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Journal of Chromatography A, 917 (2001) 147–158

JOURNAL OF CHROMATOGRAPHY A

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Validatibility of a capillary isoelectric focusing method for impurity quantitation

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Received 13 October 2000; received in revised form 15 February 2001; accepted 16 February 2001

Abstract

A strategy is presented for examining the validatability of a capillary isoelectric focusing (cIEF) method, intended for quantitation of product-related impurities in a protein drug substance, according to guidelines published by the International Conference on Harmonization (ICH). The results of this study demonstrate the suitability of cIEF as an analytical method for the quantitation of two product-related impurities in a protein drug substance: a monodeamidated degradation product and an aggregated form of the parent molecule. A range of impurity levels was generated by spiking the isolated impurity species, into a representative production lot of the drug substance. Six impurity spike levels (0.5–12% impurity for deamidated species and 0.5–8% impurity for aggregated species) were analyzed in triplicate. Measurement of impurity peak area percent in the spiked samples provided the data for computing specificity, accuracy, precision, linearity and limit of quantitation (LOQ) for the impurities. Accuracy, defined as the agreement of peak area percent for impurity species with the theoretical impurity percentage from the spike ratio, was 85–96% for the deamidated species and 73–97% for the aggregated species. A linear relationship was found between the measured area percent and the theoretical percent impurity for both impurity species (coefficient of determination, r^2=0.9994 for deamidated species and =0.9827 for aggregated species). Precision (repeatability) studies demonstrated a low relative standard deviation (RSD) value (<6%) at all spike levels for both impurity species. Intermediate precision and reproducibility were evaluated by simulating many of the multivariable testing conditions expected during the life cycle of an analytical method, such as multiple equipment and laboratories. Repeated analyses of the drug substance under these varied conditions, yielded RSD values of <20%, for both impurity species. The LOQ, defined as the lowest impurity level where both accuracy and precision were achieved, was assigned at the 0.5% impurity level for both impurity species. This work illustrates a successful strategy in applying the ICH validation guidelines for impurity analytical methods to a cIEF method. Moreover, the data demonstrate the ability of cIEF to be used reliably as an analytical method for impurity quantitation.

Keywords: Isoelectric focusing; Validation; Pharmaceutical analysis; Proteins

1. Introduction

Isoelectric focusing (IEF) allows the separation of proteins across a pH gradient, formed by carrier ampholytes under the influence of an electric field. Protein molecules form tightly focused bands at their

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0021-9673/01/$ – see front matter
PII: S0021-9673(01)00662-8

isoelectric point, the pH at which their net charge is zero [1]. The high resolution offered by this technology has led to the accepted belief that IEF is the most effective technique in assessing charge heterogeneity in protein molecules [2]. The use of slab gels for IEF, however, is known to suffer from a lack of accuracy and reproducibility for quantitative measurement of the separated charged components. On the other hand, capillary IEF (cIEF) has proven its ability to combine the resolving power of IEF with advantages of capillary electrophoresis (CE) in quantitation and automation [2–8]. The ease in blending narrow and broad range ampholytes allows the method to focus proteins with minimal isoelectric point (p*I*) differences, into tight discrete zones, in a relatively short time [4,5]. Focused proteins are forced across a detection window by either chemical mobilization or hydrodynamic force, without a loss in resolution [5]. The quantitative characteristics of cIEF are provided by on-line detection via UV or other modes, and computerized electronic data analysis. All of the above characteristics allow cIEF to function as a highly quantitative and reproducible analytical method. As an example, separation and quantitative measurement of monoclonal antibody variants, differing by a single amino acid, has been accomplished by cIEF, with a high level of precision [6]. More extensive validation of cIEF methods, for monoclonal antibodies and protein drug products, has been reported, in which the method precision, linearity, accuracy and robustness were shown to meet target values established to demonstrate the suitability of the assay [7,8]. Recently, a high efficiency cIEF method has been shown to be useful for a rapid resolution of peptides with the minimum resolvable p*I* difference of ~0.01 [3]. These reports indicate the high level of reliability for the quantitative abilities of cIEF-based analytical methods.

Analytical method validation in pharmaceutical analysis is a key requirement for commercial manufacturing and registration of the product. It determines that the performance characteristics of a method meet the requirements of its intended analytical application [9]. Guidelines for validation of analytical methods have been published in the United States Pharmacopeia [9], by the US Food and Drug Administration (FDA) [10,11], and in published reviews [12–15]. The tripartite consensus guidelines published by the International Conference on Harmonization (ICH) have established a uniform understanding of the performance characteristics which are evaluated in the course of validation [16,17]. The subset of performance characteristics which require investigation in the course of validation, as well as the strategy for designing appropriate experiments, are based upon the intended purpose of the analytical method. Thus, different validation requirements are outlined by the ICH guidelines for each of assay, impurity and identity methods [16]. Strategies for adapting the ICH guidelines to cIEF analytical methods have been successfully implemented [6–8]. Much of the published literature on cIEF validation focuses on assay or identity method, whereas validation of cIEF impurity methods has not been widely reported.

This report describes studies that examine the validatability of a cIEF method for quantitative measurement of product-related impurities found in production lots of a protein drug substance. Product-related impurities for protein biotechnology products are described in the ICH guidelines as molecular variants which arise from processing or during storage [18]. Examples of product-related impurities include truncated and aggregated forms, as well as, impurities resulting from chemical modification of amino acids. One such amino acid modification is the deamidation of an asparagine or glutamine residue to aspartate and glutamate, respectively [19]. Monodeamidated impurities differ by a single amino acid from the parent molecule. As such, these impurities are difficult to quantitatively measure by high-performance liquid chromaography (HPLC) methods, such as ion exchange, which do not always offer sufficient resolving power to discriminate between these variants. On the other hand, cIEF is an ideal technique to measure deamidated impurities, since this method offers both the necessary resolution as well as the performance characteristics required of a quantitative analytical method. The cIEF impurity method described in this report resolves the parent molecule from two product related impurities, deamidated and aggregated forms, present in the drug substance. The separation is due to the differences in the charge of the impurities compared to the parent molecule.

The protein molecule used in this study was a

myelopoietin (MPO) of molecular mass ≈35 000 [20]. Myelopoietins are a family of engineered dual interleukin-3 (IL-3) and granulocyte colony-stimulating factor (G-CSF) receptor agonists that are superior in comparison to the single agonists in their ability to promote the growth and maturation of hematopoietic cells of the myeloid lineage. The experiments described here were designed to determine the suitability of the cIEF method for quantitative measurement of each impurity species related to the myelopoietin drug substance. Accordingly, the performance characteristics which were investigated in the course of this study were those as mentioned in the ICH guidelines for impurity methods (specificity, accuracy, precision, linearity and quantitation limit) [16]. Therefore, this work presents a strategy for adapting the ICH guidelines for quantitative impurity methods to a cIEF technique.

2. Experimental

2.1. Materials

For cation-exchange HPLC, sodium phosphate monobasic was purchased from Sigma (St. Louis, MO, USA, catalog No. S-9683). Sodium chloride was obtained from J.T. Baker (Phillipsburg, NJ, USA, catalog No. 3624-01). ProPacWCX-10 weak cation-exchange columns were purchased from Dionex (Sunnyvale, CA, USA, catalog No. 54993). Centriplus centrifugal ultrafiltration devices (10 000 M_r cut-off membrane) were purchased from Millipore (Bedford, MA, USA, catalog No. 4421). Dialysis Spectra/Por membrane tubing (12 000–14 000 M_r cut-off) was purchased from Spectrum Medical Industries (Houston, TX, USA, catalog No. D1615-4).

For cIEF separations, anolyte, catholyte and cathodic mobilizing solutions, were purchased from Bio-Rad Labs. (Hercules, CA, USA, catalog Nos. 148-5029, 148-5028, 148-5030, respectively). The following ampholyte solutions were purchased from Amersham Pharmacia (Piscataway, NJ, USA): Ampholine, p*I* 5.0–7.0, 0.4 g/ml (catalog No. 80-1125-91), Ampholine, p*I* 6.0–8.0, 0.4 g/ml (catalog No. 80-1125-93), Pharmalyte, p*I* 5.0–8.0, 0.36 mequiv./ml pH (catalog No. 17-0453-01), Pharmalyte, p*I* 3–10, 0.36 mequiv./ml pH (catalog No. 17-0456-01), Ampholine, p*I* 3.5–10.0, 0.4 g/ml (catalog No. 80-1125-87). Synthetic p*I* markers (pI 7.0, 6.6 and 6.5) were purchased from Bio-Rad Labs. (catalog Nos. 148-2105, 148-2106, and 148-2107). Methylcellulose was purchased from Sigma (catalog No. M-0512). Neutral capillaries were purchased from Beckman Coulter (Fullerton, CA, USA, eCAP capillaries, catalog No. 47441) and MicroSolv (Eatontown, NJ, USA, Zeroflow capillaries, catalog No. 04650-ZF).

2.2. Reagent preparation

2.2.1. Methylcellulose

A 0.65% (w/v) methylcellulose solution (MC) was prepared by adding 0.65 g of methylcellulose to 100 ml deionized water which was pre-warmed to 80°C. The solution was gently mixed at room temperature for 5 min and then under cold running water for another 5 min. This was followed by mixing for 2 h at 2–8°C and then for 1 h at room temperature using a magnetic stir bar. Finally, the solution was passed through a 0.45-μm filter, Corning Science Products (NY, USA, catalog No. 430768).

2.2.2. Ampholyte mixtures

Broad and narrow range mixtures were initially prepared separately. The mixture of broad range ampholytes contained a 1:1 ratio of Pharmalyte, p*I* 3–10, and Ampholine, p*I* 3.5–10. The mixture of narrow range ampholytes contained a 1:1:1 ratio of Pharmalyte, p*I* 5.0–8.0, Ampholine, p*I* 5.0–7.0, and Ampholine, p*I* 6.0–8.0.

Ampholyte solution (broad:narrow ratio of 3:7) was prepared by combining 12.5 μl of mixed broad range ampholytes, 29.2 μl of mixed narrow range ampholytes and 500 μl of 0.65% MC solution (2× ampholyte–MC mixture). The solution served as a diluent to dilute protein samples from an initial concentration of 3–5 mg/ml, to a working concentration of 0.25 mg/ml. The dilution of protein, in preparation for cIEF analysis, was achieved by combining 50 μl of the 2× ampholyte–MC mixture with an aliquot of concentrated protein solution, calculated to achieve a final 0.25 mg/ml protein concentration in a 100 μl working volume. Deion-

ized water was used to adjust the final volume to 100 μl. Blank samples contained equal volumes of water and 2× ampholyte–MC mixture, with no protein addition. The p*I* marker solutions (3%, v/v) were prepared by adding 3 μl of each concentrated p*I* marker, 50 μl of 2× ampholyte–MC mixture and deionized water was used to adjust the final volume to 100 μl.

2.2.3. Preparation of degraded samples

A highly deamidated form of drug substance was prepared by maintaining a drug substance sample (pH 8.2) at 2–8°C for >6 months prior to isolation of the deamidated impurity peak. A highly aggregated form of the drug substance was prepared by dialyzing drug substance sample overnight at 2–8°C against 10 m*M* sodium acetate solution, pH 4.3 through a 12 000–14 000 M_r cut-off membrane in a polypropyelene beaker. The degraded forms of the drug substance (deamidated or aggregate impurity), were isolated by cation-exchange HPLC.

2.3. Cation-exchange HPLC procedure

Cation-exchange HPLC was performed with a Perkin-Elmer quaternary pump (Model 410), autosampler (Model ISS-200) and UV–Vis detector (Model 785A). The ProPac WCX-10 column (25 cm×4.0 mm I.D.) was equilibrated with 25 m*M* sodium phosphate buffer, pH 6.0. Protein (40–300 μg) was injected and eluted with a 0–188 m*M* sodium chloride gradient over 25 min. Emerging peaks were detected at 214 nm and collected. Deamidated or aggregate impurity fractions, isolated from cation-exchange HPLC, were concentrated and buffer exchanged into 10 m*M* sodium acetate buffer at pH 4.3 with Centriplus ultrafiltration devices (10 000 M_r cut-off). Protein determination of the fractions was performed by UV absorbance (at 280 nm).

2.4. cIEF procedure

Isolated impurity species from cation-exchange HPLC, were blended with the drug substance, at ratios designed to yield a range of six impurity levels for each of the deamidated and aggregate impurity species, respectively. After blending, theoretical percent impurity values were calculated based on 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. These blended impurity samples were then analyzed by the cIEF method to determine the empirical impurity level, based upon the measured area percent for the integrated impurity peak. The inclusion of p*I* marker in each cIEF analysis allowed for alignment and, thus, provided a clearer comparison of electropherograms from different analytical runs as the spiked peaks of same impurity in the blended samples had identical relative migration times.

A Model P/ACE MDQ capillary electrophoresis unit with a UV–Vis detector (Beckman Coulter) was used for cIEF. Capillaries were either eCAP neutral capillary (Beckman Coulter) or Zeroflow capillary (MicroSolv), of 40.2 cm (30 cm to detector)×50 μm I.D.

The following steps were programmed using the P/ACE System MDQ software (version 2.3, Beckman Coulter) to operate the MDQ instrument for each cIEF run. Prior to each analysis, the capillary was rinsed with anolyte solution and then by deionized water at 30 p.s.i. for 3 min per rinse (1 p.s.i.= 6894.76 Pa). The capillary was then filled with protein or blank samples using a pressure rinse (20 p.s.i. for 3 min). In addition, a pressure injection (5 p.s.i. for 5 s) of a p*I* marker (p*I* 6.5, 3%, v/v, in ampholyte solution) followed the sample or blank injection. Focusing was accomplished by applying a constant voltage of 25 kV for 20 min, from anolyte to catholyte solution (normal polarity). Chemical mobilization was performed at 25 kV for another 30 min, from anolyte to cathodic mobilizing solution. Detection was at 280 nm. Capillary cartridge temperature was set at 25°C and the sample tray temperature was set at 12.5°C.

Data from each cIEF run were analyzed via the P/ACE System MDQ software. The following parameters were routinely reported: peak area counts (area units under each integrated peak), peak area percent (ratio of area counts for each respective integrated peak and the sum of the area counts of all integrated peaks, multiplied by 100) and relative

migration time (ratio of the migration time for each respective integrated peak and the migration time of the reference p*I* 6.5 marker).

3. Results and discussion

3.1. Overview of experimental strategy

Previous work has demonstrated the utility of cIEF as a method for verifying the identity, as well as providing quantitative analysis, of protein therapeutic reagents [6,7]. The work reported here demonstrates the use of cIEF as a method for quantitative analysis of product-related impurities for a protein drug substance. According to the ICH guidelines topics Q3A and Q3B, analytical impurity methods are used to determine the acceptability of manufactured drug batches against the impurity specification of the drug substance or drug product [21]. Thus, validation of these analytical methods serves to establish their suitability for quantitation of the impurities for the commercial release of manufactured product. The selection of impurities to be included in drug substance or drug product specifications is based upon the profile of typical impurity species observed in manufacturing batches. Thus, validation of these procedures should focus on the quantitation of these impurity species. The cIEF assay reported here, accordingly, focuses on the quantitation of two defined impurity peaks, present in the drug substance. To examine this method's validatability for such quantitation, these impurity species were isolated, identified and then spiked into the drug substance. Triplicate cIEF analyses were performed for each spike level, for both impurity species. The method specificity, accuracy, linearity, precision, and limit of quantitation (LOQ) were established from the measurement of the impurity peak area percent for spiked samples. Intermediate precision and reproducibility were established by repeated analysis of the unspiked drug substance, using multiple equipment set-ups over multiple days in two different laboratories.

3.2. Specificity

Fig. 1A illustrates an electropherogram obtained from cIEF analysis of a representative production lot of the drug substance and demonstrates two minor peaks with longer migration times than the parent peak. This indicates that the impurities are more acidic than the parent molecule. FDA guidance for method system suitability requirements specify that well-separated peaks, with resolution, R_s, >2 between the peak of interest and the closest eluted peak, are essential for reliable quantitation [10]. Each of the two impurity peaks in the drug product cIEF profile met this specification for ''well-separated peaks'', which can also be visibly confirmed from the figure inset. A p*I* marker (p*I* 6.5) was routinely

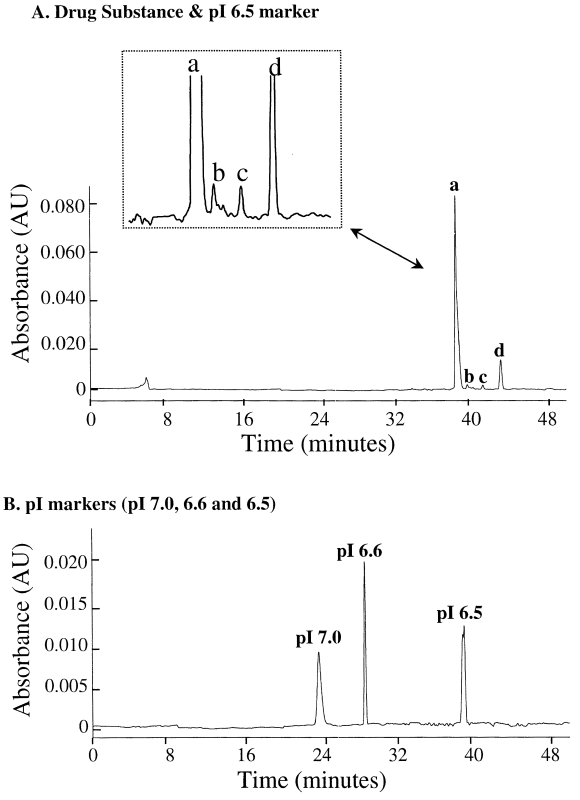

Fig. 1. Impurity profile of a representative drug substance lot by cIEF in a neutral capillary (A) and separation of markers with p*I* values of 6.5, 6.6 and 7.0 (B). Peaks in (A): a=parent molecule, b=aggregated species, c=deamidated species, d=p*I* 6.5 marker. Method conditions: sample concentration in (A), 0.25 mg/ml; injection length for p*I* marker, 5 s in (A) and 9 s in (B); injection pressure in p*I* marker, 5 p.s.i. in (A) and 9 p.s.i. in (B); capillary dimensions, 30 cm×50 μm I.D.; focusing time, 20 min; mobilization time, 30 min; focusing and mobilization voltage, 25 kV.

co-injected with protein samples in order to align electropherograms from different analyses. The p*I* marker peak (peak d in Fig. 1A) was observed following the second impurity peak.

Fig. 1B demonstrates the separation of three markers having p*I* values of 6.5, 6.6, and 7.0, using the cIEF method. Capillary was filled with a ''blank'' sample, containing ampholytes and deionized water in a 1:1 mixture. The ''blank'' sample was co-injected with a solution containing all three p*I* markers. The migration times of the p*I* 6.5 and 6.6 markers differed by more than 10 min. Accordingly, analytes with p*I* values between 6.5 and 6.6 would likely have excellent specificity with this method. Separation of analytes with minimal p*I* differences is achievable by the use of narrow p*I* range ampholytes [4] and this strategy was followed in the cIEF method described (see Section 2.2.2). Thus, the experimental conditions for this method were well-suited for separating the parental peak and two impurity peaks, which migrated between the p*I* 6.5 and 6.6 markers.

Evidence that these two minor peaks represent impurity species was provided from spiking the drug substance with impurities which were isolated by cation-exchange HPLC. Fig. 2A–C display cation-exchange HPLC chromatograms of a representative drug substance lot as well as degraded samples containing elevated levels of two impurity peaks. These degraded samples were created by exposure to alkaline and acidic pH conditions, respectively. The major impurity species found in the degraded samples were isolated by the same cation-exchange method and identification of the isolated species was provided by orthogonal methods. Independent analysis indicated >95% purity for the deamidated impurity sample and >90% purity for the aggregated impurity sample. In Fig. 2A, the identity of the impurity isolated from the base-degraded sample was found by Edman sequencing to be a monodeamidated parent molecule and the impurity isolated from the acid-degraded sample in Fig. 2B was identified through size-exclusion HPLC to be an aggregated form of the parent molecule (data not shown).

As shown by the cIEF electropherograms in Fig. 3A and B, each of the above two isolated impurity species were separately added to the drug substance, which otherwise exhibited them at low levels. The

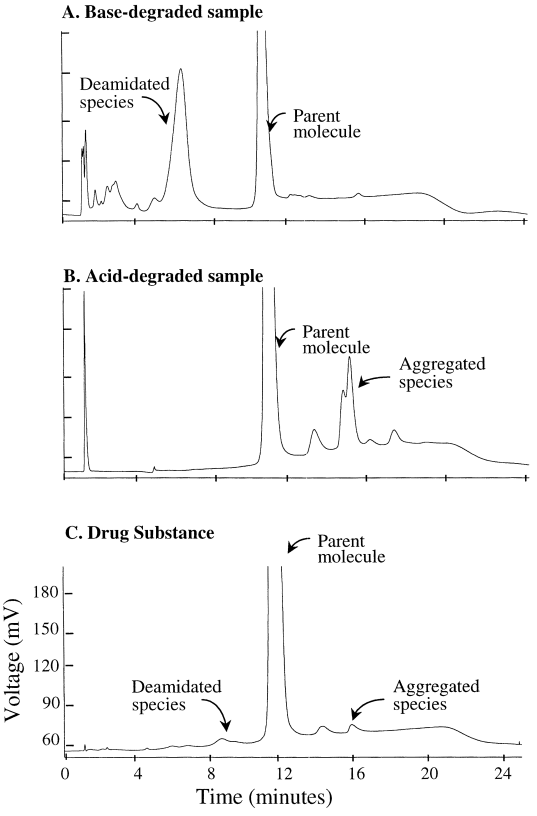

Fig. 2. Cation-exchange HPLC analysis of drug substance degradation products. Protein load, 40 μg; column, Dionex WCX-10, 25 cm×4.0 mm I.D.; gradient, 0–188 m*M* NaCl in 25 min; column buffer, 25 m*M* sodium phosphate, pH 6.0. Degraded samples (A and B), were prepared by exposure to alkaline and acidic conditions, respectively. (C) is the drug substance control sample. *x*- and *y*-axis scales are same for figures (A)–(C).

two impurity peaks were individually augmented by the addition of aggregated and deamidated impurity species. The cIEF profiles of the spiked samples were matched with that of unspiked drug substance (Fig. 3C). The two impurity peaks from the unspiked drug substance were found to align well with their counterparts in the respective spiked samples. These data therefore 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 with closely related structures (ICH Topic Q2B). The fact that the cIEF method can separate the parental protein from im-

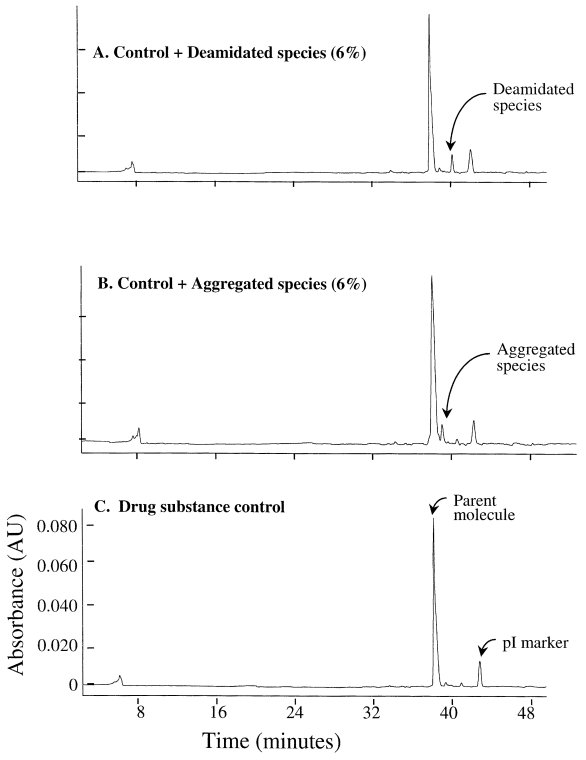

Fig. 3. Analysis by cIEF of drug substance spiked separately with deamidated and aggregate impurity species. Independent analysis indicated >95% purity for the deamidated impurity sample and >90% purity for the aggregated impurity sample. *x*- and *y*-axis scales are same for figures (A)–(C). Method conditions as in Fig. 1.

purities with closely related structures ascertains its specificity as an impurity method.

3.3. Accuracy

A widely adopted approach for determining accuracy of a method is by measuring the recovery of known amounts of the test substance, after spiking into blank matrices. For impurity methods, recovery is typically measured at three concentrations that span the expected impurity content of a sample [10,11,16,20]. In the case of protein impurities, however, it is often impractical to spike the impurity into a blank matrix since this will usually result in significant sample loss at low impurity concentrations due to reasons such as adsorption on the different surfaces of contact like the capillary, sample vial, etc. Therefore, to determine the accuracy for the cIEF impurity method, each of the two impurity species was separately blended with the drug substance. After blending, a theoretical percent impurity was calculated based on the protein concentration of the parent protein and impurity species, and the mixing ratio of the two species. In this study, six impurity levels were generated through this blending strategy over a range which encompassed the specification levels for product-related impurities that were published internally by the quality assurance group. A similar approach for spiking has been used for measuring trace enantiomer content in pharmaceuticals [22]. Recovery of the impurity was determined by comparing the area percent for each impurity peak versus the theoretical percent impurity. This calculation of accuracy for an impurity method is quite different from that of assay methods, which generally examine area or mass recovery. For the latter type of method, the strength of the parental molecule is the primary focus and so recovery is usually determined by comparing the empirical mass of active pharmaceutical ingredient interpolated from its peak area to its known mass by weight. However, for an impurity method, the percentage of impurity species relative to the parental species is the critical information required in order to release a manufactured product against an impurity specification. Therefore, the peak area percent of the impurity species, rather than the absolute peak area (or mass), is of more significance. Thus, accuracy testing for an impurity method is designed to rule out a deviation of the empirical peak area percent from the theoretical percent impurity in the spiked sample. Such deviations would often result from tailing of parental peak into the much smaller impurity peak or when the signal of the impurity species approaches baseline noise.

The data for recovery of the deamidated and aggregated impurity species are presented in Table 1. Six impurity spike levels were generated by blending the deamidated or aggregated impurity species with the drug substance. Three cIEF analyses were performed for each impurity level. The averaged area percent values for spiked samples were corrected by subtracting from them the peak area percent values for the unspiked drug substance. The corrected area percent value was compared to the theoretical per-

Table 1
Measurement of peak area percent of deamidated and aggregate impurity species in spiked samples by cIEF

Theoretical impurity (%)	Average ($n=3$)	RSD (%)	Recovery (%)
	(A) Area percent measurement for deamidated impurity		
12	10.46	0.69	87.2
6	5.12	5.35	85.3
3	2.56	1.14	85.3
2	1.93	0.73	96.5
1	0.93	2.09	93.0
0.5	0.48	0.76	96.0
	(B) Area percent measurement for aggregate impurity		
8	5.84	2.13	73.0
6	4.84	2.99	80.7
3	2.92	1.13	97.3
2	1.85	1.05	92.5
0.5	0.40	4.75	80.0

cent impurity (based on the blending ratio) for each spike level and the ratio of these two quantities was reported as recovery. Data were used to compute repeatability (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 >90% for the 0.5–2% impurity levels, and >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%, and then abruptly declined to <80% for impurity level >6%. With the exception of the 73% recovery for the highest aggregated impurity level of 8%, the recovery values were >80%.

3.4. Linearity

Linearity is defined by the ICH guidelines as the ability of the assay to obtain results proportional with the amount of analyte in the sample [16]. Often, linearity is assessed by plotting injected mass versus measured area counts (or a mass value interpolated from an external standard). In our case, the primary focus was quantitative assessment of the percent impurity. Therefore, linearity was determined through regression analysis of corrected area percent values versus the theoretical percent impurity values, at each spike level, for both impurity species. The data are plotted in Fig. 4A and B and the coefficients of determination, r^2, were calculated to be $=0.9827$ and $=0.9994$ for aggregated and deamidated impurities, respectively.

3.5. Precision

Precision is the measure of the closeness of results among repeated analyses under normal operating conditions [13,16]. ICH guidelines indicate three separate levels at which precision should be determined during analytical method validation: repeatability, intermediate precision and reproducibility [17]. Repeatability reflects the closeness of results among repeated measurements over a short time interval, under the same conditions. In testing intermediate precision, one introduces additional laboratory variations such as repeated analyses on different days, different instruments, HPLC columns or CE capillaries, or by different analysts. Reproducibility is a measure of precision between analyses performed in different laboratories. Precision is usually expressed as the RSD for the set of repeated analyses [13].

3.5.1. Repeatability

The assessment of method precision for impurity methods is generally identical to that of assay methods. Repeatability, the closeness of results between repeated analyses, is often evaluated at three concentrations, in triplicate analyses, over the specified range (ICH Topic Q2B) [17]. In this work, repeatability was assessed by calculating the RSD of

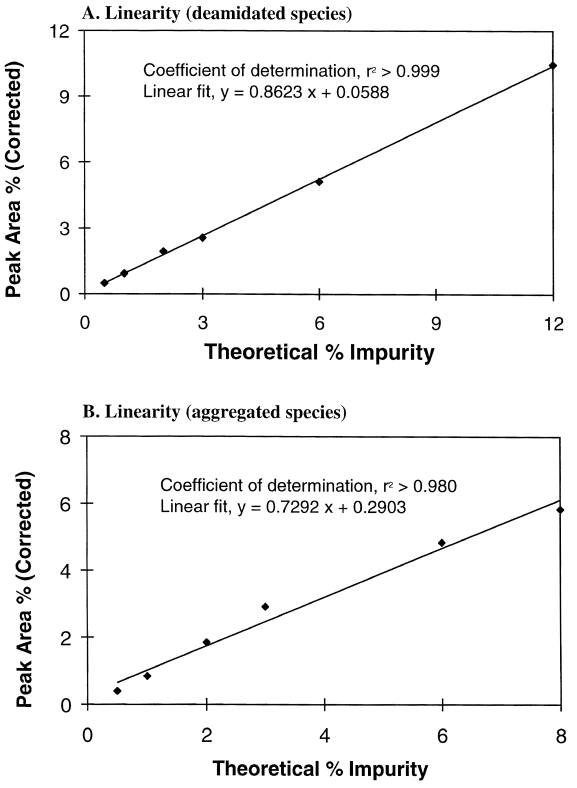

Fig. 4. Evaluation of linearity for a range of six impurity spike levels, for each of two impurity species. Drug substance was spiked with impurity species to create a range of impurity levels (theoretical % impurity: deamidated impurity, 0.5–12; aggregated impurity, 0.5–8). The theoretical % impurity values were plotted against empirical area percent values (averages of triplicate analyses). Area percent values were corrected by subtraction of area from unspiked drug substance. Method conditions as in Fig. 1.

the peak area percent, from triplicate analyses of each spiked sample. These calculations were performed for six spike levels of aggregated and deamidated impurity species. The data presented in Table 1 for the aggregated and deamidated impurity species demonstrate RSD values of <6% for the peak area percent for all spike levels.

3.5.2. Intermediate precision and reproducibility

Evaluation of both intermediate precision and reproducibility is intended to demonstrate that the normal experimental variabilities, which are expected in production environments, will not adversely affect the method's performance. Simulation of these variable experimental conditions is therefore an integral part of analytical method validation. A successful validation, achieved by demonstrating the closeness of analytical results across these variable conditions, establishes the suitability of the method for multiple personnel, equipment, over an extended time period (intermediate precision) and in multiple laboratories (reproducibility) [12–14,17]. Experiments evaluating these variable testing conditions can be combined through a matrix design and the cumulative effect of all variables on the consistency of analytical results is then reported. Therefore, in this work, repeated testing of a drug substance in multiple equipment, capillaries, test dates and laboratories, was conducted in a matrix design and the data are presented in Tables 2 and 3.

Table 2 lists the intermediate precision evaluation for deamidated and aggregated impurity species. Analysis of the drug substance by cIEF was conducted in triplicate on each of 3 days using three separate capillaries. Two Beckman eCAP neutral capillaries from one vendor lot and one capillary from a second lot were used. Averages and RSD values for peak area percent are reported for the triplicate analyses set per day (i.e., per capillary). In addition, cumulative average and RSD values are reported for all analyses ($n=9$) for each impurity species, as a measure of intermediate precision. The cumulative RSD value was obtained from calculating an average and standard deviation for the individual nine replicate results. For each individual triplicate set, the RSD values for peak area percent, was <4% for each impurity species. For the combined group of nine analyses, RSD values of <11% and <15% were obtained for the deamidated and aggregated impurity species, respectively. These cumulative RSD values quantitatively illustrate the intermediate precision performance of the cIEF impurity method.

Table 3 presents the reproducibility calculation for the deamidated and aggregated impurity species. cIEF analyses of drug substance were conducted on two CE instruments, using two separate capillaries per instrument. In addition, these experiments also evaluated different capillary lots as well as capillary vendors, yielding a total of four experimental conditions ($n=12$ replicates, due to triplicate analyses per condition). Standard deviation (SD) and RSD values for peak area percent and peak area counts are

Table 2
Intermediate percision evaluation for determidated and aggregate impurity species

Experimental variation		Peak area percent value				
		Replicate 1 (n=3)	Replicate 2 (n=3)	Replicate 3 (n=3)	Within-day average	Within-day RSD (%)
Deamidated impurity						
Day 1	Lot 1 capillary a	1.45	1.49	1.48	1.45	1.56
Day 2	Lot 1 capillary b	1.51	1.51	1.57	1.53	2.26
Day 3	Lot 2 capillary c	1.20	1.19	1.26	1.22	3.11
Cumulative (n=9): average peak area %=1.41 and RSD=1.050%						
Aggregate impurity						
Day 1	Lot 1 capillary a	2.10	2.11	2.16	2.12	1.51
Day 2	Lot 1 capillary b	2.71	2.71	2.70	2.71	0.21
Day 3	Lot 2 capillary c	2.02	1.99	1.97	1.99	1.26
Cumulative (n=9): average peak area %=2.27 and RSD=14.49%						

Lots 1 and 2: Beckman eCAP neutral capillaries (two production lots).

reported for each triplicate experimental set and cumulative values are reported for all analyses (n=12), as a measure of reproducibility, for each impurity species. In addition, Table 3 also displays the SD and RSD values for peak area counts and relative migration time (RMT), defined as relative to the migration time for the p*I* 6.5 marker, for both the deamidated and aggregated impurity species. For peak area percent, a cumulative RSD value (for n=12 analyses) of <20%, was obtained, for both impurity species. For peak area counts, the RSD values, within experimental sets (n=3), was <11%. A much higher cumulative RSD value (>25%) for peak area counts (for deamidated impurity) is due to the use of multiple instruments and capillaries, which inherently produce divergent UV signal. The peak area percent value is a more useful index of precision as it is not influenced significantly by multiple experimental conditions. In addition, percent impurity is the reported endpoint information for an

Table 3
Reproducibility calculation for deamidated and aggergate impurity species

Experimental variation Instrument No.	Capillary	Area percent (n=3)		Area counts (n=3)		Relative migration time (n=3)	
		Mean±SD	RSD (%)	Mean±SD	RSD (%)	Mean±SD	RSD (%)
(A) Peak area percent, peak area count and relative migration time for deamidated impurity							
1	Lot 1 Capillary a	1.58±0.08	5.26	19 371±1809	9.34	0.94±0.00	0.11
	Lot 2 capillary b	1.54±0.01	0.75	15 228±268	1.76	0.95±0.00	0.12
2	Lot 2 capillary c	2.14±0.03	1.40	29 309±577	1.97	0.96±0.00	0.12
	Lot 3 capillary d	2.26±0.03	1.11	26 257±113	4.31	0.96±0.00	0.00
	Cumulative (n=12)	1.88±0.34	18.01	22 541±5872	26.05	0.95±0.01	0.69
(B) Peak area percent, peak area count and relative migration time for aggregate impurity							
1	Lot 1 capillary a	2.76±0.20	7.26	33 817±2493	10.33	0.91±0.00	0.17
	Lot 2 capillary b	2.41±0.07	2.90	23 758±888	3.74	0.92±0.00	0.19
2	Lot 2 capillary c	1.75±0.17	9.44	23 952±2353	9.82	0.93±0.01	0.59
	Lot 3 capillary d	2.89±0.04	1.40	26 257±1131	4.31	0.93±0.00	0.06
	Cumulative (n=12)	2.45±0.48	19.42	26 946±4671	17.34	0.92±0.01	0.65

Lots 1 and 2: Beckman eCAP neutral capillaries (two production lots). Lot 3: Micro Slov zero flow column. RMT: Relative migration time, by comparison to the migration time of the p*I* 6.5 marker. Instruments 1 and 2 are located in different laboratories.

impurity assay and so it is most relevant to assess the closeness of peak area percent values when validating method precision. The cumulative RSD value for RMT was <1% (n=12) even with the use of three capillary lots and two different vendors. These data underscores the importance of including a p*I* marker as a migration time reference peak, since absolute peak migration times were observed to shift as much as 3 min between days (data not included).

The data presented in Tables 2 and 3 demonstrate a hierarchy of performance variance. The lowest RSD values were seen within repeated analyses on a single capillary, intermediate RSD values were obtained between capillaries (Table 2) and the highest RSD values were obtained between instruments (Table 3). These findings give credence to the ICH organization for precision studies, which distinguishes between repeatability, intermediate precision and reproducibility [16,17].

3.6. LOQ

In the ICH guidelines, the methods for calculating the LOQ for an analyte, are delineated [17]. The LOQ can be determined by examining the lowest analyte level for which suitable accuracy and precision is obtainable, through signal-to-noise measurements, or from a ratio of the standard deviation of the response and the slope of the calibration curve. The former most definition of the LOQ was followed in this study. In the case of the cIEF method, acceptable accuracy and repeatability were obtained for both impurity species at all impurity levels except the 8% aggregated impurity level which yielded low recovery values (<75%). Therefore, the lowest impurity level tested (0.5%), was assigned as the LOQ.

4. Conclusions

Validatability of a cIEF method for impurity quantitation was examined by spiking product-related impurities into the drug substance at multiple impurity levels. Table 4 summarizes the performance of the method with respect to the various validation attributes outlined in the ICH guidelines.

The method presented here demonstrates accurate quantitation of low levels of impurities with minor changes in molecular structure, such as deamidation and aggregation, which may be difficult to achieve with other analytical techniques. It is evident that the method performance demonstrates satisfactory precision (repeatability) and linearity for each impurity species. The LOQ was determined at the 0.5% impurity level and specificity was shown with regard to two different impurity species. Intermediate precision and reproducibility studies with the drug substance, demonstrated consistent performance over different testing conditions, representative of the life cycle of the method.

Although the method satisfactorily met the various validation criteria, large RSD values in peak area and peak area percent were obtained upon changes in instruments and capillary manufacturers. Further, it follows from Table 4 that to achieve a performance

Table 4
Summary of the performance of a cIEF method for impurity quantitation with respect to the various validation attributes

Validation attribute	Performance	
	Deamidated impurity	Aggregate impurity
Specificity	Baseline resolution from the parent peak as in Fig. 3 (R_s>2)[a]	Baseline resolution from the parent peak as in Fig. 3 (R_s>2)[a]
Accuracy	Recovery was >90% for 0.5–2% impurity levels and >85% for impurity levels of 3–12% in Table 1	Recovery increased to 97% as impurity levels went from 0.5 to 3% and then declined to <80% for impurity levels >6% as in Table 1
Linearity	Coefficient of determination, r^2=0.9994	Coefficient of determination, r^2=0.9827
Precision	Cumulative RSD value (for n=12 analyses) was 18% for the peak area percent, 26% for the peak area counts and 0.7% for the relative migration times as shown in Table 3	Cumulative RSD value (for n=12 analyses) was 19% for the peak area percent, 17% for the peak area counts and 0.7% for the relative migration times as shown in Table 3
LOQ	Lowest impurity level (with RSD <5%) was 0.5%.	Lowest impurity level (with RSD <5%) was 0.5%.

[a] FDA guidance for method system suitability requirements specify that well-separated peaks, with resolution, R_s, >2 between the peak of interest and the closest eluted peak, are essential for reliable quantitation [10].

level comparable to that of HPLC methods, an improved precision would be required. All these gaps suggest the need for further improvements in robustness of the cIEF method.

It can be concluded on the basis of results presented here that this cIEF method is suitable for impurity quantitation for a recombinant protein sample. Further, it is shown that the ICH validation criteria can be determined and, based on our experiences with other impurity methods, this cIEF method would be validatable for most situations.

Acknowledgements

The authors would like to thank R.L. Dufield, J.R. Borgmeyer and C.S. Adams for their help in impurity identification, R.E. Shell and S.I. Allen for their help in impurity isolation, J.J. Buckley for his help in cation-exchange method development, and G.S. Bild and R.M. Leimgruber for helpful discussions.

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