The number of biotechnology products on the market and, particularly, in development has increased enormously during the past decade. Biological products generally are more complex than their small-molecule counterparts. Some product variants — such as deamidated, clipped, aggregated, or misfolded species — could be present in the process stream and even in the final products. Regulatory agencies expect manufacturers to demonstrate their ability to produce an identical product from batch to batch. With the structural complexities of various types of biological products, confirming batch-to-batch reproducibility requires the use of a wide range of analytical tools based upon different separation mechanisms to obtain acceptable characterization of the product. Fortunately, the advent of modern analytical techniques makes this process possible.

Isoelectric focusing is an example of a modern analytical technique often used for monitoring consistency and stability and for quantifying the levels of different variants present in batches of a biotechnology product. In this technique, analytes are separated across a pH gradient formed by carrier ampholytes under the influence of an electric field. Different analytes form tightly focused bands at their isoelectric point, the pH at which their net charge is zero (1). In particular, capillary isoelectric focusing combines the resolving power of isoelectric focusing with advantages of capillary electrophoresis in quantitation and automation, and it has emerged as a very effective and useful technique for assessing charge heterogeneity in protein molecules (2–5).

Santora and co-workers (3) used capillary isoelectric focusing, with a high level of precision, to separate and quantitatively measure monoclonal antibody variants that differed by a single amino acid. Other researchers have reported the validation of capillary isoelectric focusing methods for monoclonal antibodies and protein drug products (2,4,5). They found that method precision, linearity, accuracy, and robustness met target values established to demonstrate the suitability of their assays. These reports indicate capillary isoelectric focusing's high level of reliability as a quantitative analytical method. Scientists have been successful in implementing strategies for adapting International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines to capillary isoelectric focusing analytical methods (2–5). Many of the publications about capillary isoelectric focusing validation focus on assay or identity methods, whereas the validation of capillary isoelectric focusing impurity methods has not been reported widely.

In this “Validation Viewpoint” column, we look at various requirements that must be met for validating a capillary isoelectric focusing method intended for the quantitative measurement of product-related impurities found in production lots of a protein drug substance. The ICH guidelines describe product-related impurities for protein biotechnology products as molecular variants that arise from processing or that appear during storage. Examples of product-related impurities include truncated and aggregated forms and impurities resulting from the chemical modification of amino acids. One amino-acid modification is the deamidation of an asparagine or glutamine residue to aspartate or glutamate, respectively. As such, these impurities are difficult to measure quantitatively by high performance liquid chromatography (HPLC) methods, such as ion exchange, which do not always offer sufficient resolving power to discriminate between these variants. On the other hand, capillary isoelectric focusing is an ideal technique to measure deamidated impurities, because this method offers both the necessary reso-
lution and the performance characteristics required of a quantitative analytical method. The capillary isoelectric focusing impurity method described in this column resolves the parent molecule from two product-related impurities — deamidated and aggregated forms — present in the drug substance. The separation is the result of differences in the charge of the impurities compared with the parent molecule.

General Method Validation Requirements

Guidelines for validation of analytical methods have been published by the U.S. Pharmacopeia (6) and the U.S. Food and Drug Administration (FDA) (7,8), as well as in published reviews (9,10). The tripartite consensus guidelines published by ICH have established a uniform understanding of the performance characteristics that are evaluated in the course of validation (11,12). The subset of performance characteristics that require investigation in the course of validation and the strategy for designing appropriate experiments are based upon the intended purpose of the analytical method. Thus, different validation requirements are outlined by ICH guidelines for assay, impurity, and identity methods (11). Requirements for impurity methods include the estimation of specificity, accuracy, linearity, repeatability, intermediate precision and reproducibility, and limit of quantitation. The importance of each of these elements to the overall validation process can vary depending upon the objective of the method. As a result of the ICH process, USP guidelines have been updated to include ICH terms, definitions, and methodology.

Specificity: The specificity of an analytical method can be defined as its ability to assess unequivocally the analyte in the presence of components that could be present (6,11,12). FDA guidance for method system-suitability requirements state that the peaks should be well-separated for reliable quantitation with a resolution ($R_s$) greater than 2 between the peak of interest and the closest eluted peak (7).

Accuracy: The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a conventional true value, or an accepted reference value and the value found (6,11,12). It is commonly estimated by measuring the recovery of known amounts of the test substance, after spiking into blank matrices. For impurity methods, the recovery typically is measured at three concentrations that span the expected impurity content of a sample (7,8,11,13). In the case of protein impurities, however, it often is impractical to spike the impurity into a blank matrix because spiking usually will result in significant sample loss at low impurity concentrations because of adsorption on contact surfaces such as capillaries and sample vials.

Linearity: The linearity of an analytical procedure is its ability within a given range to obtain test results that are directly proportional to the concentration or amount of analyte in the sample (6,11,12). Often, linearity is assessed by plotting injected mass versus measured area counts or a mass value interpolated from an external standard.

Precision: The precision of an analytical procedure expresses the closeness of agreement or degree of scatter between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions (6,11,12). USP–ICH guidelines indicate three separate levels at which precision should be determined during analytical method validation: repeatability, intermediate precision, and reproducibility (11,12).

Repeatability: Repeatability expresses the precision under the same operating conditions during a short interval of time (6,11,12). Repeatability often is evaluated at three concentrations in triplicate analyses throughout the specified range.

Intermediate precision and reproducibility: Intermediate precision expresses within-laboratory variations such as different days, analysts, and equipment, and reproducibility expresses the precision between laboratories such as collaborative studies, usually applied to standardization of methodology (6,11,12). The simulation of these variable experimental conditions is an integral part of successful analytical method validation. Experiments that evaluate these variable testing conditions can be combined through a matrix design, and analysts can report the cumulative effect of all variables on the consistency of analytical results.

Limit of quantitation: The limit of quantitation of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy (11,12).
Validating a Capillary Isoelectric Focusing Method

Recently, we published a study that presented a successful strategy for applying validation guidelines for impurity quantitation by a capillary isoelectric focusing method (2). In this study, we blended isolated impurity species from cation-exchange HPLC with the drug substance at ratios designed to yield a range of six impurity levels for each of the deamidated and aggregate impurity species. After blending, we calculated the theoretical percent impurity values based upon the protein concentration of the parent protein and impurity species and the mixing ratio of the two species. The ranges of theoretical impurity levels for blended samples were 0.5–12% and 0.5–8% for the deamidated and aggregate impurity species, respectively. We next analyzed these blended impurity samples by a capillary isoelectric focusing method to determine the empirical impurity level based upon the measured area percent for the integrated impurity peak. The method specificity, accuracy, linearity, precision, and limit of quantitation were established from the measurement of the impurity peak area percentage for spiked samples. We established the intermediate precision and reproducibility by repeated analysis of the unspiked drug substance using multiple equipment setups during multiple days in two laboratories.

Specificity: Figure 1a shows an electropherogram obtained from capillary isoelectric focusing analysis of a representative production lot of the drug substance. It demonstrates two minor peaks with longer migration times than the parent peak, which indicate the relatively acidic nature of the impurities. As Figure 1a shows, the impurity peaks are well separated from the parent peak. Figure 1b depicts the separation of three markers with pI values of 6.5, 6.6, and 7.0 using the capillary isoelectric focusing method. The migration times of the pI 6.5 and 6.6 markers differ by more than 10 min, meaning that this method will exhibit good specificity for separation of analytes with pI values between 6.5 and 6.6. Because the parent peak and the impurities for our case migrate in this area of the pI, this method is well suited for this separation.

To determine the identity of the two minor impurity peaks, we degraded the drug substance by exposure to alkaline and acidic pH conditions, respectively. The resulting impurities were isolated using cation-exchange chromatography (2). Independent analysis indicated greater than 95% purity for the deamidated impurity sample and greater than 90% purity for the aggregated impurity sample. As the electropherograms in Figures 2a and 2b show, each of the two isolated impurity species were added separately to the drug substance, which otherwise exhibited them at low levels. The two impurity peaks were augmented individually by the addition of aggregated and deamidated impurity species. The capillary isoelectric focusing profiles of the spiked samples were matched with that of unspiked drug substance (Figure 2c). The two impurity peaks from the unspiked drug substance aligned well with their counterparts in the respective spiked samples. These data establish the identity of these two impurity peaks in drug substance, as aggregated and deamidated forms of the parent molecule. In validating an impurity method, the specificity of the method is proven by adequate resolution between critical components, especially those components with closely related structures (12). The fact that the method could separate the parental protein from impurities with closely related structures confirms its specificity as an impurity method.

Accuracy: In this study, we blended each of the two impurity species separately with the drug substance. Then we calculated the theoretical percent impurity from protein concentration of the parent protein and impurity species and the mixing ratio of the two species. We looked at six impurity levels that were generated throughout the range of interest and determined the recovery of the impurity by comparing the measured area percentage for each impurity peak versus the theoretical percentage impurity. Our thinking was that for an impurity method, the peak area percentage of the impurity species is of more signifi-
cance than the absolute peak area or mass. The latter variable is used more commonly for an assay method.

Tables I and II present data for recovery of the deamidated and aggregated impurity species, respectively. We conducted three capillary isoelectric focusing analyses for each impurity level. The averaged area percentage values for spiked samples were corrected by subtracting from them the peak area percentage values for the unspiked drug substance. We compared the corrected area percentage value with the theoretical percentage impurity (based upon the blending ratio) for each spike level and reported the ratio of these two quantities as the recovery. Data were used to compute repeatability (relative standard deviation [RSD] values) and accuracy (measured area percent versus theoretical percent impurity). For both impurity species, recovery was lowest at the higher impurity levels. For the deamidated impurity, recovery was greater than 90% for the 0.5–2% impurity levels and greater than 85% for impurity levels of 3–12%. For the aggregated impurity, the recovery improved to 97% as the impurity level increased from 0.5% to 3% but then abruptly declined to less than 80% for impurity levels greater than 6%.

Figure 2: Capillary isoelectric focusing analysis of drug substance spiked separately with deamidated and aggregate impurity species. Shown are (a) a control sample with the deamidated species (6%), (b) a control sample with the aggregated species (6%), and (c) the drug substance control. Independent analysis indicated greater than 95% purity for the deamidated impurity sample and greater than 90% purity for the aggregated impurity sample. Method conditions were the same as for Figure 1.
With the exception of the 73% recovery for the highest aggregated impurity level of 8%, the recovery values were greater than 80%.  

**Linearity:** The primary focus for us was quantitative assessment of the impurity percentage. Therefore, we determined the linearity through regression analysis of corrected area percentage values versus the theoretical percent impurity values, at each spike level, for both impurity species (2). As Table III shows, we calculated the coefficients of determination ($r^2$) to be 0.9827 and 0.9994 for aggregated and deamidated impurities, respectively.

### Table I: Measurement of peak area percentage of deamidated species in spiked samples by capillary isoelectric focusing (2)

<table>
<thead>
<tr>
<th>Theoretical Impurity (%)</th>
<th>Average* (%)</th>
<th>RSD (%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>10.46</td>
<td>0.69</td>
<td>87.2</td>
</tr>
<tr>
<td>6</td>
<td>5.12</td>
<td>5.35</td>
<td>85.3</td>
</tr>
<tr>
<td>3</td>
<td>2.56</td>
<td>1.14</td>
<td>85.3</td>
</tr>
<tr>
<td>2</td>
<td>1.93</td>
<td>0.73</td>
<td>96.5</td>
</tr>
<tr>
<td>1</td>
<td>0.93</td>
<td>2.09</td>
<td>93.0</td>
</tr>
<tr>
<td>0.5</td>
<td>0.48</td>
<td>0.76</td>
<td>96.0</td>
</tr>
</tbody>
</table>

* $n = 3$.

### Table II: Measurement of peak area percentage of aggregate impurity species in spiked samples by capillary isoelectric focusing (2)

<table>
<thead>
<tr>
<th>Theoretical Impurity (%)</th>
<th>Average* (%)</th>
<th>RSD (%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>5.84</td>
<td>2.13</td>
<td>73.0</td>
</tr>
<tr>
<td>6</td>
<td>4.84</td>
<td>2.99</td>
<td>80.7</td>
</tr>
<tr>
<td>3</td>
<td>2.92</td>
<td>1.13</td>
<td>97.3</td>
</tr>
<tr>
<td>2</td>
<td>1.85</td>
<td>1.05</td>
<td>92.5</td>
</tr>
<tr>
<td>1</td>
<td>0.84</td>
<td>2.83</td>
<td>84.0</td>
</tr>
<tr>
<td>0.5</td>
<td>0.40</td>
<td>4.75</td>
<td>80.0</td>
</tr>
</tbody>
</table>

* $n = 3$.

### Table III: Summary of the performance of a capillary isoelectric focusing method for impurity quantitation with respect to the various validation attributes (2)

<table>
<thead>
<tr>
<th>Validation Attribute</th>
<th>Deamidated Impurity</th>
<th>Aggregate Impurity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Specificity</strong></td>
<td>Baseline resolution from the parent peak, as in Figure 2 ($R_s &gt; 2$)*</td>
<td>Baseline resolution from the parent peak, as in Figure 2 ($R_s &gt; 2$)*</td>
</tr>
<tr>
<td><strong>Accuracy</strong></td>
<td>Recovery was greater than 90% for 0.5–2% impurity levels and greater than 85% for impurity levels of 3–12%, as in Table I</td>
<td>Recovery increased to 97% as impurity levels went from 0.5% to 3% and then declined to less than 80% for impurity levels greater than 6%, as in Table II</td>
</tr>
<tr>
<td><strong>Linearity</strong></td>
<td>Coefficient of determination ($r^2 = 0.9994$)</td>
<td>Coefficient of determination ($r^2 = 0.9827$)</td>
</tr>
<tr>
<td><strong>Precision</strong></td>
<td>Cumulative RSD value ($n = 12$ analyses) was 18% for the peak area percentage, 26% for the peak area counts, and 0.7% for the relative migration times</td>
<td>Cumulative RSD value ($n = 12$ analyses) was 19% for the peak area percentage, 17% for the peak area counts, and 0.7% for the relative migration times</td>
</tr>
<tr>
<td><strong>Limit of quantitation</strong></td>
<td>Lowest impurity level (RSD &lt; 5%) was 0.5%</td>
<td>Lowest impurity level (RSD &lt; 5%) was 0.5%</td>
</tr>
</tbody>
</table>

* FDA guidance for method system-suitability requirements specify that well-separated peaks, with $R_s > 2$ between the peak of interest and the closest-eluted peak, are essential for reliable quantitation (7).
with minor changes in molecular structure, such as deamidation and aggregation, which can be difficult to achieve with other analytical techniques. The capillary isoelectric focusing method satisfactorily met the various validation criteria, including the precision (repeatability), linearity, limit of quantitation, specificity, intermediate precision, and reproducibility in different testing conditions representative of the method’s life cycle. However, we obtained large RSD values in peak area and peak area percentage after changes in instruments and capillaries.

It follows from Table III that achieving a performance level comparable to that of HPLC methods would require improved precision. All these gaps suggest the need for further improvements in robustness of the capillary isoelectric focusing method. We can conclude on the basis of our results presented here that capillary isoelectric focusing is suitable for impurity quantitation for a recombinant protein sample and that a capillary isoelectric focusing method can be validated using the approach outlined in this “Validation Viewpoint” column.

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The columnists regret that time constraints prevent them from responding to individual reader queries. However, readers are welcome to submit specific questions and problems, which the columnists could address in future columns. Direct correspondence about this column to “Validation Viewpoint,” LCGC, 859 Willamette Street, Eugene, OR 97401, e-mail lcgcedit@lcgcmag.com.