Stage 4 Harmonization
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⟨1055⟩ BIOTECHNOLOGY-DERIVED ARTICLES—PEPTIDE MAPPING

Delete the following:

▲This chapter provides guidance and procedures used for characterization of biotechnology-derived articles by peptide mapping. This chapter is harmonized with the corresponding chapter in *JP* and *EP*. Portions of the chapter that are not harmonized with the other two pharmacopeias are marked by the symbol ⧫. Other characterization tests, also harmonized, are shown in *Biotechnology-Derived Articles—Amino Acid Analysis* ⟨1052⟩,*Capillary Electrophoresis* ⟨1053⟩, *Biotechnology-Derived Articles—Isoelectric Focusing* ⟨1054⟩, *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩, and *Biotechnology-Derived Articles—Total Protein Assay* ⟨1057⟩.

INTRODUCTION

⧫Peptide mapping is an identity test for proteins, especially those obtained by rDNA technology. It involves the chemical or enzymatic treatment of a protein, resulting in the formation of peptide fragments, followed by separation and identification of the resultant fragments in a reproducible manner. It is a powerful test that is capable of identifying single amino acid changes resulting from events such as errors in the reading of complementary DNA (cDNA) sequences or point mutations. Peptide mapping is a comparative procedure because the information obtained, compared to a Reference Standard or Reference Material similarly treated, confirms the primary structure of the protein, is capable of detecting whether alterations in structure have occurred, and demonstrates process consistency and genetic stability. Each protein presents unique characteristics that must be well understood so that the scientific and analytical approaches permit validated development of a peptide map that provides sufficient specificity.

This chapter provides detailed assistance in the application of peptide mapping and its validation to characterize the desired protein product, to evaluate the stability of the expression construct of cells used for recombinant DNA products, to evaluate the consistency of the overall process, and to assess product stability, as well as to ensure the identity of the protein product or to detect the presence of protein variant. ⧫The validation scheme presented differentiates between qualification of the method at an early stage in the regulatory process, the Investigational New Drug (IND) level, and full validation in support of New Drug Application (NDA), Product License Application (PLA), or Marketing Authorization Application (MAA). The validation concepts described are consistent with the general information chapter *Validation of Compendial Procedures* ⟨1225⟩ and with the International Conference on Harmonization (ICH) document *Analytical Methods Validation*.⧫

THE PEPTIDE MAP

Peptide mapping is not a general method, but involves developing specific maps for each unique protein. Although the technology is evolving rapidly, there are certain methods that are generally accepted. Variations of these methods will be indicated, when appropriate, in specific monographs.

A peptide map may be viewed as a fingerprint of a protein and is the end product of several chemical processes that provide a comprehensive understanding of the protein being analyzed. Four major steps are necessary for the development of the procedure: isolation and purification of the protein, if the protein is part of a formulation; selective cleavage of the peptide bonds; chromatographic separation of

the peptides; and analysis and identification of the peptides. A test sample is digested and assayed in parallel with a Reference Standard or Reference Material. Complete cleavage of peptide bonds is more likely to occur when enzymes such as endoproteases (e.g., trypsin) are used instead of chemical cleavage reagents. A map should contain enough peptides to be meaningful. On the other hand, if there are too many fragments, the map might lose its specificity because many proteins will then have the same profiles.

Isolation and Purification

Isolation and purification are necessary for analysis of bulk drugs or dosage forms containing interfering excipients and carrier proteins and, when required, will be specified in the monograph. Quantitative recovery of protein from the dosage form should be validated.

Selective Cleavage of Peptide Bonds

The selection of the approach used for the cleavage of peptide bonds will depend on the protein under test. This selection process involves determination of the type of cleavage to be employed—enzymatic or chemical—and the type of cleavage agent within the chosen category. Several cleavage agents and their specificity are shown in *Table 1*. This list is not all-inclusive and will be expanded as other cleavage agents are identified.

Table 1. Examples of Cleavage Agents

Type	Agent	Specificity
Enzymatic	Trypsin, EC 3.4.21.4	*C*-terminal side of Arg and Lys
	Chymotrypsin, EC 3.4.21.1	*C*-terminal side of hydrophobic residues (e.g., Leu, Met, Ala, aromatics)
	Pepsin, EC 3.4.23.1 and EC 3.4.23.2	Nonspecific digest
	Lysyl endopeptidase (Lys-C endopeptidase), EC 3.4.21.50	*C*-terminal side of Lys
	Glutamyl endopeptidase; (from *S. aureus* strain V8), EC 3.4.21.19	*C*-terminal side of Glu and Asp
	Peptidyl-Asp metalloendopeptidase (Asp-N endoproteinase), EC 3.4.24.33	*N*-terminal side of Asp
	Clostripain, EC 3.4.22.8	*C*-terminal side of Arg
Chemical	Cyanogen bromide	*C*-terminal side of Met
	2-Nitro-5-thiocyanobenzoic acid	*N*-terminal side of Cys
	o-Iodosobenzoic acid	*C*-terminal side of Trp and Tyr
	Dilute acid	Asp and Pro
	BNPS-skatole	Trp

Pretreatment of Sample

Depending on the size or the configuration of the protein, different approaches in the pretreatment of samples can be used. For monoclonal antibodies, the heavy and light chains will need to be separated before mapping. If trypsin is used as a cleavage agent for proteins with a molecular mass greater than 100,000 Da, lysine residues must be protected by citraconylation or maleylation; otherwise, too many peptides will be generated.

Pretreatment of the Cleavage Agent

Pretreatment of cleavage agents, especially enzymatic agents, might be necessary for purification purposes to ensure reproducibility of the map. For example, trypsin used as a cleavage agent will have to be treated with tosyl-L-phenylalanine chloromethyl ketone to inactivate chymotrypsin. Other methods, such as purification of trypsin by HPLC or immobilization of enzyme on a gel support, have been successfully used when only a small amount of protein is available.

Pretreatment of the Protein

Under certain conditions, it might be necessary to concentrate the sample, or to separate the protein from added substances and stabilizers used in the formulation of the product if these interfere with the mapping procedure. Physical procedures used for pretreatment can include ultrafiltration, column chromatography, and lyophilization.

Other pretreatments, such as the addition of chaotropic agents (e.g., urea) can be used to unfold the protein prior to mapping. To allow the enzyme to have full access to cleavage sites and permit some unfolding of the protein, it is often necessary to reduce and alkylate the disulfide bonds prior to digestion.

Digestion with trypsin can introduce ambiguities in the tryptic map as a result of side reactions occurring during the digestion reaction, such as nonspecific cleavage, deamidation, disulfide isomerization, oxidation of methionine residues, or formation of pyroglutamic groups created from the deamidation of glutamine at the *N*-terminal side of a peptide. Furthermore, peaks may be produced by autohydrolysis of trypsin. Their intensities depend on the ratio of trypsin to protein. To avoid autohydrolysis, solutions of proteases may be prepared at a pH that is not optimal (e.g., at pH 5 for trypsin), which would mean that the enzyme would not become active until diluted with the digest buffer.

Establishment of Optimal Digestion Conditions

Factors that affect the completeness and effectiveness of digestion of proteins are those that could affect any chemical or enzymatic reactions.

pH—The digestion mixture pH is empirically determined to ensure the optimal performance of the given cleavage agent. For example, when using cyanogen bromide as a cleavage agent, a highly acidic environment (e.g., pH 2, formic acid) is necessary; however, when using trypsin as a cleavage agent, a slightly alkaline environment (pH 8) is optimal. As a general rule, the pH of the reaction milieu should not alter the chemical integrity of the protein during the digestion and should not change during the course of the fragmentation reaction.

Temperature—A temperature between 25° and 37° is adequate for most digestions. The temperature used is intended to minimize chemical side reactions. The type of protein under test will dictate the temperature of the reaction milieu because some proteins are more susceptible to denaturation as the

temperature of the reaction increases. For example, digestion of recombinant bovine somatropin is conducted at 4° because at higher temperatures it will precipitate during digestion.

Time—If a sufficient amount of sample is available, a time course study is considered in order to determine the optimum time to obtain a reproducible map and avoid incomplete digestion. Time of digestion varies from 2 to 30 hours. The reaction is stopped by the addition of an acid that does not interfere with the tryptic map, or by freezing.

Amount of Cleavage Agent—Although excessive amounts of cleavage agent are used to accomplish a reasonably rapid digestion time (i.e., 6 to 20 hours), the amount of cleavage agent is minimized to avoid its contribution to the chromatographic map pattern. A protein-to-protease ratio between 20:1 and 200:1 is generally used. It is recommended that the cleavage agent be added in two or more stages to optimize cleavage. Nonetheless, the final reaction volume remains small enough to facilitate the next step in peptide mapping—the separation step. To sort out digestion artifacts that might be interfering with the subsequent analysis, a blank determination is performed using a digestion control with all the reagents except the test protein.

Chromatographic Separation

Many techniques are used to separate peptides for mapping. The selection of a technique depends on the protein being mapped. Techniques that have been successfully used for the separation of peptides are shown in *Table 2*.

Table 2. Techniques Used for the Separation of Peptides

Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC)
Ion-Exchange Chromatography (IEC)
Hydrophobic Interaction Chromatography (HIC)
Polyacrylamide Gel Electrophoresis (PAGE), nondenaturating
Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)
Capillary Electrophoresis (CE)
Paper Chromatography—High Voltage (PCHV)
High-Voltage Paper Electrophoresis (HVPE)

In this section, a most widely used reverse-phase HPLC (RP-HPLC) method is described as one of the procedures of chromatographic separation.

The purity of solvents and mobile phases is a critical factor in HPLC separation. HPLC-grade solvents and water that are commercially available are recommended for RP-HPLC. Dissolved gases present a problem in gradient systems where the solubility of the gas in a solvent may be less in a mixture than in a single solvent. Vacuum degassing and agitation by sonication are often used as useful degassing procedures. When solid particles in the solvents are drawn into the HPLC system, they can damage the sealing of pump valves or clog the top of the chromatographic column. Both pre- and post-pump filtration are also recommended.

Chromatographic Column

The selection of a chromatographic column is empirically determined for each protein. Columns with 100 Å or 300 Å pore size and silica support can give optimal separation. For smaller peptides, octylsilane chemically bonded to totally porous silica particles 3 to 10 µm in diameter (L7) and of octadecylsilane chemically bonded to porous silica or ceramic microparticles 3 to 10 µm in diameter (L1) column packings are more efficient than the butyl silane chemically bonded to totally porous silica particles 5 to 10 µm in diameter (L26) packing.

Solvent

The most commonly used solvent is water with acetonitrile as the organic modifier to which less than 0.1% of trifluoroacetic acid is added. If necessary, add isopropyl alcohol or *n*-propyl alcohol to solubilize the digest components, provided that the addition does not unduly increase the viscosity of the components.

Mobile Phase

Buffered mobile phases containing phosphate are used to provide some flexibility in the selection of pH conditions, because shifts of pH in the 3.0 to 5.0 range enhance the separation of peptides containing acidic residues (e.g., glutamic and aspartic acids). Sodium or potassium phosphates, ammonium acetate, phosphoric acid, and a pH between 2 and 7 (or higher for polymer-based supports) have also been used with acetonitrile gradients. Acetonitrile-containing trifluoroacetic acid is also used quite often.

Gradient Selection

Gradients can be linear, nonlinear, or include step functions. A shallow gradient is recommended in order to separate complex mixtures. Gradients are optimized to provide clear resolution of one or two peaks that will become “marker” peaks for the test.

Isocratic Selection

Isocratic HPLC systems using a single mobile phase are used on the basis of their convenience of use and improved detector responses. Optimal composition of a mobile phase to obtain clear resolution of each peak is sometimes difficult to establish. Mobile phases for which slight changes in component ratios or in pH significantly affect retention times of peaks in peptide maps should not be used in isocratic HPLC systems.

Other Parameters

Temperature control of the column is usually necessary to achieve good reproducibility. The flow rates for the mobile phases range from 0.1 to 2.0 mL per minute, and the detection of peptides is performed with a UV detector at 200 to 230 nm. Other methods of detection have been used (e.g., postcolumn derivatization), but they are not as robust or as versatile as UV detection.

System Suitability

The section *System Suitability* under *Chromatography* ⟨621⟩ provides an experimental means for measuring the overall performance of the test method. The acceptance criteria for system suitability depend on the identification of critical test parameters that affect data interpretation and acceptance. These critical parameters are also criteria that monitor peptide digestion and peptide analysis. An indicator that the desired digestion endpoint was achieved is the comparison with a Reference Standard or Reference Material, which is treated exactly as the article under test. The use of a USP Reference

Standard in parallel with the protein under test is critical in the development and establishment of system suitability limits. In addition, a specimen chromatogram should be included with the USP Reference Standard or Reference Material for additional comparison purposes. Other indicators may include visual inspection of protein or peptide solubility, the absence of intact protein, or measurement of responses of a digestion-dependent peptide. The critical system suitability parameters for peptide analysis will depend on the particular mode of peptide separation and detection, and on the data analysis requirements.

When peptide mapping is used as an identification test, the system suitability requirements for the identified peptides cover selectivity and precision. In this case, as well as when identification of variant proteins is done, the identification of the primary structure of the peptide fragments in the peptide map provides both a verification of the known primary structure and the identification of protein variants by comparison with the peptide map of the USP Reference Standard or Reference Material for the specified protein. The use of a digested USP Reference Standard or Reference Material for a given protein in the determination of peptide resolution is the method of choice. For an analysis of a variant protein, a characterized mixture of a variant and a Reference Standard can be used, especially if the variant peptide is located in a less-resolved region of the map. The index of pattern consistency can be simply the number of major peptides detected. Peptide pattern consistency can be best defined by the resolution of peptide peaks. Chromatographic parameters—such as peak-to-peak resolution, maximum peak width, peak area, peak tailing factors, and column efficiency—may be used to define peptide resolution. Depending on the protein under test and the method of separation used, single peptide or multiple peptide resolution requirements may be necessary.

The replicate analysis of the digest of the USP Reference Standard or Reference Material for the protein under test yields measures of precision and quantitative recovery. Recovery of the identified peptides is generally ascertained by the use of internal or external peptide standards. The precision is expressed as the relative standard deviation (RSD). Differences in the recovery and precision of the identified peptides are expected; therefore, the system suitability limits will have to be established for both the recovery and the precision of the identified peptides. These limits are unique for a given protein and will be specified in the individual monograph.

Visual comparison of the relative retention times, the peak responses (the peak area or the peak height), the number of peaks, and the overall elution pattern is completed initially. It is then complemented and supported by mathematical analysis of the peak response ratios and by the chromatographic profile of a 1:1 (v/v) mixture of sample and USP Reference Standard or Reference Material digest. If all peaks in the sample digest and in the USP Reference Standard or Reference Material digest have the same relative retention times and peak response ratios, then the identity of the sample under test is confirmed.

If peaks that initially eluted with significantly different relative retention times are then observed as single peaks in the 1:1 mixture, the initial difference would be an indication of system variability. However, if separate peaks are observed in the 1:1 mixture, this would be evidence of the nonequivalence of the peptides in each peak. If a peak in the 1:1 mixture is significantly broader than the corresponding peak in the sample and USP Reference Standard or Reference Material digest, it may indicate the presence of different peptides. The use of computer-aided pattern recognition software for the analysis of peptide mapping data has been proposed and applied, but issues related to the validation of the computer software preclude its use in a compendial test in the near future. Other automated approaches have been used that employ mathematical formulas, models, and pattern recognition. Such approaches, for example the automated identification of compounds by IR spectroscopy and the application of diode-array UV spectral analysis for identification of peptides, have been proposed. These methods have limitations due to inadequate resolutions, co-elution of fragments, or absolute peak response differences between USP Reference Standard or Reference Material and sample fragments.

The numerical comparison of the retention times and peak areas or peak heights can be done for a selected group of relevant peaks that have been correctly identified in the peptide maps. Peak areas can be calculated using one peak showing relatively small variation as an internal reference, keeping in mind that peak area integration is sensitive to baseline variation and is likely to introduce error into the analysis. Alternatively, the percentage of each peptide peak height relative to the sum of all peak heights can be calculated for the sample under test. The percentage is then compared to that of the corresponding peak of the USP Reference Standard or Reference Material. The possibility of autohydrolysis of trypsin is monitored by producing a blank peptide map, that is, the peptide map obtained when a blank solution is treated with trypsin.

The minimum requirement for the qualification of peptide mapping is an approved test procedure that includes system suitability as a test control. In general, early in the regulatory process, qualification of peptide mapping for a protein is sufficient. As the regulatory approval process for the protein progresses, additional qualifications of the test can include a partial validation of the analytical procedure to provide assurance that the method will perform as intended in the development of a peptide map for the specified protein.

Analysis and Identification of Peptides

This section gives guidance on the use of peptide mapping during development in support of regulatory applications.

The use of a peptide map as a qualitative tool does not require the complete characterization of the individual peptide peaks. However, validation of peptide mapping in support of regulatory applications requires rigorous characterization of each of the individual peaks in the peptide map. Methods to characterize peaks range from *N*-terminal sequencing of each peak followed by amino acid analysis to the use of mass spectroscopy (MS).

For characterization purposes, when *N*-terminal sequencing and amino acid analysis are used, the analytical separation is scaled up. Because scale-up might affect the resolution of peptide peaks, it is necessary, using empirical data, to ensure that there is no loss of resolution due to scale-up. Eluates corresponding to specific peptide peaks are collected, vacuum-concentrated, and chromatographed again, if necessary. Amino acid analysis of fragments may be limited by the peptide size. If the *N*-terminus is blocked, it may need to be cleared before sequencing. *C*-terminal sequencing of proteins in combination with carboxypeptidase digestion and matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) MS can also be used for characterization purposes.

The use of MS for characterization of peptide fragments is by direct infusion of isolated peptides or by the use of on-line LC-MS for structure analysis. In general, it includes electrospray and MALDI-TOF analyzers as well as fast atom bombardment (FAB). Tandem MS has also been used to sequence a modified protein and to determine the type of amino acid modification that has occurred. The comparison of mass spectra of the digests before and after reduction provides a method to assign the disulfide bonds to the various sulfhydryl-containing peptides.

If regions of the primary structure are not clearly demonstrated by the peptide map, it might be necessary to develop a secondary peptide map. The goal of a validated method of characterization of a protein through peptide mapping is to reconcile and account for at least 95% of the theoretical composition of the protein structure.

♦THE USE OF PEPTIDE MAPPING FOR GENETIC STABILITY EVALUATION

A validated peptide map can be used to assess the integrity of the predicted primary sequence of a protein product (i.e., its genetic stability). It can also be used to determine lot-to-lot consistency of the biotechnology-derived product process. Furthermore, the performance of the protein expression of the

production system is best assessed by peptide mapping of the expressed protein. Peptide maps of protein produced at various times of the protein expression process, including a point well beyond the normal protein expression time, compared with those of a USP Reference Standard or Reference Material, will evaluate the genetic stability of the expression system as a function of time.

Variant protein sequences can arise from a genetic variation at the DNA level (point mutation) or as an error in the translation process. A validated peptide map is the best approach to the detection of protein variants. However, the limitations of the peptide mapping itself must be taken into consideration. The detection of a structured variant is possible only if the corresponding peptide variant is easily isolated and characterized. To establish genetic stability will require the use of a battery of biochemical methods, provided that the variants have properties different from those of the "normal" protein.

VALIDATION

Critical Factors

Validation of peptide mapping requires that a protocol be designed, outlining in detail the experiment to be conducted and the criteria for acceptance of the map. Criteria for acceptance of mapping include detection limit, specificity, linearity, range, accuracy, precision, and reagent stability. Reproducibility of the peptide map is a critical element in the utilization of such a map as an identity test and for confirming genetic stability. Those technical aspects of peptide mapping that influence the reproducibility of the map will be discussed.

The setting of limits, with respect to quantification (peak area or height) and identification (retention times) for the selected group of relevant peaks is based on empirical observations. These limits detect significant differences between the sample and USP Reference Standard or Reference Material within a series of analyses.

Another critical issue is the recovery of peptides and its impact on peak area determination and reproducibility and on the establishment of acceptance criteria. The recovery criteria address all aspects of test methodology, from digestion to chromatographic conditions. Determination of peptide recovery includes quantitative amino acid analysis, spike addition, radiolabeling, and UV summation. An overall recovery of about 80% is considered satisfactory. Recovery of individual peptides is more problematic and is handled on a case-by-case basis. The critical factors considered in the validation of a peptide map are as follows.

Written Test Procedures

These procedures include a detailed description of the analytical method in which reagents, equipment, sample preparation, method of analysis, and analysis of the data are defined.

Validation Protocol

A protocol is prepared that contains a procedure for test validation.

Acceptance Criteria

The criteria can be minimal at the early stages, but need to be better defined as validation studies progress.

Reporting of Results

Results from the validation study are documented with respect to the analytical parameters listed in the validation protocol.

Revalidation of the Test Procedure

If the method used requires alteration that could affect the analytical parameter previously assessed in the validation of the procedure, the test procedure must be revalidated. Significant changes in the processing of the article, in laboratories performing the analysis, in formulation of the bulk or the finished products, and in any other significant parameter will require revalidation of the methods.

Requirements

Precision

Intratest Precision—This is a measure of the reproducibility of peptide mapping. The two critical steps in peptide mapping are fragmentation (i.e., digestion) and separation of peptides. An acceptable precision occurs where the absolute retention times and the relative peak areas are constant from run to run, and the average variation in retention time is small relative to that of a selected internal reference peak. The reproducibility of the map can be enhanced if a temperature-controlled column oven is used, if an extensive equilibration of the system is performed prior to the start of the test, if a blank (control digest mixture without protein) is run first to minimize "first run effects," and if a USP Reference Standard or a Reference Material digest is interspersed periodically with test samples to evaluate chromatographic drift.

The criteria for validation of the fragmentation step are similar to those described below for separation of peptides, but they are met for consecutive tests of a series of separately prepared digests of the protein under test.

The criteria for validation of the separation of peptides step include the following:

1. The average standard deviation of the absolute retention times of all major peaks for a set of consecutive tests of the same digest does not exceed a specified acceptance criterion.
2. The average standard deviation of absolute peak area for all fully resolved major peaks does not exceed a specified percentage.

Intertest Precision—This is a measure of the reproducibility of the peptide mapping when the test is performed on different days, by different analysts, in different laboratories, with reagents or enzymes from different suppliers or different lots from the same supplier, with different instruments, on columns of different makes or columns of the same make from different lots, and on individual columns of the same make from the same lot. Although it would be desirable, from a scientific perspective, to validate all of these variables in terms of their impacts on precision, a practical approach is to validate the test using those variables most likely to be encountered under operational conditions. Additional variables can be included when needed.

The experimental design allows the analyst to make comparisons using peak retention times and areas that are expressed relative to a highly reproducible internal reference peak within the same chromatogram. The relative peak area is expressed as the ratio of the peak area to that of the internal reference peak. The relative retention time can be expressed as the difference between the absolute retention time and that of the reference peak. The use of relative values eliminates the need to make separate corrections for differences due to injector-to-injector volumes, units of measure for peak areas, column dimensions, and instrument dead volumes. The variability in the retention times and peak areas for the *Intertest Precision* experiments is expected to be slightly higher than the variability observed for *Intratest Precision*.

Robustness

Factors such as composition of the *Mobile Phase*, protease quality or chemical reagent purity, column variation and age, and digest stability are likely to affect the overall performance of the test and its reproducibility. Tolerances for each of the key parameters are evaluated and baseline limits established in case the test is used for routine lot release purposes.

Mobile Phase—The composition of the *Mobile Phase* is optimized to obtain the maximum resolution of peptides throughout the elution profile. A balance between optimal resolution and overall reproducibility is desired. A lower pH might improve peak separation but might shorten the life of the column, resulting in lack of reproducibility. Peptide maps at a pH above and below the pH of the procedure are compared to the peptide map obtained at the pH of the procedure and checked for significant differences; they are also reviewed with respect to the acceptance criteria established in the validation protocol.

Protease Quality or Chemical Reagent Purity—A sample of the USP Reference Standard or Reference Material for the protein under test is prepared and digested with different lots of cleavage agent. The chromatograms for each digest are compared in terms of peak areas, shape, and number. The same procedure can be applied to other critical chemicals or pretreatment procedures used during sample preparation, such as reducing and carboxymethylation reagents.

Column Considerations—Column-to-column variability, even within a single lot, can affect the performance of the column in the development of peptide maps. Column size may also lead to significant differences. A USP Reference Standard or Reference Material of the protein under test is digested and the digest is chromatographed on different lots of column from a single manufacturer. The maps are then evaluated in terms of the overall elution profile, retention times, selectivity resolution, and recovery. To evaluate the overall lifetime of the column in terms of robustness, perform a peptide mapping test on different columns and vary significantly the number of injections (e.g., from 10 injections to 250 injections). The resulting maps can then be compared for significant differences in peak broadening, peak area, and overall resolution. As a column ages, an increase in back pressure might be observed that might affect the peptide maps.

A sensible precaution in the use of peptide mapping columns is to select alternative columns in case the original columns become unavailable or are discontinued. Perform a peptide mapping test using equivalent columns from different manufacturers, and examine the maps. Differences in particle shape and size, pore size and volume, carbon load, and end-capping can lead to significant differences in retention times, elution profile selectivity, resolution, and recovery. Slight modifications in the gradient profile may be required to achieve equivalency of mapping when using columns from different manufacturers.

[Note—The equivalency between instrumentation used for the validation of the test and for routine quality control testing should be considered. It might be preferable to use the same HPLC system for all applications. Otherwise, equivalency of the systems is determined, which may require some changes in the chromatographic test conditions.]

Digest Stability—The length of time a digest can be kept before it is chromatographed, as well as the conditions under which the digest is stored before chromatography, is assessed. Several aliquots from a single digest are stored at different storage conditions and chromatographed. These maps are then evaluated for significant differences.

Reproducibility

Determination of various parameters indicated above is repeated using the same USP Reference Standard or Reference Material and test sample in at least two different laboratories by two analysts

equipped with similar HPLC systems. The generated peptide maps are evaluated for significant differences.

Add the following:

INTRODUCTION

Proteins can exist as large complex structures, with some molecules in the population displaying heterogeneity in their amino acid sequence due to improper assembly, degradation or post-translational modification. The high molecular mass of proteins combined with their complexity makes it particularly challenging to chemically identify an intact protein product using a single analytical method. It is possible to cleave the test protein into smaller fragments which can be identified with sufficient mass resolution to determine the amino acid sequence of the protein. This process is the basis of the protein identification technique commonly known as peptide mapping. The peptide mapping technique involves a digestion step in which the protein is selectively cleaved at amide bonds between specific amino acid residues to yield a predictable set of peptides. Analytical chromatographic separation, detection, and identification of the peptide mixture reveal information on the amino acid sequence of the protein which can be used to identify the protein. Peptide mapping is a comparative procedure; the results from the test protein are contrasted with the results of the reference standard or material similarly treated to determine the identity of the test protein. This comparative identification confirms that the primary structure of the test protein matches that of the reference protein.

Peptide mapping's ability to detect gross alterations in the primary structure has resulted in many applications for the determination of protein quality which are outside the scope of this chapter. The purity of the test protein with regard to amino acid misincorporation or other misassembly such as disulfide bond scrambling, post-translational modifications, and degradation can be determined using a quantitative peptide map. Peptide mapping comparison during scale up or manufacturing changes can support studies of process consistency. Additionally, peptide mapping can be used to determine the degree and specific amino acid location of modifications such as glycosylation and conjugation (e.g., degree of pegylation). The focus of this chapter will be on the use of peptide mapping for the chemical identification of a protein product where specificity is the primary attribute of the analytical method.

DEVELOPMENT OF A PEPTIDE MAPPING IDENTITY TEST PROCEDURE—POINTS TO CONSIDER

Prior to development of an identity test method procedure it is important to understand the application and level of specificity required to differentiate the identity of the test protein from other products processed in the same facility. In some instances orthogonal methods may be required to differentiate samples of structurally related proteins. Each protein presents unique characteristics that must be well understood so that the scientific approach used during development of the peptide map procedure will result in an analytical method that can be validated with sufficient specificity. The amino acid sequence of the test protein should be evaluated in order to select pretreatment and cleavage conditions resulting in optimal peptide length for analysis. Depending on application, complete or nearly complete sequence coverage is important, because there may be no prior knowledge of the alterations to the protein during development. The following points should be considered during development of a peptide mapping analytical technique. These elements are also presented graphically in Figure 1.

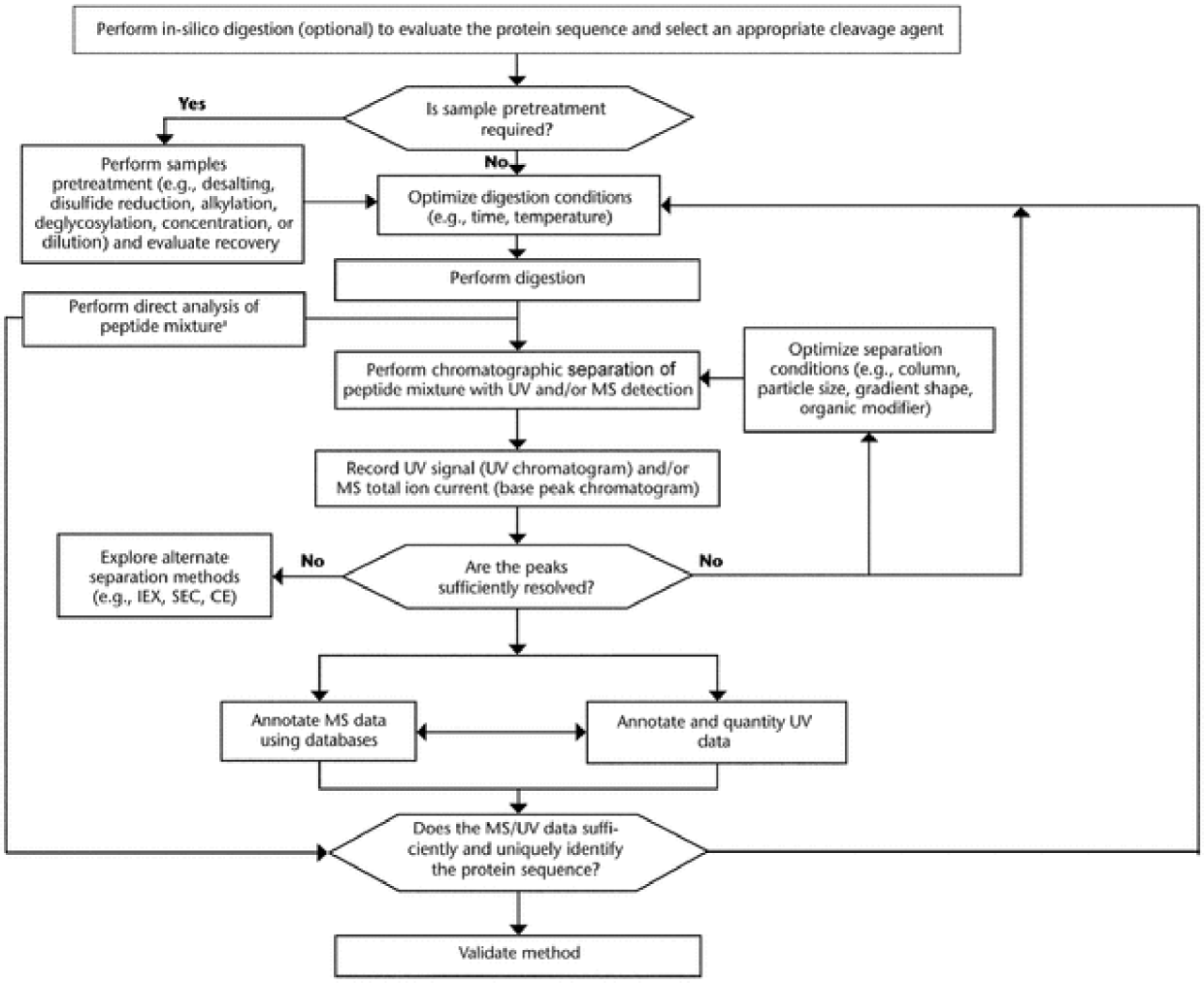

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Figure 1: Identify Peptide Map Method and Target Performance Parameters

PRETREATMENT

Isolation and purification may be necessary for analysis of bulk drugs, dosage forms, or reference standards or materials containing interfering excipients or carrier proteins. Residual interfering substances may impact enzymatic cleavage efficiency and appearance of the peptide map. The impact of residual substances or the sample purification process on the final test peptide map should be assessed during the development process.

The tertiary structure of proteins may hinder full access of the cleavage enzyme to all cleavage sites resulting in unacceptable sequence coverage. The treatment of proteins with chaotropic agents (e.g., guanidinium chloride, urea) and surfactants (e.g., sodium dodecyl sulfate) can be used to unfold the protein prior to digestion. Denaturing agents can affect enzyme activity and additional purification (e.g.diafiltration) or dilution steps may be needed prior to digestion. It may be necessary to reduce and alkylate the disulfide bonds prior to digestion in order to allow the enzyme to have full access to cleavage sites; however, the cysteine-to-cysteine linkage information is then lost. Common reagents for disulfide reduction include dithiothreitol and trialkylphosphine compounds such as tris (2-carboxyethyl) phosphine.

Reagents for alkylating reduced cysteines include iodoacetamide, iodoacetic acid, and 4-vinylpyridine. The use of alkylating agents may create adducts which will impact the chromatographic separation and alter molecular weight of affected peptide.

Since peptide mapping is a comparative procedure; any purification or pretreatment steps performed on the test protein must also be performed on the product reference standard or material. The impact of residual substances, purification procedures, or pretreatment of the protein on method specificity and precision should be investigated during development and considered for inclusion in robustness studies conducted for method validation.

DIGESTION

The choice of a cleavage technique is protein-dependent. Some of the more common cleavage agents, both enzymatic and chemical, and their specificity are shown in *Table 1*. There may be specific reasons for using other cleavage agents or combinations of methods.

Table 1. Examples of Cleavage Agents

Type	Agent	Specificity
Enzymatic	Trypsin, EC 3.4.21.4	C-terminal side of Arg and Lys
	Chymotrypsin, EC 3.4.21.1	C-terminal side of hydrophobic residues (e.g., Leu, Met, Ala, aromatics)
	Pepsin A (Pepsin), EC 3.4.23.1	Low-specificity digest
	Lysyl endopeptidase (Lys-Cendopeptidase), EC 3.4.21.50	C-terminal side of Lys
	Glutamyl endopeptidase (Glu-C endoproteinase; V8 protease); (from S. aureus strain V8), EC 3.4.21.19	C-terminal side of Glu and Asp
	Peptidyl-Asp metalloendopeptidase (Asp-N endopro-teinase), EC 3.4.24.33	N-terminalside of Asp
	Clostripain (Arg-C endopeptidase),EC3.4.22.8	C-terminal side of Arg
Chemical	Cyanogen bromide	C-terminal side of Met
	2-Nitro-5-thiocyanobenzoic acid	N-terminal side of Cys
	O-Iodosobenzoic acid	C-terminal side of Trp and Tyr
	Dilute acid	Asp and Pro
	3-Bromo-3-methyl-2-(2-nitrophenylthio-3H-indole (BNPS-skatole)	Trp

Factors that impact the effectiveness and reproducibility of protein digestion include pH, digestion buffer, temperature, time, and ratio of digest enzyme/reagent to protein. The optimal digestion mixture pH is generally determined by the enzyme or reagent. Chemical stability of the peptides including the

amino acid side chains and protein modifications at the selected pH must be considered. For example, a highly acidic environment (e.g., pH 2, formic acid) is necessary when using cyanogen bromide as a cleavage agent; however, a slightly alkaline environment (pH 8) is optimal when using trypsin as a cleavage agent.

The optimal temperature is dependent on the cleavage reagent; for example, most enzymes have optimum activity in a range of 25°–37° . The temperature can define the specificity of the enzyme to some extent. In these cases the adjustment of the temperature can be used to optimize the digestion conditions for certain proteins. Ideally, the digestion temperature will minimize sample-related chemical side reactions, such as deamidation, and protein aggregation while maximizing the susceptibility of the test protein to digestion while maintaining the activity of the cleavage agent.

It is necessary to ensure the digestion time is sufficient for intended use to avoid variable digests. A simple time-course study should be performed to ensure sufficient digestion with minimal peptide fragments resulting from partial digestion. Time of digestion varies from minutes to days and aliquots of a single reaction may be appropriately stabilized for analysis to determine the time required for complete digestion of the protein.

A sufficient cleavage agent should be used to attain the desired level of digestion within a practical time period (i.e., 2–20 h), while the amount of cleavage agent is minimized to avoid its contribution to the peptide map. For an enzymatic digest, the protein-to-protease mass ratio between 20:1 and 200:1 is generally used. In cases where the cleavage agent is unstable, the cleavage efficiency may be improved by making multiple additions of cleavage agent. Enzymes may be bound to a solid support to allow the use of higher relative amounts of protease while avoiding enzyme autolysis contamination and contribution of enzyme fragments to the peptide map. Chemical cleavage reagents are usually used in significant molar excess, and may need to be removed at the end of the digestion.

The optimal concentration of the test protein in the digestion should be empirically determined. The concentration should be low enough to minimize the potential aggregation of intact and partially digested proteins but must be sufficient to result in acceptable limit of detection of peptides following chromatographic separation with the selected detection method. Sample dilution or sample concentration by techniques such as centrifugal filtration may be required. Any dilution or concentration steps performed on the test protein must also be performed on the product reference standard or material. Protein recovery should be evaluated for any concentration step and the impact of dilution or concentration on method specificity and precision should be investigated during development and considered for inclusion in robustness studies conducted for method validation.

The digestion step can introduce ambiguities in the peptide map as a result of side reactions, such as nonspecific cleavage, deamidation, disulfide isomerization, oxidation of methionine residues, carbamylation of lysine residues, or formation of pyroglutamic groups created from the deamidation of glutamine at the *N*-terminus of a peptide. Autolysis may introduce extraneous peaks produced by the proteolytic enzyme digesting itself. The intensities of autolysis peptide peaks are dependent on the enzyme to substrate ratio and the modifications and quality of the enzyme used. To avoid autolysis reagent solutions of proteolytic enzymes should be prepared at a pH which inhibits enzyme activity or the reagent solutions should be prepared immediately before use. Modified enzymes, where changes are made to the protease to prevent autolysis, may be used. Commercial preparations of trypsin (often called "proteomics grade") are available in which the lysine residues of the enzyme have been methylated or acetylated to reduce the number of autolytic cleavage sites. To identify digestion artifacts, a blank determination is performed using a digestion control with all the reagents except the test protein.

SEPARATION

Chromatographic separation of the peptide mixture resulting from the digestion step is meant to resolve its complexity so that a valid interpretation of the data is meaningful and reproducible. The complexity of the peptide map will ultimately dictate the optimal set of chromatography conditions, column, and mobile phases. Method optimization experiments will be required to obtain the highest quality reproducible chromatogram. The molecular weight of the test protein will also influence the complexity of the map and the optimal separation.

Many techniques (e.g., ion-exchange high performance liquid chromatography [HPLC], hydrophobic interaction HPLC and capillary electrophoresis) have been used to separate peptides for peptide map analysis. However, reversed phase HPLC (RP-HPLC) is the most common method for the peptide mapping separation step and will be the focus of this chapter.

The selection of a chromatographic column is empirically determined for each protein. Columns with different pore sizes (80–1000Å) or nonporous based on silica, polymeric, or hybrid supports have been shown to give adequate separation. Columns with particle sizes <2 µm are available and are typically more efficient than those with 3–5 µm particle sizes. Generally, octyl or octadecylsilyl bonded phases are ideal for peptides. Octadecylsilane (C18) with 300Å or smaller pores is the most commonly employed bonded phase for the peptide mapping separation step.

The most common mobile phase for the RP-HPLC separation of peptides is water with acetonitrile as the organic modifier; however other organic modifiers such as methanol, isopropyl alcohol, or *n*-propyl alcohol can be employed. Solvents such as the propyl alcohols in the mobile phase may be useful for separating samples that contain many highly hydrophobic peptides; however, it should be noted that hydrophilic or small peptides may possibly elute in a column void volume. Mobile phase additives such as acids, bases, buffer salts, and ion-pairing reagents are generally needed to produce high quality chromatographic separations of peptides. The most common mobile phase additive has been trifluoroacetic acid (TFA) with typical concentrations of 0.05%–0.2% being employed. The use of phosphate as an additive is less common but can be useful in cases where UV detection is used. Volatile acids and salts can be used in the mobile phase to improve compatibility with mass spectrometer detection. While TFA has a significant positive impact on the quality of peptide separation, sensitivity with mass spectrometer detection can suffer with TFA due to ion suppression. Formic acid, acetic acid, or combinations of these with TFA increase mass spectrometer sensitivity by reducing ion suppression. Temperature control of the chromatographic column is necessary to achieve good reproducibility. The column temperature may be used to optimize peptide separation or improve the retention or elution of certain peptides since the resolution typically increases with temperature for a reversed-phase column.

DETECTION

While RP-HPLC is the most common separation method employed with peptide mapping for identity testing, the most common detection method is ultraviolet (UV) light absorption at 214 nm. The peptides resulting from protein digestion may not contain amino acids with aromatic side chains that absorb light at higher wavelengths (e.g., 280 nm) so detection at 214 nm (i.e., wavelength where peptide bonds absorb light) is essential to ensure sequence coverage of the protein while taking care to minimize background due to the mobile phase. Other detection methods may also be suitable.

The limitation of UV detection is that it provides no peptide structural information. Mass spectrometry is a useful detection method which provides mass information to aid in identification of peptides, as well as selectivity in cases when peptides co-elute. In most applications, the RP-HPLC effluent can be directly introduced into the mass spectrometer, provided that the mobile phase is compatible. Specific mobile phase considerations are dependent on the ionization method selected. Electrospray ionization (ESI) is the most common method for the introduction of proteins and peptides into the mass analyzer, and volatile, water-solvent mixtures provide the greatest ionization efficiency. Peptide mapping by ESI-MS is

most often performed in positive ion mode. Formic acid or acetic acid are commonly added to the mobile phase to reduce pH and thereby enhance protonation of the peptides. Buffers and salts should be minimized since they can reduce signal, and nonvolatile salts can deposit in the source. As mentioned previously, TFA should be avoided because it can result in ion suppression, a type of matrix interference, which may reduce the signal of some peptides, particularly when ESI is used. Ion suppression may also reduce the ionization efficiency of glycosylated peptides, resulting in reduced sensitivity. It is thus important to optimize conditions in order to achieve results for both UV and MS detection.

DATA ANALYSIS

Peptide mapping is a comparative procedure. To determine if the test protein is the desired protein of interest, the test protein's peptide map must be compared to the peptide map of the reference standard or material generated using identical pre-treatment, separation and detection procedures. Visual comparison of the retention times, the peak responses (the peak area or the peak height), the number of peaks, and the overall elution pattern is the first step of the procedure. It is a best practice to conduct a further non-subjective analysis of the peak response ratios of the critical peaks and the peak retention times. If all critical peaks in the test protein digest and in the reference standard or material digest have the same retention times and peak response ratios, then the identity of the test protein is confirmed. For example, peptide mapping tests for monoclonal antibody samples often include a common Fc peptide that is used as a reference peak. This reference peptide can be spiked into the sample digest and then peak response ratios of the critical peaks and retention times can be examined in comparison with the predefined acceptance criteria. The method of comparison selected should depend on the complexity of the resulting peptide map and the specificity required for the particular identity test application (e.g. differentiation between different protein products manufactured at the same facility or differentiation of variants of the same protein product).

When high specificity is required, a mass spectrometer can be used for routine analyses to provide insight into peptide modifications, truncations, missed cleavages, impurities, and unresolved co-eluting peak(s) under a single peak.

POINTS TO CONSIDER PRIOR TO VALIDATION

During the development of the peptide mapping procedure, knowledge and experience are gained that lead to selection of system suitability criteria and analytical method validation acceptance criteria. A final review of the procedure prior to validation can ensure that the procedure is ready for validation, reducing risk of failure to meet criteria. As a general procedure, peptide mapping may encompass a significant range of experimental designs, applications, and requirements for performance. As a consequence, in a general text, it is not possible to set out specific system suitability or validation criteria. The following elements are suggested for evaluation prior to starting the validation.

It should be noted that the scope of this document does not include routine application of mass spectroscopic (MS)–based peptide mapping applications; however, the application of mass spectrometry for structural identification of peptides during the development of peptide mapping methods is a best practice. Mass spectrometric detection can be utilized to evaluate the following performance parameters.

Coverage

Coverage refers to the percentage of the amino acid sequence identified in the peptide map to the target protein sequence. Although no specific figure can be identified for all applications, in many cases, coverage approaching 95% has been found to be an acceptable performance target for a peptide mapping procedure.

Specific Bond Cleavages

The specific bonds cleaved by the chosen enzyme or chemical digestion procedure should be identified and listed.

Major Peaks

The major peptides recovered from the specific bond cleavages should be identified and listed.

Partial Cleavages

Peptide bonds susceptible to partial or incomplete cleavage and their associated chromatographic peaks or signals should be identified.

Minor/Non-specific Cleavages

The extent of cleavage at non-specific bonds should be identified and limited or controlled.

Protease-derived Peaks

If a protease is used for the test protein digestion then any peaks above background derived from the protease should be identified and, where appropriate, limited.

Undigested "Core" Protein

Undigested or partially digested protein (often called "core") should be identified and limited.

Mean Peptide Length

It describes the peptide set produced by the combination of the chosen protease and/or chemical cleavage reagent and the test protein. This is a trade-off between smaller peptides, which show a higher level of structural selectivity with peptide mapping but produce a more complex map with more peaks, and longer peptides which produce simpler maps but with less resolving capacity for structural variants. No specific peptide length is suitable for all applications, but a mean peptide length of 10–20 residues is often considered appropriate.

Resolution Capacity

Resolution capacity refers to the capacity of the separation system to resolve the peptide set generated by the protease or chemical cleavage reagent. For example, a digest may produce 30 peptides but only 20 peaks due to co-elutions or nonrecoveries. Problematic separations should be identified and resolved by appropriate chromatographic procedures and, if necessary, controlled by the use of peptide reference standard or material or system performance criteria.

System Suitability Criteria Selection

System suitability criteria should be developed to ensure that the elements of the procedure for protein digestion, separation, and detection have successfully provided a structural identification of the test protein at the level of unambiguity required for the application. System suitability criteria evaluated during routine analysis for identity tests will typically include an assessment of the reference protein digest chromatogram and may include such performance characteristics as:

- Qualitative similarity to reference chromatogram
- Extent of digestion
- Partial cleavages
- Non-specific cleavages
- Peak heights/signal-to-noise ratio
- Peak shape
- Peak retention time
- Resolution of specific peaks

For test method procedures that require sample isolation, purification, or concentration. a sample recovery criteria should be determined and included as part of the system suitability assessment. In cases where digestion artifacts may be present, assessment of a blank digestion control may be needed to demonstrate a lack of interference.

VALIDATION

Before validating a peptide mapping procedure, the procedure should have been developed to its final form and documented with system suitability criteria. Each time the procedure is performed the results are evaluated against the system suitability criteria to determine if the procedure has successfully provided reproducible results consistent with previous testing instances. Pre-approved acceptance criteria often evolve based on the system suitability criteria of the procedure. The elements of the analytical validation protocol are as follows:

Specificity

Method performance requirements will vary depending on the application of the identity test method and may require a risk assessment to understand what degree of specificity is needed to differentiate the identity of the test protein from other products processed in the same facility. Peptide mapping is a comparative technique confirming that the primary structure of the test protein matches that of the reference protein. Specificity is established by the comparison of the peptide maps of a suitable reference standard or material and samples of structurally related proteins. The selection of comparator samples should be selected based on a risk assessment of other products processed in the same facility and should be documented in the validation protocol. In order to minimize the inherent variability of the test, the procedure is executed on reference standard or material and test protein during the same testing instance. A peptide mapping test design that analyzes the test protein digest, reference standard or material digest, and a 1:1 (v/v) comixture of the test protein and reference standard or material after digestion is a useful specificity validation experiment. Occasionally a peak can appear in a test protein's peptide map that elutes at a slightly different retention time than the corresponding peak in the reference standard or material peptide map, leading the analyst to judge the peaks as nonidentical. Testing a co-mixture sample during the specificity validation experiment can demonstrate that two peaks are identical if they co-elute in the co-mixture peptide map and confirm the identity. Chemically modified forms of the reference standard or material can be produced by exposure to conditions of pH, temperature, or chemical agents known to cause alteration of the primary structure. These alterations typically include deamidation of asparagine and glutamine residues, oxidation of methionine, histidine, or tryptophan residues, and acid catalyzed cleavage of peptide bonds. Peptide maps of a chemically modified reference standard or material and the reference standard or material can be compared based on predetermined acceptance criteria to demonstrate if the specificity of the peptide mapping procedure is affected by amino acid side chain modifications.

Precision

To facilitate the determination of the precision (repeatability and intermediate precision) of the peptide mapping procedure, an empirical method of quantifying peak responses (peak areas or peak heights) and peak retention factor should be part of the procedure. One approach is to make peak response and peak retention time comparisons that are expressed relative to a highly reproducible reference peak within the same chromatogram. The precision results obtained during the analytical procedure validation are reported and should meet the acceptance criteria of the validation. Failure of the precision results to meet the acceptance criteria can lead the analyst to reassess the digestion and/or separation steps in the procedure.

Robustness

Robustness may be evaluated during the development of analytical procedures. It is not necessarily repeated, but it may be included as part of method validation. Factors such as composition of the mobile phase, protease quality or chemical reagent purity, column variation and age, digestion temperature, and digest stability are likely to affect the overall performance of the test and its reproducibility. Tolerances for each of the key parameters are evaluated and baseline limits established in case the test is used for routine lot release purposes.

Variations in purification, pretreatment, dilution, or concentration procedures of the protein sample can have impact on recovery, test system and the chromatogram. The variations and their impacts should be identified during the development process and controlled. Impact of residual substances remaining after sample preparation on method specificity and precision should be considered. Critical parameters identified during development should be included in robustness studies conducted for method validation.

Many protein fragmentation strategies employ the use of proteolytic enzymes. As a result, the digestion portion of the peptide mapping procedure is inherently more sensitive to minor variation of test parameters. These parameters may include all or a subset of the following: digestion pH, buffer, buffer concentration, ionic strength, digestion temperature, digestion kinetics, test protein concentration, protease quantity, protease quality, and the stability of the digest. Using a design-of-experiments approach, the identified critical parameters are systematically studied to understand their impact on method variability. Those digestion parameters where small variations have been shown to impact the precision of the peptide mapping procedure should be carefully controlled within the test procedure using operating ranges established and validated by these studies.

To evaluate the protease quality or chemical reagent purity a sample of the reference standard or material is prepared and digested with different lots of cleavage agent. The chromatograms for each digest are compared in terms of peak areas, peak shape, and number. The same procedure can be applied to other critical chemicals or pretreatment procedures used during sample preparation, such as reducing and *S*-carboxymethylation reagents.

The length of time a digest can be held before proceeding to the separation step of the procedure, as well as the conditions under which the digest is stored before separation, are assessed. Several aliquots from a single digest are stored under different storage conditions and resolved by the chromatographic method. These maps are then evaluated for significant differences.

During the separation step column-to-column variability, even within a single column lot, can affect the performance of the peptide mapping procedure. To evaluate column lot differences, reference standard or material of the protein of interest is digested and the digest is subjected to separation using different column lots from a single manufacturer. The resulting peptide maps are then evaluated in terms of the overall elution profile, retention times, and resolution according to predetermined acceptance criteria.

To evaluate the lifetime of a column in terms of robustness, a single digest of the reference standard or material can be analyzed using the peptide mapping procedure with columns that vary by the injection number history (e.g., 10 injections per column to 250 injections per column). The resulting peptide maps can then be compared for significant differences in peak broadening and overall resolution. As a column ages, an increase in back pressure might be observed that can affect the peptide map. System suitability or assay validity criteria can be designed to be diagnostic of column aging or other events that may affect the peptide mapping results.

SUMMARY

The peptide mapping procedure consists of multiple steps possibly including protein isolation, denaturation, chemical modification (e.g., blocking sulfhydryl groups) if necessary, protein digestion, peptide separation and detection, and data analysis. Each step should be optimized during development

to result in a well-qualified analytical procedure for the peptide mapping identity test. In combination with the use of a suitable reference standard or material, system suitability criteria should be chosen that evaluate if all the steps in the procedure worked together properly to produce a successful peptide map of that reference standard or material that is consistent with the validation of the analytical procedure. When properly developed, validated, and performed, the analytical peptide mapping procedure can be used to verify the identity of the test protein which is a critical quality attribute of the product.♦▲ (USP 1-Dec-2024)

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