Single sample preparation and triplicate injections for each lot cartridge; 4% pharmalytes (broad:narrow = 20:80) and focused for 12 min.

3.4.8. Sample stability during analysis

The IgG used in the study has p*I* values above 8. It requires the use of high pH range pharmalytes. The pH of sample matrix was measured to be 8.3. Therefore, analytes were exposed to basic pH environment, which leaves them susceptible to deamidation. The stability of the sample in autosampler (2–8 °C) was extrapolated from intermediate precision. A single sample was analyzed within 3 days. The R.S.D. for p*I* values was 0.2 or less and R.S.D. for area percent distribution for acidic, main and basic species was 1.4, 0.6 and 5.7, respectively, within 3 days. This data indicated that the sample is stable within the time frame of the sample analysis.

3.4.9. Stability indicating properties

A key requirement of a method in formulation development is its stability indicating property. Generally, in a formulation development laboratory, active pharmaceutical ingredients are placed in a wide variety of different buffer species and excipients in physiologically acceptable pH ranges and concentrations. The resulting formulations are put in stability chambers in a variety of conditions. Samples are withdrawn at different time intervals and analyzed for detection of new species or increasing amount of degradation products. To that end, this method was evaluated using IgG for its use as stability indicating assay. Fig. 7 is the analysis of IgG under thermal stressed (40 °C) and desired (5 °C) storage conditions for up to 6 weeks. These data show the parent species decreasing and acidic species increasing with

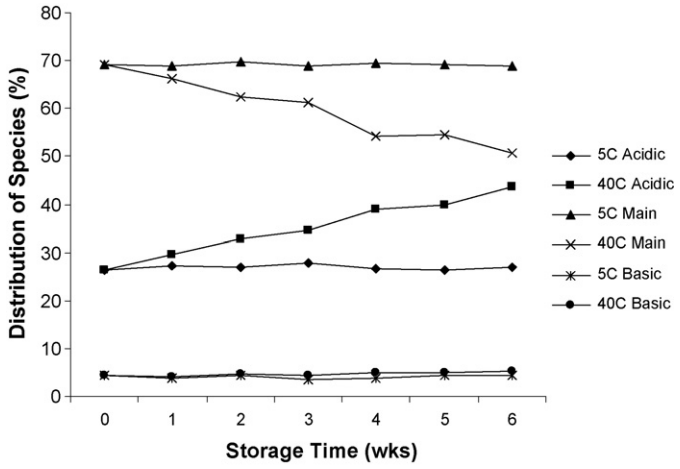

Fig. 7. IgG Stability analyses by iCE 280 Analyzer: IgG was placed in 5 and 40 °C for up to 6 weeks and analyzed at each time point and results indicate that the method is stability indicating. Analysis condition is described in Section 2.

time under stressed condition. Increase in acidic species under stressed condition in solution is consistent with protein deamidation. This was further demonstrated by earlier HPLC/MS analysis of tryptic digest of the stressed sample. Further, the parent species remained unchanged with time under the desired storage condition (5 °C). This demonstrates that the method is stability indicating.

3.5. Technology comparison

Up to now, large number of papers has been published using cIEF as a tool for monitoring the charge heterogeneity of biologically active molecules [1,2,8,9,13,14]. At least two papers reported validation of the cIEF methods for use in GMP environment [11,22]. The majority of the methods were performed using traditional CE instruments with two steps: focusing and mobilization. The advantages of using Imaged cIEF over two step cIEF include: Sample throughput can be increased significantly. Generally, the focusing step after the sample is introduced to the capillary for two step cIEF takes about 5–10 min, but the mobilization step can take from 20 to 30 min [13,14,20,25]. Elimination of mobilization step could at least double the sample throughput. In early candidate selection, pre-formulation, and formulation development, greater than 15 separate formulations are typically evaluated per study under 5 different storage conditions. Thus, at each time point at least 75 samples are pulled and analyzed. By doubling the sample analysis throughput, the analysis time is cut in half. Additionally, elimination of mobilization step would also improve the reproducibility of the method. The detection in most traditional CE instruments for cIEF is at a fixed point towards the outlet of the capillary. The focused protein zones need to be mobilized to pass through the detection window. The mobilization step is generally achieved hydrolytically, chemically, or by pressure. This step is often the cause for loss of resolution and reproducibility [10]. Finally, method development could be simplified significantly and the development time could be shortened. In the past, cIEF method development for two step cIEF needed to optimize the resolution of charge related species first, then different mobilization approaches were applied to see how to push the separated protein zone passing through the detector window without losing the resolution. This is a time consuming process. A review by Harris and Chess [23] stated that more than 80 polypeptide pharmaceuticals are marketed in the United States, and there are an additional 350 polypeptides in clinical trials today. It is clear that simplifying the method development procedure and reducing method development time are a must, especially for the early stages of the project. The iCE280 analyzer software can be operated in a 21 CFR, Part 11 compliant manner and instrument is user friendly. Therefore, it can be operated under GMP and non-GMP conditions by a trained analytical scientist or formulator.

Ion exchange HPLC will continue to be one of the most powerful techniques for analyzing the charge variants of protein molecules. The separation mechanism for cIEF and ion exchange HPLC are based on the same principles. The robustness of HPLC is generally considered better than cIEF. The experiences of scientists working with HPLC are far richer than cIEF because HPLC instruments have been well developed. However, the throughput for Imaged cIEF is better than ion exchange HPLC in many cases. A typical analysis can be done in less than 20 min with Imaged cIEF. HPLC analyses may take longer. This is exemplified by the analysis of Genotropin®. The ion exchange HPLC analysis for the measurement of deamidated species showed in Fig. 6 took 56 min per run; but Imaged cIEF analysis of the same sample took 15 min or less.

4. Conclusion

Separations of charge related heterogeneity of three categories of representative protein molecules in the pharmaceutical industries were investigated using iCE280 Analyzer. The results demonstrated that charge related microheterogeneity of glycosylated and non-glycosylated proteins can be readily resolved. Separation of pegylated protein from its charge related species using iCE280 Analyzer, perhaps including traditional CE instruments as well remains challenging. Even if the peak shape as discussed earlier were acceptable, the band broadening may prevent it from picking up lower levels of degradation products.

The major sources of charge related species were identified using iCE280 Analyzer in conjunction with enzymatic digestion, HPLC, and mass spectrometry techniques. The method was simple and quick. It is well suited for early candidate selection, pre-formulation and formulation development, as well as characterization under limited resources, especially for project stages where no extensive analytical method development and characterization work is performed and understanding degradation pathways to design stable formulations is required.

The limited assay performance evaluation conducted using IgG molecule demonstrated that good linear response, precision, specificity, and robustness were achieved. Limit of detection and limit of quantitation were similar to those reported in the literature [13]. This work supports the use of iCE280 Analyzer in both non-regulated and regulated environments.

The major advantage of using iCE280 Analyzer for cIEF analysis over traditional CE instruments is that method can be

developed easily and quickly as general platform for a class of biomolecules with minimum modification to suit each individual molecule with different p*I*s. Additionally, the operation is simple and throughput is high. This is especially important during early formulation screening where large numbers of solution conditions need to be investigated in a relatively short time.

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