Tackling the Challenging Development and Validation of Peptide Methods

Claire E. Brook, Ph.D.
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Outline

• Introduction to peptides
• Developing suitable methods for peptides
• Validating peptide methods
• Interactive exercise
Introduction to Peptides

- What are peptides?
- Why are peptides interesting?
- Examine the (sometimes) unique challenges of developing methods for peptides
- Look at U.S. Pharmacopeia (USP) and European Pharmacopeia (EP) requirements for peptide analysis
- Learn the differences that occur in the analysis of peptides and small molecules

What are Peptides?

- Two or more amino acids linked by a peptide bond
- Condensation of –COOH of one amino acid and the –NH₂ of another
- Polymeric chain of up to 50 amino acids
- Amino acid chains longer than 50 are proteins
- A specific peptide has a unique amino acid sequence
- Two condensed peptides make up a polypeptide
Amino Acid Structure

• Four species attached to a central carbon
  – Acid (-COOH)
  – Amine (-NH₂)
  – Hydrogen
  – Side chain (-R)

• Different side chains make different amino acids

• Glycine is the most simple with –R = -H, and all but glycine have a chiral center and are optically active

• L-amino acids are the natural form
Formation of the Peptide Bond

Aspartame

- Dipeptide of aspartic acid and modified phenylalanine
- Aspartic acid and phenylalanine not sweet, but as aspartame 200x sweeter than sugar (sucrose)
- Phenylalanine warning label
- Transformation of Phe-Tyr
Peptide General Structure

Peptide Classification

Peptides are often classified based upon their source:

- Milk
- Ribosomal
- Non-ribosomal
- Peptones
- Peptide fragments
Peptide Pros and Cons

PEPTIDE PROS AND CONS

ADVANTAGES
High activity
High specificity
Little unspecific binding to molecular structures other than desired target
Minimization of drug-drug interactions
Less accumulation in tissues
Lower toxicity
Often very potent
Biological & chemical diversity

DISADVANTAGES
Low oral bioavailability
Injection required
Less stable
Difficult delivery; challenge to transport across membranes
Challenging & costly synthesis
Solubility challenges
Risk of immunogenic effects
Cleared from body quickly

SOURCES: Frost & Sullivan, D&M Publications, Roche, SAM Ventures, and Richard DiMarchi

C&E News March 14, 2005 Volume 83, Number 11

Common Peptides in Use

Table X: Potential peptides and protein drug candidates for oral-colon-specific delivery systems.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Therapeutic Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylin</td>
<td>Diabetes and nutrition regulation</td>
</tr>
<tr>
<td>Antiviral oligonucleotides</td>
<td>Cancer and AIDS</td>
</tr>
<tr>
<td>Calcitonin</td>
<td>Paget's disease of bone, hypercalcemia</td>
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<tr>
<td>Cidoacin</td>
<td>Immunosuppressant</td>
</tr>
<tr>
<td>Desmopressin</td>
<td>Pituitary diabetes insipidus</td>
</tr>
<tr>
<td>Epinephrin</td>
<td>Arterial associated with chronic renal failure</td>
</tr>
<tr>
<td>Etanercept</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>Glucagon</td>
<td>Chronic intractable hypoglycemia</td>
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<tr>
<td>Glucoribose</td>
<td>Endothelins, inlarity</td>
</tr>
<tr>
<td>Insulin</td>
<td>Type I diabetes</td>
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<tr>
<td>Interferon</td>
<td>Prophylaxis for hepatitis, malignancy</td>
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<tr>
<td>Lanzoprazole</td>
<td>Intestinly, prostate carcinoma</td>
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<tr>
<td>Asparaginase</td>
<td>Neutropenia</td>
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<tr>
<td>Moiprimadine</td>
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<tr>
<td>Octreotide</td>
<td>Pancreatitis, acromegaly</td>
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<tr>
<td>Protekin</td>
<td>Endothelins and inlarity</td>
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<tr>
<td>Semeradin</td>
<td>Endothelins and inlarity</td>
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<td>Setodomin</td>
<td>Paget's disease of bone, hypercalcemia</td>
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<td>Somatopin</td>
<td>Turner's syndrome, dihydro</td>
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<td>Unitekin</td>
<td>Intensity</td>
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<tr>
<td>Vasopressin</td>
<td>Pituitary diabetes insipidus</td>
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Drugs Today 1999, 35(7): 537
Pharmacopoeial Peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Source</th>
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<tbody>
<tr>
<td>Bacitracin, Bacitracin zinc</td>
<td>Fermentation</td>
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<tr>
<td>Calcitonin acetate, salmon</td>
<td>Chemical synthesis, rDNA technology</td>
</tr>
<tr>
<td>Colimembehate sodium</td>
<td>Semi-synthetic, fermentation</td>
</tr>
<tr>
<td>Desmopressin acetate</td>
<td>Chemical synthesis</td>
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<tr>
<td>Felypressin acetate</td>
<td>Chemical synthesis</td>
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<tr>
<td>Glucagon, human</td>
<td>rDNA technology</td>
</tr>
<tr>
<td>Glucagon, porcine &amp; bovine</td>
<td>Pancreas extraction</td>
</tr>
<tr>
<td>Gonadorelin acetate, hydrochloride</td>
<td>Chemical synthesis</td>
</tr>
<tr>
<td>Gonadorelin acetate</td>
<td>Chemical synthesis</td>
</tr>
<tr>
<td>Gramicidin</td>
<td>Fermentation</td>
</tr>
<tr>
<td>Insulin aspart, lispro</td>
<td>rDNA technology</td>
</tr>
<tr>
<td>Insulin, bovine &amp; porcine</td>
<td>Pancreas extraction</td>
</tr>
<tr>
<td>Insulin, human</td>
<td>Pancreas extraction and enzymatic modification</td>
</tr>
<tr>
<td>Leuprolin acetate</td>
<td>Chemical synthesis</td>
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<tr>
<td>Octreotide acetate</td>
<td>Chemical synthesis</td>
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<tr>
<td>Polymyxin B sulphate</td>
<td>Fermentation</td>
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<td>Proline acetate</td>
<td>Chemical synthesis</td>
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<tr>
<td>Somatostatin acetate</td>
<td>Chemical synthesis</td>
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<tr>
<td>Tetracosactide acetate</td>
<td>Chemical synthesis</td>
</tr>
<tr>
<td>Vasopressin</td>
<td>Fermentation</td>
</tr>
<tr>
<td>Vasopressin</td>
<td>Chemical synthesis and phytoextraction</td>
</tr>
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</table>

Quality specifications for peptide drugs: a regulatory-pharmaceutical approach
Journal of Peptide Science Volume 15, Issue 11, Pages 697 - 710

Schematic of Peptide Synthesis

Dan Cojocari, Dept. of Medical Biophysics, University of Toronto 2009
Why Peptides Have Special Analytical Requirements

• There are different production possibilities:
  – Chemical synthesis
  – Fermentation
  – Tissue extraction
  – rDNA technology

• All have impurities resulting from their preparation

• Impurities from the preparation method will likely vary

• Side chain covalent bonds – di-cys

• Zwitterions – pH a real concern when developing methods

Peptide Challenges

• Solubility is often one of the major challenges for researchers who work with peptides

• Each amino acid exhibits its own physical characteristics

• Amino acids such as leucine, isoleucine, and valine are very hydrophobic

• Other amino acids like lysine, histidine, and arginine are hydrophilic
Solution Strategies

• As a general rule, peptides should first be dissolved in distilled, preferably sterile water. If solubility still remains a problem, try the following steps:
  
  • Sonication

  • Add small amounts of dilute aqueous acetic acid for basic peptides or aqueous ammonia for acidic peptides

  • Dissolve the peptide to the highest possible concentration, and then diluted with water or buffer to the working concentration. (Note: be careful to add buffer after the peptide is completely in solution to reduce the chance of aggregation)

Peptide Storage

• Peptides are often in lyophilized powder form and often hygroscopic

• Absorption of water will decrease stability of the peptide and may reduce overall peptide content

• For best results:
  
  - Store in closed dry environment with desiccants

  - Always store lyophilized peptide in a freezer at least -20°C for maximum stability

  - Avoid using a frost-free freezer. Changes in moisture and temperature may affect peptide stability
Developing Suitable Methods for Peptides

- Methods required for peptide analysis
- Example standard USP/EP methods
- Transfer in-house of USP/EP methods
- Developing methods from scratch
- New developments in analytical technology for peptides

Quality Assurance

- Two components - Quality Assurance (QA) and Quality Control (QC)
- QA oversees the whole process
- QC provides analytical testing development and validations
- QC early on is responsible for providing ways to assess the purity and structure of peptides and its stability.
- QC is the focus of this look at peptides.
Quality Control

- Integral to manufacturing - from raw material, through the production process to the final product for release
- API’s are typically regulated by:
  - ICH (International Conference on Harmonization)
  - FDA (Food and Drug Administration)
  - EMEA (European Agency for the evaluation of Medical Products)
- Quality control produces methods to evaluate the peptides and is the source for reference standards

General EP Requirements for Peptide Analysis

- Identification
  - Confirmation of the peptide
- Tests
  - Limit tests on process and drug related organic impurities
  - Inorganic impurities
  - Residual solvents
  - Microbiological
- Assay
  - Single method also stability indicating
### Typical Peptide Impurities

<table>
<thead>
<tr>
<th>Impurity Type</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typical Peptide Impurities</td>
<td>Defined by pharmaceutical manufacturers</td>
<td>USP, BP, JP</td>
</tr>
</tbody>
</table>

Quality specifications for peptide drugs: a regulatory-pharmaceutical approach.

Assay

- Must be able to detect enantiomers, deletions, deamidations/acetylations
- Looking for >97% purity with no single impurity >1%
- Often have to synthesize the individual impurities
- Stability indicating separation and detection of degradants, may use heat stress to create these

Example Assay Chromatogram
Close-up of Example Chromatogram

Final Product & Release Testing
Fully Characterizing the Peptide

• Identification - No single simple test to identify the peptide (Nuclear magnetic resonance not simple), so usually a combination including mass spectrometry, amino acid analysis and liquid chromatography is used.

• Mass Spectrometry - Looks for the monoisotopic mass. Requires resolution of <1a.m.u. Can be used to determine the amino acid sequence.

• Amino Acid Analysis - Peptide hydrolysis, then High-Performance Liquid Chromatography (HPLC) ion exchange and then ninhydrin post-column. Limitations: everything must be quantified and a quantified reference used. This is difficult as the National Institute of Standards and Technology standard is accurate between 2.9 and 4.8% depending on the amino.

• HPLC - Requirements for developing assay for in-process

Example of Residual Solvent Method

• 1mL of Class II A was added to a 100mL volumetric flask and then brought to volume with reagent water and mixed

• 1ml of the solution was transferred to a 20 ml headspace vial containing 5 ml of reagent grade water

• The vial was sealed and mixed prior to analysis
Inorganic Impurities

- Sulphated ash/residue on ignition/heavy metals largely missing from recent monographs
- Palladium (residual catalyst) is of particular interest and suitable levels are determined from expected daily dosage amounts (typically 1-10ppm of Palladium allowed)
- A full metal analysis is likely to be incorporated into future monographs (Inductively coupled plasma optical emission spectrometry or mass spectrometry) due to the likelihood of peptides binding trace metal amounts
- Also heavy metals can be determined by thioacetamide, which is sensitive, specific and reproducible for determination and encompasses all elements of interest.
Water and Acetic Acid

- Required as both are indicative of long-term stability of the peptide, also large percentage, so required for formulation and economic determination.

- Water – Karl Fisher coulometric
  Loss on Drying (non-synthetic peptides)

- Acetic – Liquid chromatography or Ion chromatography
  Typically 3-14% depending on peptide with EP and USP 2.0 to 8.0%
  But is related to the number of positively charged aminos in the peptide and is normalized with the weight.

- Strange example – Gonadorelin acetate – EP 4-7.5%, USP 8-12%
  (mono and diacetate forms)
Absorbance - Ultraviolet (UV) Region

- EP requires the UV absorbance to be determined at 278 nm for 4 peptides, mainly looking for tryptophan
- Goserelin and leuprorelin no longer require this testing as they are both very similar in structure
- Largely another obsolete method for determining the presence of impurities
Microbiology

• Less likely to be found in synthetics, but would still be involved in product breakdown or contamination

• Total aerobic microbial count should be less than 10^3 CFU/g

• Total yeast and mold count should be less than 10^2 CFU/g

• Must meet the EP/USP definition if defined as sterile

Immunogenicity & Bioidentity

• Only in the EP for insulin from animal pancreas

• This was the historical method for production, but not as usual for this to be the manner of production

• More of a problem for proteins, but truncated peptides could be more so than parental peptides, so if <1% impure considered OK

• Bioidentity in 4 USP monographs, not for EP (abandoned 20 years ago)

• Most common is rabbit testing with insulin to check response

• If fully chemically derived, the manufacturing defines the product making bioidentity obsolete
Optical Rotation & Other Tests

- Appears to be largely an historical requirement
- In the EP 12, (mainly synthetic peptides) monographs require OK but have a large variation in the result (2-6 degrees absolute)
- No longer major format for defining impurities in synthetics
- Usually product specific, e.g. pH or solution appearance
- Probably relates to the final formulation format of the product

Mass Balance

- Assay + counterion = 100% (+/- 5%)
- Shows no significant quantities for inorganics or other impurities
- A lot of peptides are hygroscopic so this is tough
Validating Peptide Methods

- Writing clear validation protocols and reports
- Validating a suitable assay method for in-process and/or final product testing
- Discerning the differences between assay/purity methods for stability trials
- Discovering the genesis of common impurities in all methods
- Validating methods that stand the test of time

Method Development

Screen → Optimize → Pre-validation → Validation

Screen:
- Column
- pH, buffer
- Modifier
- \( \lambda_{\text{Max}} \)

Optimize:
- Gradient
- Temperature
  - Column
  - Sample

Pre-validation:
- Specificity
- Accuracy
- Precision
- Robustness

Validation:
- Linearity & range
- Accuracy
- Intermediate Precision
- LOD
- Robustness
Standard Addition

- Need to look at many examples before starting validation
- Must ensure homogeneous samples with validation
- Ensure ability to accurately add the amount required
  - Not too small an amount that accuracy is compromised
  - Need to obtain analyte in highest purity possible
  - If there are impurities in analyte, they don't compromise the analytical method

Standard Addition

- Especially useful if there is no possibility of an uncontaminated 'blank'

![Graph showing the relationship between amount added and response.](image)
Typical Peptide Monograph

Acetate

C_{55}H_{75}N_{17}O_{13} \cdot xC_2H_4O_2 \cdot yH_2O \quad 1182.3

Luteinizing hormone-releasing factor acetate (salt) hydrate.
5-Oxo-L-prolyl-L-histidyl-L-tryptophyl-L-seryl-L-tyrosylglycyl-L-leucyl-L-arginyl-L-prolylglycinamide acetate (salt) hydrate \[52699-48-6; 33515-09-2]\.

> Gonadorelin acetate is a synthetic polypeptide hormone having the property of stimulating the release of the luteinizing hormone from the hypothalamus. It contains not less than 80 percent by weight of C_{55}H_{75}N_{17}O_{13}, the remainder being acetic acid and water.

NOTE—Gonadorelin acetate is extremely hygroscopic. Protect from exposure to moisture, and store in a desiccator.

Packaging and storage—Preserve in tight, well-sealed containers, protected from moisture. Store at a temperature of not more than 8.

Labeling—Label it to indicate it is for veterinary use only.

Typical Peptide Monograph (cont’d)

Identification-

A: The monoisotopic mass by Mass Spectrometry is 1181.6 \pm 1 mass units.

B: The retention time of the major peak in the chromatogram of the Test solution corresponds to that in the chromatogram of the Standard solution, as obtained in the test for Related compounds.

Specific Rotation: between –54 and –66, at 20, calculated with reference to the peptide content determined in the Assay.
Test solution: 10 mg per mL, in 1% (v/v) acetic acid.

Water: not more than 7.0%, determined by directly introducing not less than 2 mg of the solid substance into the titrator.
Typical Peptide Monograph (cont’d)

Related compounds—

Standard solution—Dissolve an accurately weighed quantity of USP Gonadorelin Acetate RS in water to obtain a solution having a known concentration of about 0.5 mg per mL.

System suitability solution—Dissolve an accurately weighed quantity of USP Gonadorelin Acetate Related Compound in water to obtain a solution having a known concentration of about 0.5 mg per mL. Mix equal volumes of this solution and the Standard solution.

Test solution—Dissolve an accurately weighed quantity of Gonadorelin Acetate in water to obtain a solution having a known concentration of about 0.5 mg per mL.

System 1—
Solvent 1—Mix 1 mL of trifluoroacetic acid with 1 L of water. Pass through a 0.45-µm filter, and degas.
Solvent 2—Mix 1 mL of trifluoroacetic acid with 1 L of acetonitrile.
Solution A—Prepare a mixture of Solvent 1 and Solvent 2 (95:5).
Solution B—Prepare a mixture of Solvent 2 and Solvent 1 (60:40).

Chromatographic system - The HPLC is equipped with a 220-nm detector and a 4.6-mm × 25-cm column that contains 5-µm packing L1. The flow rate is approximately 1.5 mL per minute. Chromatogram programming information included.
**Writing the Protocol**

- Determine the signatures required – QA, QC, R&D, Manufacturing
- Include EVERYTHING needed
- Clearly define requirements to pass validation
- Write clear and detailed instructions (inc. calculations)
- Note that any additional experiments can be added as required
- Utilize a 2nd analyst and if possible a 2nd instrument and column
- Include people that will be involved in running the method in the validation
- Define suitable chemicals and equipment
- Before signing protocol have 2nd analyst review
- Review time requirements

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**Report Writing**

- Make as clear as possible
- Use the protocol as a template
- Define the pass/fail criteria and list if the run fulfills the criteria
- Include example chromatograms/spectrograms/pertinent information
- Outline full information on equipment used, chemical lots, columns, etc.
- Detail the notebooks where the information is stored, ensure they are completed
- List where the data files are stored
- Create validation pack including spreadsheets, calculations, copies of notebook pages
- Have 2nd analyst review before sending for signatures to complete
## Projected Monograph Proposal for Peptides

<table>
<thead>
<tr>
<th>Quality attribute</th>
<th>Typical method</th>
<th>Typical acceptance limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appearance</td>
<td>Visual</td>
<td>None or visible</td>
</tr>
<tr>
<td>Solubility</td>
<td>Solubility test</td>
<td>Solubility is required</td>
</tr>
<tr>
<td>Specific activity</td>
<td>Specific activity test</td>
<td>Specific activity is required</td>
</tr>
<tr>
<td>General purity test</td>
<td>RP-HPLC/UV</td>
<td>Retention time analysis</td>
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<tr>
<td>Counter ion content</td>
<td>Ion Chromatography</td>
<td>Counter ion content is required</td>
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<tr>
<td>Water content</td>
<td>Karl Fisch</td>
<td>Water content is required</td>
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<tr>
<td>For use in injectable preparation</td>
<td>YMC plate column</td>
<td>Counter ion content is required</td>
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<tr>
<td>Assay of peptide-free water</td>
<td>Phenomenex Lux Column</td>
<td>HPLC analysis of water is required</td>
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<tr>
<td>Buffers</td>
<td>EMF test</td>
<td>pH is required</td>
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<tr>
<td>Stability</td>
<td>Stability test</td>
<td>Stability is required</td>
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<tr>
<td>Synthesis-specific purity test</td>
<td>SEC on HPLC/UV</td>
<td>Counter ion content is required</td>
</tr>
</tbody>
</table>

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