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Analytical Method Validation for Biopharmaceuticals, Part 4

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This fourth and final installment of the series, beginning in section 4.3.

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This contribution, because of its length, will appear in approximately, six, consecutive issues of JVT, in unequal installments, every other month, but will use the same references and Figure numbers/captions throughout. Each installment, as for the rest of each Journal issue, will remain available online at the usual website as you receive newer sections. Each section will also be available to download for subscribers.

Part 4, that below, to appear in the June, 2017 issue of JVT, will deal with the following sections: 4.3. Purity/Impurities, Peptides and Biopharmaceuticals, Stability Indicating Properties for Purity Methods, 5. Conclusions, and 6. Acknowledgements. The list of references for all sections, Parts 1-3 already having appeared, is located in this same part of the JVT/IVT website, and should be close by.

4.3. Purity/Impurities, Peptides and Biopharmaceuticals, Stability Indicating Properties for Purity Methods

Every therapeutic program that expects to file a BLAmust “include a full description of the manufacturing methods, including analytical procedures that demonstrate if the manufactured product meets prescribed standards of identity, quality, safety, purity, and potency.” In addition, in the ICH Q6B, which is focused on specification testing of biotechnology products, the requirements for methods are that they should address quantity, purity/impurities, potency and identity (108). Accordingly, the FDA Guidance on Analytical Procedures and Methods Validation for Drugs and Biologics (July, 2015) therefore requires assessment of purity, which is a common requirement for biologics (17).

It is also clear that the sponsors of any therapeutic program, large or small molecule, must provide validated analytical procedures to address the critical qualities (CQA = critical quality attributes) of the product. However, for large molecules such as biologics, the specific process of method validation remains a challenging task, as many of the existing guidance documents on method validation are open to interpretation on how analytical assays for biologics should be validated. While the industry would benefit from harmonized, easily interpreted guidance documents for biologics, a fear of preventing or hindering critical medicines from reaching the public, coupled with the difficulty of harmonizing guidance across the diverse types of molecular constructs under clinical evaluation, pose significant barriers to further definitive regulatory guidances. As a result, the official guidances remain nebulous (at best). On the other hand, because there are FDA approved biologics, there must be a ‘well-traveled’ path for companies to achieve approvals. Information can be gleaned from a published survey of UK companies on methods and performance characteristics (109). Approvals are granted because there is an understanding of the types of methods and validation procedures that are required. Seemingly, this unspoken “trade secret” of understanding can be thought of as the groundwork for the approval process. Some insight might be gained from external publications of method validations. With this information, one may be able to find the ‘well-traveled’ path to product approval, in the absence of clear, concise, and definitive government or regulatory documents. It is hoped that published examples of validated methods can minimally serve as best practices, while completely recognizing that there must be variation to these studies to achieve approval.

This section provides some examples from the literature that discuss the stability-indicating properties of purity methods for biologics, with a focus on best practices that can be gleaned from previously published work. The scope of this discussion includes methods assessing purity, such as reversed phase, size-exclusion, hydrophobic interaction, and ion exchange chromatography. A brief description of these techniques can be found under the umbrella of a guidance document via the publication from CDER (110). While only a few validations for protein purity methods have been published, together they provide a good basis for best practices to guide future studies.

ICH Q2(R1), a foundational guidance for method validation, states that there are four common kinds of methods that require validation: 1) Identification tests; 2) Quantitative tests for impurity content; 3) Limit tests for the control of impurities; and 4) Quantitative tests of the active moiety in samples of drug substance or drug product or other selected component(s) in the drug product (53). Purity methods would be broadly covered in the last three types. Identification methods are discussed in another section of this review. Regardless of the terminology, the fact remains that the agency submission package must address the issue of purity, which is required by all regulatory agencies for release and stability indicating analytical methods. However, the question remains: how is this accomplished to successfully pass regulatory review?

Before proceeding further, we should recall that the precise definition of the method validation “exercise” has not been clearly harmonized for biopharmaceutical products between the industry and regulators

worldwide. There are likely many reasons for this lack of alignment, including the complexity of biopharmaceutical analytes, the relatively rapid technological change driving the biotechnology industry, and the increasing capability to “hone in” on important molecular attributes using modern high resolution analytical techniques.

While there is a lack of clarity around the validation procedure, especially for relatively complicated methods, such as many of the purity methods, a rather clear definition of the attribute of purity is provided in the Code of Federal Regulations: (Title 21, SubChapter F, SubPart A, Section 600.3, Biologic Products: Definitions; the definition of purity). “Purity means relative freedom from extraneous matter in the finished product, whether or not harmful to the recipient or deleterious to the product”. Purity includes but is not limited to relative freedom from residual moisture or other volatile substances and pyrogenic substances. A purity method therefore is defined as any analytical procedure that is able to determine the presence and quantity of “extraneous matter”. As related to biologics, the definition of the extraneous matter will be discussed below as related to Q6B. The purity method in order to prove “fit for purpose” must have, in addition to precision, specificity, and linearity sections, a well-developed, accuracy section with limits of detection and quantitation.

The genesis of a purity method must start with robust and thorough method development, which is the key to a successful method validation and long term method performance. The development experiments will offer an understanding of the method’s performance and capabilities. This understanding, in turn, will drive the establishment of a scientifically sound validation protocol. Therefore, upon completion of method development, the published regulatory guidances should be consulted, as they are the basis from which all method validations are derived. Nonetheless, these guidances have shortcomings, in that they cannot provide specific, direct guidance for validation. Guidelines, such as ICH Q2(R1) (Validation of Analytical Methods) and Q6B (Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products), enumerate the “general terms and definitions…” associated with method validation, but these are not sufficient for providing detailed validation guidance.

Furthermore, another deficiency of the regulatory guidances is a simple omission - the ICH guidance on the treatment of impurities was not written to address impurities in biologics (111). Biologics, in contrast to conventional drugs,may have impurities that cannot be isolated. Potential impurities can be due to the process of biologics manufacturing, such as peptides stemming from clipped fragments of the intact protein, host cell proteins or residual proteins from the purification (Protein A columns) and nucleic acid components (112). Posttranslational modifications, such as deamidated or isoaspartate residues, or cyclic congeners (i.e., pyroglutamate), should not be considered impurities (as long as these modifications have no appreciable impact on safety or efficacy), but rather, as product-related species (108, 113). However, protein aggregates, even dimeric forms, should be thought of as impurities. IEX has become the gold standard method for measuring charge variants (114). The disulfide isoforms (not always separable), an indicator of properly folded protein, are viewed as related species forms, and it is necessary to determine if one form is more potent than the others. A less potent species might be considered as a product-related impurity under these circumstances due to its lower bioactivity. Lastly, in addition to assessing protein posttranslational modifications, glycan profiling methods, can employ hydrophobic interaction liquid chromatography (HILIC), ion exchange or reversed phase techniques, where the glycan profile should separate these related substances (115). It is possible that a minor glycan species could be viewed as an impurity, in cases where glycans are identified that might be “non-human”, and therefore might represent an immunological risk (e.g; alpha-gal linkages).

Regardless of the technique, a purity and/or impurity method must be fit for purpose, and so therefore it must be able to quantify these minor components, as well as present an accurate determination of the minor, as well as the major, components. To prove that a purity method is fit for purpose, the validation of

the method must contain the appropriate performance characteristics. Apostol et al. 2012, which referenced the validation characteristics of a Table in ICH Q2(R1), provides helpful insights to define the elements of purity method validation (33). Validation of a purity method must include an evaluation of the method's accuracy (column recovery, sample recovery), and proper determinations of the limits of detection and quantitation. Additional performance characteristics would be precision, specificity, linearity and range, as appropriate for purity methods. It is important to point out that, unlike small molecules, large molecules may never have an absolute purity assessment. All purity results are relative to a well characterized standard, with a purity measured through orthogonal techniques to the one in validation. This is in sharp contrast to conventional drug products, because the absolute purity of biotechnological and biological products is difficult to determine, even with orthogonal techniques, as the results are method-dependent (108). An alternate approach using MS was published by Adou et al. (116).

It is generally acknowledged that no pharmaceutical company will have the resources to isolate every impurity for every biologic in their pipeline. Therefore, an understanding of the identities and sources of these impurities should be pursued. The ICH infers "if impurity or degradation product standards are unavailable, specificity may be demonstrated by comparing the test results of samples containing impurities or degradation products to a second, well-characterized procedure e.g., pharmacopoeial method or other validated analytical procedure (independent procedure). As appropriate, this should include samples stored under relevant stress conditions: light, heat, humidity, acid/base hydrolysis and oxidation (53,108). Properly conducted, forced degradation studies can provide much useful information relating to the source of an impurity or product-related species. Typically these experiments are run in the method development phase, so general pathways of degradation can be explored by a variety of techniques (e.g., MS).

The literature published on method validation of chromatography based methods for biopharmaceuticals remains scant and diverse, as each sponsor is reluctant to divulge the process that worked for them. It therefore remains unknown which validation methodologies available in the literature were acceptable to regulatory authorities or not. With that stated, nine publications are presented and reviewed here that are the result of a search of the existing literature. Each publication highlights and illustrates a chromatographic technique in the light of at least one best practice used that should be considered for future activities.

Moussa et al. treaded carefully through the landscape of revising a pharmacopeial method involving HPLC validation for a reversed phase method for insulin, using bulk and the final dosage form as samples. It should be noted, first off, that while therapeutic proteins have grown in complexity, we intend to use this work by Moussa and co-workers as a paradigm for more complex examples (68). The driver and purpose for this work was to modify the USP chromatographic conditions to develop and validate a method possessing a shorter run time, and the demonstration of robustness for an industrial application was required. To claim therefore that the method was appropriately validated, these authors were required to fully validate the revised method. This requirement is defined and clearly explained in Q6B, and is applicable when there are changes to the product's matrix, process manufacture, synthetic improvements, or revisions to the method of analysis that are outside of the scope of the robustness section of the previous method. Then, the analytical method must be revalidated. This revalidation is considered as best practice, and also a regulatory requirement, if the validated method is modified or altered beyond the existing validation performance characteristics.

The authors' goal was to shorten the run time of the method but retain the essential aspects of the initial validation. Results reported in this publication are the precision, intermediate precision, robustness, accuracy, linearity, specificity, limit of detection/quantitation, and solution stability studies. Clearly, these elements are in the guideline's spirit, and so they are also appropriate for inclusion in method validation.

Each has been tailored to fit the product undergoing study. The results indicated that they accomplished their primary goal, in that the retention time of insulin (main peak) was indeed shortened by 10 mins, and in the process of demonstrating specificity, the peaks of the additives were separated and identified (phenol and m-cresol). The accuracy was almost quantitative at 99.76% recovery, as based on the USP standard. The existence and use of the USP standard greatly aided this validation, as many biologics do not have a standard where the purity is assigned as 100%. In the case where there is no commercial or USP certified standard, the assays must be referenced to a well-characterized lot, where the purity has been checked by orthogonal methods. The use of a well-characterized lot in the validation is considered an important best practice.

The ensuing dosage analysis for specificity (40 IU/mL to 60 IU/mL) also gave excellent accuracy, with recovery percentages >99%, as compared to the FDA set range of 100%±2%. Overall, the recovery was determined between 80-120 IU/mL, and the %RSD was calculated based on three triplicate analyses at four levels (12 data points). Accuracy based on these runs was assessed at 99.8%±1.5% recovery. In this publication, the linearity was evaluated over the same range of 20-120% with a determination coefficient of 0.9993, thereby satisfying the USP requirement of >0.99. The %RSD was in fact much lower than 2%, coming in between 0.1% and 0.2%, with an overall precision of 4.9% at the 100 IU/mL dose. Lastly, the stability of insulin was assessed over 5 days with the new method; the overall %RSD was 0.8%. These investigators thereby confirmed that the revised shortened method was as suitable as the compendial method by analyzing the samples using both methods. Their final conclusion was that the validated, modified method can be used for routine quality control procedures with no interference from vial additives (68).

Rea et al. published their work regarding a so-called ‘mock’ validation of an ion exchange method for charge variants of a monoclonal antibody (45). The purpose of this validation was to enable the assessment of product stability. The authors quickly pointed out, and appropriately so, that any validation of a purity/stability method must be performed according to a pre-defined validation protocol with acceptance criteria. The method chosen to use in this validation study was previously published by Rozhkova (102). A critical component of a validation is the robustness of the method, as applied to a different but “equivalent” instrument. By using HPLC instruments from different manufacturers, it demonstrates to a reviewer that the method is potentially capable of inter-site transfer. An often overlooked and sometimes misunderstood aspect of validation is that the instruments used must be qualified for the assay, meaning documentation must exist to verify the proper installation, operation and performance of the instrument(s)(e.g., IQ, OQ, PQ). Injection amounts were examined for linearity of response. This test is an excellent way to ensure that the analysis is not biased by the detector. The success of this robustness depended on the overall system performance, and clearly the system suitability was appropriately designed for the purpose of the method. The investigators performed a well-designed, method precision study, which incorporates intermediate precision covering several days with multiple analysts. This type of precision study design is thought to be a best practice. Fittingly, they deemed these results appropriate by the %RSD found. The RSD values were in-line with the current guidances of the FDA regarding precision (<2% of the coefficient of variation). The design of alternative studies would be possible, dependent on the method development and overall capabilities of the method. The design of the protocol, with a thorough and well-designed method precision section, is also considered a best practice.

Next these investigators looked at the suitability of the method for use as a stability-indicating method, through the use of stressed samples. The observations in changes in the integrated peak areas of the cation-exchange chromatogram regions, namely the acidic, basic and main peak regions, were used to assess the method for stability indicating suitability. A summary table was used to show the variations in the conditions. Typically with each validation, a summary table of the results should be included to

summarize each performance characteristic section, including all of the system suitability runs. A tabulation of this sort allows a full statistical analysis of the multiple runs to judge the performance of the method throughout the validation.

Navas et al. applied the ICH guidelines to the HPLC/DAD based quantification of the monoclonal antibody, rituximab (39). The goals of this exercise were the development and validation of a reversed phase HPLC method according to ICH guidelines, and to demonstrate its suitability as a stability-indicating method. This study evaluated linearity, LOD/LOQ, precision, accuracy, specificity and robustness. System suitability tests were also devised. Guidance for these characteristics, regarding acceptable criteria for intraday %RSD, was absent from ICH guidance documents, so these researchers used the ?2% as described by the FDA document on chromatographic methods. Similarly, the ICH also did not provide criteria for recovery percent for HPLC-based methods, so they adopted the FDA guidance level at 100±2% (47,54). For biologics these suggestions of guidance levels are a reasonable starting point, but the recovery ranges might be larger due to the inherent uncertainties around absolute protein quantitation. In contrast to the Rea et al. study, these investigators performed more extensive forced degradation experiments under ICH recommended conditions, including variations in pH and ionic strength, as well as exposure to temperature, oxidizing agents and UV light. This list of conditions is in the spirit of the guidelines and is considered a best practice as they are suggested by Q2(R1) (53). Navas et al. are quick to point out that the chromatographic profile of the main peak may not differ significantly with the occurrence of low level degradations, as typically seen in biologicals, such as oxidation and deamidation (39). The inability to adequately resolve the numerous modification forms of a protein by chromatography is common to large molecules (118), and it is common for peaks to contain more than one molecular variant. To deal with this purity issue, the investigators have employed a function of the ChemStation software, in order to estimate the purity of damage to the protein, as a result of the forced degradation conditions (119). More important than the evaluation of this software, this study included a description of the impact of each condition. Thermal conditions revealed no unknown peaks, and only slight changes to the main peak were reported. As mentioned already, slight changes in the main peak may indicate conformational changes in the protein (118). Nonetheless, acceptable protein recovery was obtained. For ionic strength (matrix formulation evaluation, with 0.9% saline at medicinal concentrations, i.e., that which would be used in the clinic), all samples behaved well, and no changes to the chromatograms were noticed.

Next, the researchers examined the effects of light (UV Chamber 250 nm at 765 W/m2). No changes in the chromatograms were observed for these conditions. The researchers went on to mention that Trp, Tyr and Phe residues, were the most sensitive to this condition, and that the exposure did cause a discoloration of the drug substance, but not of the formulated material. Lastly, exposure to acidic/basic pH and oxidizing conditions, caused significant decreases in the intensity of the main peak. For proteins, which are sensitive to oxidation conditions at the Trp and sulfur-containing residues, the use of high concentrations of oxidant (30% H_2O_2), as commonly done with small molecule drugs, is completely inappropriate, as the high oxidant level will cause complete oxidation of the protein and reveal no information suitable for a forced degradation study. These scientists used two mixtures of 3.3% and 33.3% diluted solutions, by volume, of a 1% H_2O_2 solution for this study. Typically, industry standards are to cause between 10% and 50% degradation of the main peak to understand the impact of the conditions. Both acidic and basic conditions caused a slightly longer retention on the column of the main component. The explanation for these alterations in retention times was related to the deamidation and oxidation of the intact species. As previously noted, these protein modifications/changes are NOT completely separable by chromatographic means, and therefore the method does not completely fulfill the requirements of the ICH. This type of result is typical for proteins; however, these researchers overcame this deficiency. They claim that the method is suitable (according to ICH Q2(R1)) to be used for a stability indicating method, as the main peak gives different peak purity values (53). These can then be used to judge each sample's, long-term stability.

And as a closing note, they stated that there is a need for on-going research in this area to improve the MAb quality control process.

Olivia et al. validated a reversed phase method for purity and employed the method to evaluate the stability of a chemically manufactured CCK-4 peptide in aqueous solution (120). Their study was designed to understand stability as a function of temperature, while keeping other conditions constant, such as pH and ionic strength. The temperature was varied so that the investigators could find the rate of degradation by the Arrhenius equation and correlate that to the shelf life. A long term stability study was also arranged to verify the findings of the stressed sample study and calculations of shelf life. While this Arrhenius, plot based procedure is not industry mainstream for stability studies, it does present an interesting and alternative way of evaluating shelf life for peptides. Due to the molecular complexities, this method would not be appropriate for larger molecules.

The method was validated with respect to specificity, linearity, accuracy, precision and LOD/LOQ, which are appropriate validation characteristics. Moreover, the results of the robustness experiments (flow rate was reduced to 0.7 mL/min from 1 mL/min; injection volume was reduced from 25 mL to 10 mL) were deemed acceptable, and therefore suitable, for the intended purpose. Linearity was examined over the narrow range of 2 to 12 mg/mL, but the exact details of the experiment are not reported (i.e., replicates and determination coefficient). For this purity method, the accuracy was virtually ideal, at 100.2%±2.02%. Precision was demonstrated with the repeatability of 0.67% RSD and inter-assay precision at 2.74% RSD. While these standard deviations may seem acceptable, it should be noted that no experimental description was provided. LOD and LOQ were calculated based on detector response, at 0.35 and 1.06 mg/mL, respectively. This value was deemed acceptable, but nonetheless, the inter-assay precision was above the 2% RSD criteria, typically observed with other reversed phase methods.

Overall this reversed phase method was critical in assessing the content of intact material and degradation products in this study. With the data from the HPLC method, the workers were able to determine that the degradation of batches was remarkably different, and they were able to relate this finding back to the manufacturer, who had changed the original process. Olivia et al. benefited from working with a smaller bioactive molecule than a MAb for the degradation pathway analysis (120). A key difference, as presented in this work, was the fact that MS-based characterization was able to elucidate the degradation pathways with high confidence. While it is best practice to identify the degradation products, this task is far more difficult with a MAb. Nevertheless, the reporting of impurity levels is critically required, and so best practice would involve a relative peak area based assessment of each peak in the chromatogram. Further investigation of the higher level impurities would be warranted. Lastly, the shelf life analysis should be evaluated as based on an appropriate, traditional stability study.

Moorhouse et al. published an insightful paper on the validation of a cation exchange (CEX) method for the fragments of MAbs, after treatment with the protease, papain (121). These investigators employed CEX as an indicator of purity, in a clever way. The molecule was a MAb, which can be onerous to manage either chromatographically or with an MS. However, these researchers developed an enzymatic treatment to digest the molecule to smaller but more revealing pieces for analysis. Nonetheless, by this clever route of analysis, the task of validation became more difficult. The enzyme is papain, which has affinity for cleaving the hinge region of the IgG1, and liberating the Fab and Fc regions. The authors stated in the abstract, “Fragmentation allowed the resolution of the variants arising from the cyclization of glutamine to pyroglutamate, at the amino terminus of the light and heavy chains (Fab-pE:Q variants), from the variants resulting from the processing of the carboxyl terminal Lys residues of the heavy chains (Fc-Lys variants)”. As a result, the validation must include experiments to show that minor fluctuations in the digestion time, digest stability conditions, reagent stability, pH and vendor sources have little impact on the overall fitness of the method. This requirement was satisfactorily demonstrated.

Similar to the Moussa et al. study (68), separation of the components (which resulted from the papain digestion) showed good specificity and excellent linearity for the intact protein and digested components, namely Fc-Lys variants and Fab-pE/Q variants. The linearity assessment of the minor components, resultant fragments of the digest, required a determination of the LOD and LOQ to complete the performance characteristics of a purity method. Accuracy was assessed in three parts: column recovery, sample recovery and the determinations of LOD and LOQ. A recovery percentage of 95.7% of total protein was acceptable, considering that the process was more involved (sample prep/digestion and separation) than the previous example. Digestion time, conditions and reagent solution stability were also evaluated. This evaluation should be performed in method development for a technique such as this one, and it should be required, so as to complete the validation. It was found that 2-2.5 h digestion in the presence of 1 mM cysteine, prepared fresh prior to use, was optimal and gave minimal non-specific clips (<1%). Digestion times of greater than 5 hours showed that the method was not robust, due to the increased amount of non-specific clips. A finding such as this should be incorporated into the method, so as to alert the analyst that the method will fail if allowed extended digestion times. In the case of extensive digestion time, the digest should be discarded and no separation data generated.

As for the chromatographic robustness, the pH and molarity of mobile phase constituents were examined. Moreover, as recommended, multiple column lots were evaluated for potential manufacturing differences. It is strongly advised that at least three lots are used, and that the manufacturer be contacted to inquire on the availability of these columns, throughout the lifecycle of the method. Other factors for chromatographic robustness that may be evaluated are column temperature, flow rate and injection amount. Each of these parameters could have a profound effect on the elution times and run profile. Overall, this paper is an excellent example of the stepwise validation covering both sample preparation and analysis. The data reported by these authors convincingly demonstrated that the CEX assay is fit for the purpose of determining the product purity of a MAb.

Cueto-Rojas et al. validated a method for the quantification of a cytokine, interferon-a 2b, using a UPLC procedure (51). In this work, the researchers were interested in obtaining a reliable method for the analysis of the cytokine from the inclusion bodies, a complex mixture of DNA, carbohydrates and lipids. This more modern method employed UPLC, a technique which uses sub-2 micron particles to achieve higher resolutions because of the higher, theoretical plate counts and flow rates possible (122-124). These scientists validated this method for linearity, precision, accuracy (recovery), LOD/LOQ, specificity, and robustness. System suitability tests were provided, and these are typical and expected for this type of method. Six injections of the API provided data from which to evaluate plate count, retention time variability, capacity factor, and tailing factor. These results showed that this column and system were

appropriate. Linearity was assessed over a ten-fold range, in concentrations with a determination coefficient of 0.9989. Accuracy showed a recovery percentage of 100.28% (%RSD 1.5%); both were well within the requirements for a purity method. Precision using API material was found to be <2%, at 1.54%. For specificity, this assay used the resolution factor. Typically, values >2 were desirable, and this method provided values of 3.152 (for the main peak and host cell proteins), and 6.156 for nearest peaks with target protein, recombinant, leucin aminopeptidase (LAP). With this data, these authors claim that the method is fit for purpose to quantify total protein content for inclusion bodies.

In a short study, Codevilla et al. determined the aggregate profile of recombinant humanized, granulocyte colony-stimulating factor (GCSF) in pharmaceutical formulations, using SEC-HPLC (55). Again, a cytokine, recombinant GCSF (MW 19 kDa), was used for the validation of a recombinant. These smaller proteins can present a challenge, as it is critical for development to find a SEC column and conditions that can efficiently separate the aggregates from the monomer peak (125). Typically, for a protein of this size, a column with a range of 5 kDa to 100 kDa would be suitable, whereas in contrast, for MAbs the effective range is a bit larger (10 kDa to 500 kDa). Nonetheless, these scientists were able to demonstrate range, linearity, accuracy, precision (0.16%) with intermediate precision (%RSD between day (n=2), for two separate preparations with two analysts of 0.56% and 0.96%), and robustness (flow rate, mobile phase pH, and detection wavelength) according to ICH guidelines. The concentration range examined was 50-150% of dose; linearity was excellent with a determination coefficient at an amazing 0.9998; and accuracy was evaluated based on 9 determinations with good recoveries (103.1%, 102.7% and 105.4% purity).

Overall, SEC methods typically perform well, but caution is appropriate, in that with some methods, the investigators try to link elution volume and size of the protein. This practice is fraught with concern, as not all proteins are completely globular in shape, and therefore may not perform according to the MW markers purchased. If the method requires a mere visual inspection of where the analyte elutes in comparison to the standards, then the method may be acceptable, but for absolute determination of molecular or aggregate size, an orthogonal technique must be used, such as analytical ultracentrifugation (AUC) or multi-angle laser light scattering (MALS) approaches. This orthogonal approach was used by Thiebaud et al. (126). In this study, a vaccine-derived conjugate was analyzed by SEC, and the method then validated according to ICH guidelines.

Shang et al. outlined the considerations that are critical for a HILIC type validation of a glycan map (127). The procedure for the analytical comparison of glycan forms on a glycoprotein has become routine these days. In brief, an enzyme (PNGase F) is used to remove the N-linked structures from the protein. The released glycans are then labeled with a fluorescent dye, for identification by separation on a HILIC based column. The FDA has become aware of the criticality of these structures, which have the potential to impact as potency and immunogenicity. According to Q6B, the guidance states, “For glycoproteins, the carbohydrate content (neutral sugars, amino sugars, and sialic acids) is determined. In addition, the structure of the carbohydrate chains, the oligosaccharide pattern (antennary profile), and the glycosylation site(s) of the polypeptide chain is analyzed, to the extent possible” (108).

The results of the experimentation were presented. Reproducibility of the enzymatic digest (a step similar to what is common to peptide map procedures), followed by labeling and separation of the glycoforms, gave a system precision of 0.1-5.2% relative peak area, based on six injections (n = 6) of a single MAb sample (two MAbs tested, overall). Total peak area results were tighter with %RSD of 0.8-4.7%. The overall method precision was tested on three MAbs, each with six individual injections per MAb, and showed 0.1-4.5% RSD relative peak area and 2.3-7.8% RSD for total peak area. These values are excellent in consideration of the steps required to get to the data generating step. While this study was only used for characterization purposes, if the procedure was ever to be used in a regulated lab, the method would need to be validated. The study serves to demonstrate the utility of this approach for glycan

comparability studies, and the current level of technology available for improved separations and quantification of glycoforms.

A study by Bartolomeo and Maisano described the validation of a reversed phase method applied to amino acid analysis of a protein (128). Amino acid (AA) analysis (AAA) is a technique with multiple applications. It can also be used to assess the identity and purity of a protein. With spectroscopic data, AAA can be used to determine extinction coefficients. The technique, in general, exists in two forms, the ion-exchange (IEX) based separation technique (post-column), relies on expensive and some outdated instrumentation. The second technique is more applicable to the modern laboratory, where the derivatization of the amino acids is performed before the column separation (i.e., pre-column derivatization)(e.g., Pico-Tag or AccQ-Tag, Waters).

This study makes use of current technology, and employs a robotic system to manipulate the many solutions required for the analysis. In one sense, this robotic based procedure highlights the forward-thinking technology that is rapidly becoming accessible to more laboratories. This use of modern technology is the best practice here. All methods should be designed with the highest state-of-the-art equipment. The impact of this technology is being felt currently, with reduced errors associated with pipetting and other manual steps. As should be common, when such a multi-step process of standards are used, from which to judge/evaluate the efficiency of the protein hydrolysis, a loss of AAs occurs during the hydrolysis, with material transfer loss prior to injection. The standards employed are standardized solutions of the individual AAs and a model protein, in this case, bovine serum albumin (BSA). The results were based on the 7 “well-recovered” AAs (129). Specificity was judged by the retention times of the sample AAs with the reference, AA standards, which typically gave excellent %RSD of 0.1% or less. Linearity was required and determined for each derivatized AA. The range was determined to be 20 to 500 picomole/mL, as based on AA standards, with determination coefficients always greater than 0.985. Accuracy was evaluated by two different parameters: absolute percent error and recovery. Absolute percent error was always found to be less than 7% and recoveries were a remarkable 97-108%. Precision was less than 2.5% RSD for each amino acid, and the percent coefficient of variance for all seven AAs was typically below 2.5%, with one exception (arginine). To complete this validation exercise, the LOD/LOQ were evaluated, based on detector responses. Due to the nature of the assay, forced degradations with this method are not applicable.

Conclusions

In general, the purity methods must be based on scientific principles, where the validation structure/performance characteristics can match the expectations set forth by the regulatory guidance document’s, appropriate level of technology. From each of these nine highlighted publications and the references therein, a best practice can be derived. In these publications we saw examples of well-designed validation exercises, with applications in validation of updated methods, changes in sample matrices, forced degradation studies, and impurity assessment. The discussion above also includes validation considerations for implementation of new or advanced technology (robotics, UPLC, MS).

5. Conclusions

In this review, we have chosen those specific publications from the refereed literature that have applied validation approaches to synthetic peptides or biotechnology expressed proteins (often, glycoproteins), antibodies or fusion proteins. We have purposely emphasized specific aspects of importance to the biopharmaceutical industry, such as system suitability and robustness, with the validation design broken down into three, distinct application areas: 1) identity (characterization methods using HPLC and/or HPLC-ESI-MS for peptides and biopharmaceuticals), with examples from reversed phase, ion exchange HPLC

and peptide mapping; 2) validation design for a protein content method, again using specific forms of HPLC/UHPLC; and 3) determination of purity and impurities, with examples from reversed phase, size exclusion, ion exchange, and HILIC. In each of these specific examples, we have included sample preparation methods, perhaps unique to each analytical approach, as well as a discussion of the details of how specific validation goals were achieved.

It is important to note that, despite the existence of guidances (some more useful than others), FDA and other regulatory agencies will make its decisions on individual submittals for recombinant products on a "case-by-case" basis, without providing initial recommendations, guidelines or requirements for what should appear in the final CMC or validation packages. A clear understanding of the characteristics of the product and the science behind the methods is therefore required to successfully validate analytical methods for protein biopharmaceuticals.

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References

For a compiled list of references for this 4-part series, please click here.
