

Analytical Techniques: Strengths & Limitations

- Analytical Characterization Programs (Process & Product)
 - Master Cell Bank (MCB)
 - Manufacturer's Working Cell Bank (MWCB)
 - Process Development: In-Process Control (IPC) Testing
 - Bulk API (active pharmaceutical ingredient)
 - Finished Product
 - Stability
 - Bioassay Method Development & Validation
 - Analytical Methods Development & Validation
 - Structure-Activity Relationships (SAR) Database and Quantitative SAR
 - Demonstrating therapeutic equivalence via analytical, PK/PD, and Surrogate Endpoints
- Analytical Equivalence:
 - Methods Overview: Purity, Potency, Safety
 - Characterization Testing
 - Bioassay Equivalence: Points to Consider
 - Comparability Protocols - Characterization Testing vs. Release Testing
- Viral Validation Methods & Limitations
- Integration of Analytical Testing Profile to Manufacturing and Stability Arenas
- Summary of Key Points

For almost a century, biologics have been regulated a world apart from classically synthesized drugs - evident in public health law, FDA policy, and industry practice - reflecting a history of substances that were complex and difficult to characterize and/or used potentially contaminated source materials. **Limited analytical tools and product-specific processes** galvanized the long-standing approach that biologics were defined by the manufacturing process. Thus, the "process = product" dogma permeated throughout biologics development, leaving it with some unique milestones. Changing the process often required additional clinical studies to demonstrate equivalency. It was probably this distinction - more than anything else - that excluded biologics from Title I but not Title II of the formal ANDA process⁴. However, this logic is being challenged with the emergence of the 'specified (aka, well-characterized) biologic.' Combined impact of FDAMA, REGO, improved bioanalytical methods, FDA's increased familiarity with recombinant DNA product safety, and GMP issues have allowed for greater harmonized review between the Center for Drug Evaluation (CDER) and CBER for specified biologic drugs. Managed health care, greater patient access to new therapies, and saving money - have created an impetus for faster approvals of both biologics and drugs driving greater consistency in their regulation - as seen with FDAMA (e.g., Section 123(f)). At the heart of these faster reviews and approvals is a comparability program based on analytical links and correlation of SAR to pivotal safety and efficacy.

Analytical Characterization Programs

- Master Cell Bank (MCB) & Manufacturer's Working Cell Bank (MWCB)
 - Plasmid fidelity
 - Host cell system stability in expression of protein over time and generations (e.g., end-of-production cells or late-extended cells)
 - Demonstrated absence of adventitious agents
- Process Development: In-Process Control (IPC) Testing
 - Process consistency and reproducibility across different same scale of production
 - Demonstrating product is not near and "edge of failure" with process parameters
 - Links of product characteristics to process control
 - Product consistency across multiple scales of production (comparability studies)
- Bulk API (active pharmaceutical ingredient)
 - Characterization testing & release testing
 - Stability of material during handling and storage
- Finished Product
 - Meeting product characteristics that relate/correlate to SAR and safety & efficacy
 - Stability of finished product
 - Compatibility with immediate container

See preceding page for detailed listing of components for each section of the analytical characterization programs. Specifics of MCB and MWCB characterization are detailed in numerous FDA, ICH, and CPMP guidances (see CD ROM with The Library). Process development is more specific to the product class. One needs to review other products from that same class to understand regulatory concerns (e.g., PKA in IGIV fractionation, host cell DNA in recombinant proteins, aggregated forms with monoclonals, etc.). Perhaps one of the most critical areas in process development is knowing what "edge of failure" parameters are and how close your validated process strays towards them. Some IGIV fractionation methods may be very vigorous in stirring and the increased agitation causes increase PKA formation - which is associated with hypotension upon infusion. Other processes may involve prolonged exposure of methionine-containing peptides to oxygen - leading to oxidized forms or inactive forms. Thus, it's vital to explore why product doesn't work as much as it is to ascertain when it does. Only then can one distinguish a "causal" versus "casual" relationship of process parameters to product performance. This also goes back to principle component analysis (PCA) and neural nets.

For bulk API and finished product, analytical support for release testing is usually three-fold: establishing a reference standard with penultimate characterization testing, establishing extensive release testing for finished product over development for comparability assessment and SAR database, and release/stability specifications for market applications. Where compendial test methods exist, there may be an additional burden of showing cross-over validation studies for non-compendial methods (e.g., using an ELISA for measles antibody instead of the neutralizing antibody test in the CFR).

Analytical Characterization Programs

- Stability
 - Meeting product characteristics over time
 - Characterization testing
 - Comparison to innovator material or pre-change material within same manufacturer
- Bioassay Method Development & Validation
 - SAR links to bioassay activity
 - Comparability to innovator material or pre-change material within same manufacturer
- Analytical Methods Development & Validation
 - Purity
 - Potency
 - Safety
 - Stability-indicating
- Structure-Activity Relationships (SAR) Database and Quantitative SAR
 - Links to manufacturing changes & process controls
 - Links to API & finished product release testing & stability
 - Links to bioassay, PK/PD data, and surrogate endpoints

Stability is a key area that supports comparability assessments. While FDA doesn't use any hard and fast rules regarding the 'amount' of stability data needed for a particular change, firms will typically have 3-month data or longer depending on how big the change. In the multisource biotech debate, it will also be interesting to see how much FDA demands in stability comparability - even though they claim comparable release characterization data is not enough. It will be up to the sponsor to anticipate these questions and have SAR data in hand with both your product and comparator product. Forced degradation studies also invaluable for generating impurity standards that can be used in bioassays and determine what percentage of aggregated, deamidated, oxidized, photolyzed, etc. product is associated with reduced activity.

Bioassay method development and validation - including immunological assays - are highly sensitive to variations in operator, technique, microtiter plate location of reference standards, etc. This will be a heavily scrutinized area by FDA - especially if you rely upon it for correlation to efficacy or surrogate endpoints. It's better to have a sensitive assay (with no relation to surrogate endpoints) than an insensitive one that relates to proposed mechanisms in vivo. If your firm lacks in-house capability, search carefully for firms that can develop and validate your assay; maybe can move in-house later.

Method validation is discussed in numerous FDA, ICH, and CPMP guidances (see The Library/Analytical Methods).

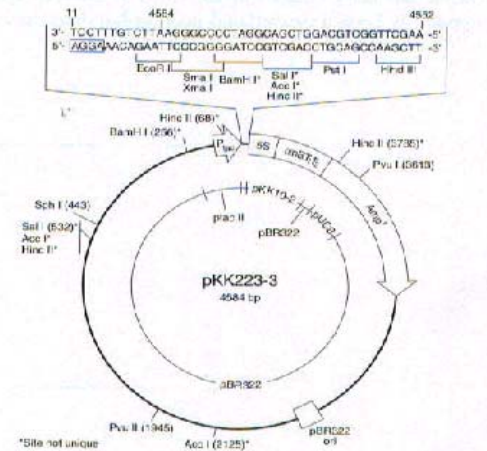
Analytical Equivalence: Methods Overview

- Products often assessed for **purity, potency, and safety** - and testing reflects this holy trinity, such as:
- **Purity (physico-chemical characterization)** of drug substance assessed by:
 - Amino acid analysis (e.g., AA sequencing (entire sequence or amino- and carboxy-terminal sequences))
 - Peptide mapping via restriction enzymatic digestion/ fragment analysis
 - Determination of sulfide linkage
 - Circular Dichroism
 - Chromatography: HPLC (chiral, reverse phase, size exclusion, ion-exchange), GC (GC-MS, GC-FID), TLC, etc.
 - Spectroscopy: MS, MALDI-TOF, EI, CI, FAB, ESI, NMR, FT-NIR,
 - Electrophoresis (reduced and non-reduced): SDS PAGE
 - Immunochemical analysis (e.g., ELISA, RIA, EIA, RID (radial immunodiffusion), flow cytometry, Western Blot, Southern Blot, Northern Blot, etc.)
 - Microheterogeneity analysis (e.g., glycosylation profiling, IgG sub-types)
- **Potency** assessed via bioassays (compendial or proprietary) and PK/PD links
- **Safety** assessed via sterility, bioburden, LAL, residual host DNA, impurities, immunogenicity, product-specific toxicities, etc.

Additional characterization may be required for products undergoing post-translational modifications such as glycosylation, sulfation, phosphorylation, or formylation. Additional characterization may be required for products derivatized with other agents, such as other proteins, toxins/ toxoids, drugs, radionuclides, or chemicals. The information should include the degree of derivatization or conjugation, the amount of unmodified product, removal of free materials, and the stability of the modified product. Safety assessments of monoclonal antibodies and some proteins include immunogenicity and formation of neutralizing antibodies or neo-antigens. Other antigen-antibody complexes may precipitate out of circulation and become deposited in pulmonary or vascular resistance beds. Some conjugated vaccines or proteins may become unlinked and separate components express a different toxicity profile versus parent compound. See table in **Attachment 1** for methods overview - applications and points to consider.

DNA Sequencing: Mutations and Revertants

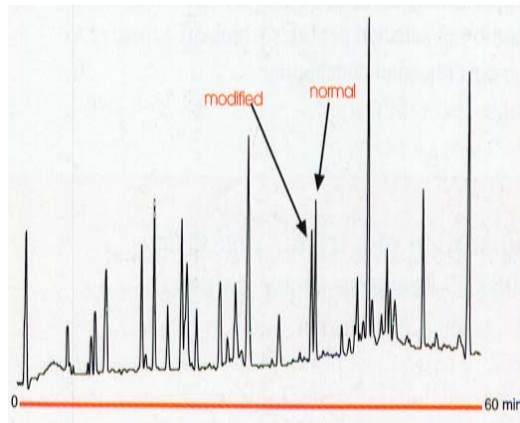
- ♦ Recommended by FDA for characterization of cell lines, expression vectors, and plasmids
- ♦ Stability of plasmid boosted through some redundancy
- ♦ Manual (radioactive) vs. automated (fluorescent), cycle vs. isothermal methods, end label vs. internal labels
- ♦ Sophisticated software available for analysis and trending; some with structural projections
- ♦ Mutation detection and localization kits also commercially available
- ♦ Templates/ kits also available for single-stranded (ss) DNA



One can also detect 'point mutations' or allelic variations via mini-sequencing methods. These employ immobilized amplified biotinylated DNA sequences on a streptavidin-coated plate. The various allelic-specific oligonucleotides have been developed through PCR technology. The advantage of this point-specific mutation is that conventional sequencing methods may be accurate, but too laborious for large numbers of samples. This specific method allows quick assessment of major allelic variations. There may also be methods to determine the genetic integrity of DNA-protein interactions *in vivo*, but it's not clear how sensitive this method may be for a given allelic variation.

High Pressure Liquid Chromatography (HPLC)

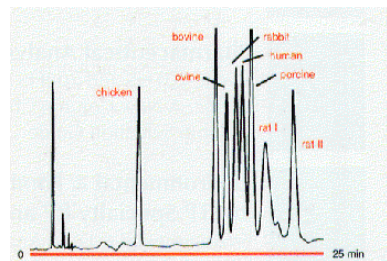
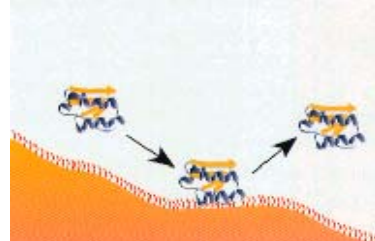
- ♦ Essential to modern analytical techniques for purity, potency, and identity/impurities
- ♦ Key features include selectivity, stability, and reproducibility of results
- ♦ Types include conventional HPLC, reverse phase (RP), ion-exchange, anion exchange, and size-exclusion (SEC)
- ♦ Polypeptides detected by UV-VIS at 210-220 nm (where peptide bond absorbs)
- ♦ UV-VIS at 280 nm used to monitor proteins with aromatic residues (e.g., tryptophan)



Above example is a RP-HPLC elution of tryptic digestion fragments of bovine somatotropin (BST) - incubated with trypsin and separated over a C18 RP-HPLC column. A fragment containing arginine at Position 98 was separated from the same fragment that was modified with isoaspartate at position 98.

Reverse Phase HPLC

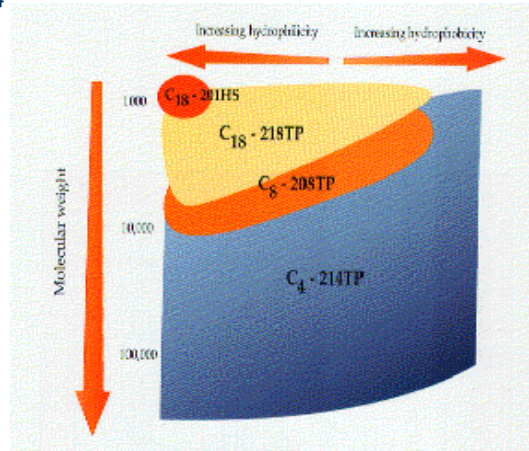
- ♦ **Excellent resolution** makes it valuable for analysis/ purification of proteins, peptides, and peptide fragments
- ♦ **Large polypeptides can't "partition"** into the stationary phase like small molecules - adsorb on to hydrophobic phase until organic phase elutes them off column
- ♦ **Quick elution** accounts for sharp peaks and high resolution
- ♦ For example, RP-HPLC resolution **sensitive** enough to separate insulin variants
- ♦ Separation via aqueous mobile phase with an **ion pairing agent** and **organic modifier**



Common ion pairing agents include: trifluoroacetic acid (TFA) [most commonly used], heptafluorobutyric acid (HFBA) [good for basic proteins], phosphoric acid, or triethylamine phosphate (TEAP). Commonly used organic modifiers include: acetonitrile, isopropanol, or ethanol [often used in large scale process applications].

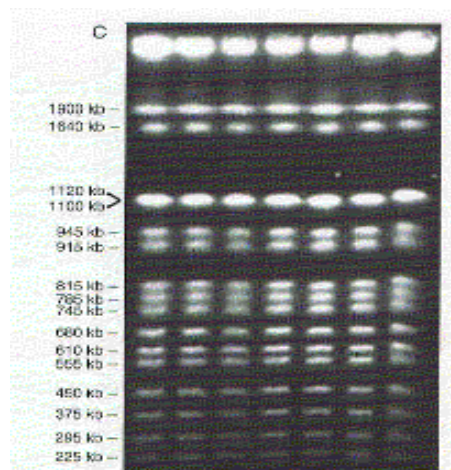
Reverse Phase HPLC: Column Selection

- ♦ **C4 columns** for polypeptides > 4 - 5,000 MW and very hydrophobic polypeptides of any size
- ♦ **C18 columns** for peptides < 4 - 5,000 MW, enzymatic digest fragments, or natural & synthetic peptides
- ♦ **C8 columns** for peptides up to 10 - 20,000 MW, enzymatic digest fragments, natural & synthetic peptides
- ♦ **Diphenyl columns** for large hydrophobic proteins or peptides with aromatic side chains
- ♦ **C18 small pore columns** for very small peptides (2-10 residues) or basic peptides



Electrophoresis

- ♦ A migration of charged particles (in solution) through a medium by application of an electric field
- ♦ Substances move at different rates depending on: electric field strength, viscosity, temperature, pH, ionic strength, dielectric constant, net charge, size, and shape
- ♦ Reduced vs. non-reduced SDS PAGE
- ♦ 2-dimensional electrophoresis
- ♦ Capillary Zone electrophoresis (CZE)



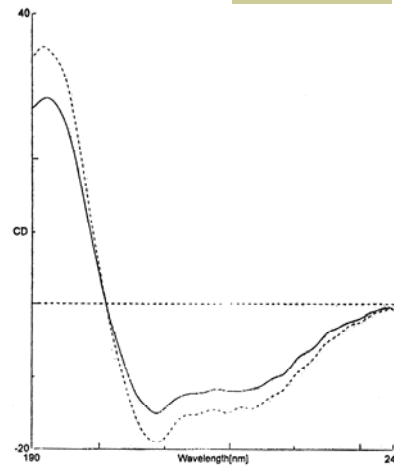
Capillary Zone Electrophoresis-Mass Spectrometry Using an Electrospray Ionization Interface

Smith, R. D., Olivares, J. A., Nguyen, N. T. and Udseth, H. R., *Anal. Chem.*, 1988, 60, 436-441

Instrumentation developed for capillary zone electrophoresis-mass spectrometry (CZE-MS) is described. The interface is based upon direct electrospray ionization from the end of the CZE capillary. The electrospray ionization source functions at atmospheric pressure and provides excellent sensitivity for wide ranges of compounds, with detection limits generally in the femtomole range (although significant improvements appear feasible). The instrumentation allows the high separation efficiencies feasible with CZE to be exploited and offers potential advantages compared with LC-MS methods, particularly when only small samples are available or high-resolution separations are necessary. The performance of the electrospray interface and the techniques and operating conditions for CZE-MS separations are described. CZE-MS separations and mass spectra are shown for mixtures that include polypeptides and quaternary ammonium salts. Separation efficiencies and detection limits vary widely from compound to compound and are shown to be sensitive to buffer selection. Separation efficiencies exceeding half a million theoretical plates are demonstrated for some compounds. Wider application and improved performance are anticipated with minimization of CZE band spread (due to adsorption and possibly other processes) and optimization of CZE buffers (for both the separation and their compatibility with electrospray ionization).

Circular Dichroism (CD)

- ♦ Sensitive method for assessing secondary structure of polypeptides and proteins
- ♦ Extensively used for monitoring conformational changes (monomer-oligomer, substrated binding, denaturation, etc.) and estimation of secondary structural content
- ♦ CD a form of light adsorption spectroscopy measuring difference in absorbance of right- and left-circularly polarized light
- ♦ Can distinguish alpha helix, parallel and anti-parallel beta sheet, turn, and others
- ♦ Reliable method for alpha-helical content but un-reliable for beta sheet content
- ♦ Example CD of recombinant human pulmonary surfactant protein C vs. native compound



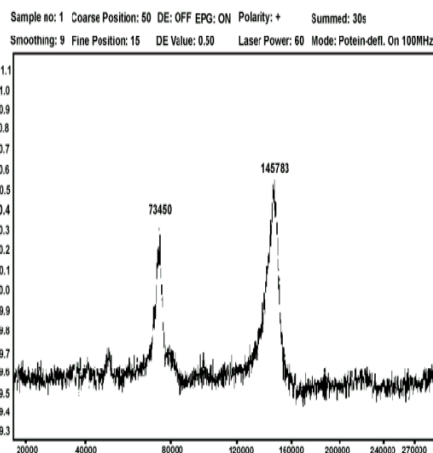
Physical principles of CD: Chromophores in asymmetric environments will interact differently with right- and left-circularly polarized light. The resulting difference observed via an optically active medium results in **optical rotation or circular birefringence** (due to different indices of refraction). The variation of optical rotation is called **optical rotary dispersion (ORD)**. Differences in absorption due to differing extinction coefficients for the two polarized rays is known as **circular dichroism**.

A significant problem with CD modeling is that the model compounds are usually infinite in length and don't mimic true secondary structures in proteins that are of finite length. In addition, the following problems also occur with any method of using CD to obtain secondary structure:

- random coils are seldom random
- Phe, Tyr, His, and Trp can contribute to peptide CD spectra
- Left handed helical structures can occur
- Disulfide bonds are very active
- Prosthetic groups are also very active

MALDI/TOF (matrix assisted laser desorption time-of-flight)

- ♦ Desorption technique similar to FAB
- ♦ Utilizes photons instead of particles to desorb analyte molecular ions, $[M+H]^+$, from a crystalline matrix
- ♦ Matrix absorbs incident radiation resulting in rapid heating of the crystal lattice
- ♦ MALDI-TOF can quantify MW > 100,000 from pmol to fmol quantities
- ♦ Distinct advantage over FAB done magnetic sector instruments



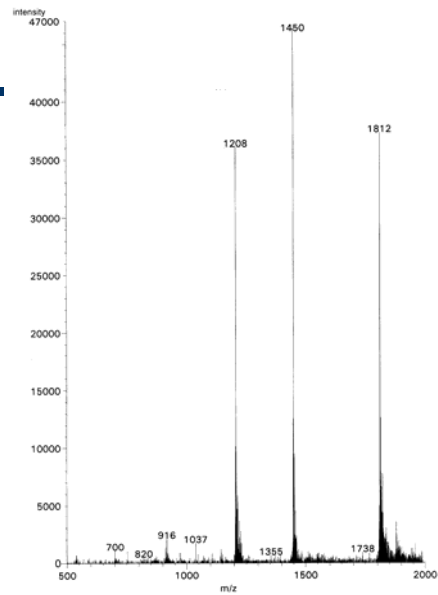
matrix assisted laser desorption ionization time-of-flight mass spectrometry

MALDI TOF MS is an innovative new tool for the analytical biochemist to obtain the molecular weight measurements from picomole amounts of biopolymers. Accurate molecular weights can be obtained quickly and easily for materials from small peptides and oligosaccharides to intact proteins larger than 100,000 Daltons.

This desorption technique first introduced in 1988 by Hillenkamp and co-workers [25] is very similar to FAB, but it utilizes photons instead of particles to desorb analyte molecular ions, $[M+H]^+$, from a crystalline matrix. The primary role of the matrix is to absorb the incident radiation which results in rapid heating of the crystal lattice on a time scale (femtoseconds) that is faster than thermal equilibration of the matrix-analyte lattice. This process results in desorption or transfer to the gas phase of matrix and intact analyte ions. The process responsible for ionization is still under current investigation and discussion but revolves around cation transfer similar to CI and FAB. MALDI, utilized in conjunction with time-of-flight (TOF) mass spectrometry, has the ability to generate molecular ions from pmol to fmol quantities of species with molecular weights in excess of 100 kDa. This is a very distinct advantage over FAB performed on magnetic sector instruments.

Mass Spectroscopy (MS)

- ♦ MS commonly used in tandem with:
 - Gas chromatography (GC-MS)
 - MALDI-TOF
 - Electron ionization (EI) (also electrospray)
 - Fast Atom Bombardment (FAB)
 - Nuclear Magnetic Resonance (NMR)
- ♦ Example of electrospray-MS chromatogram of recombinant human surfactant protein C (see right)



Virtually all forms of spectroscopy use an ionization energy source (e.g., radioisotope) to excite the bond energy of the sample, resulting in resonance or further emissions of altered energy waves via the distortion. Unique placement of bonds gives rise to highly specific emission patterns allowing an exquisite sensitivity to conformational changes or compositional shifts.

Analytical Equivalence: Characterization Testing

- Testing may be compartmentalized into three regimens: (1) intact structure, (2) post-enzymatic digestion, and (3) individual peptide sequences.
- **Entire/ Intact Structure:**
 - Chromatography: HPLC (chiral, reverse phase, size exclusion, ion-exchange), GC (GC-MS, GC-FID), TLC, etc.
 - Circular Dichroism
 - Determination of sulfide linkage
 - Spectroscopy: MS, MALDI-TOF, EI, CI, FAB, ESI, NMR, FT-NIR,
 - Immunochemical analysis (e.g., ELISA, RIA, radial immunodiffusion, flow cytometry, etc.)
 - Electrophoresis (reduced and non-reduced): SDS PAGE
 - Microheterogeneity analysis (e.g., glycosylation profiling, IgG sub-types)
- **Post-enzymatic Digestion:**
 - Chromatography: HPLC (chiral, reverse phase, size exclusion, ion-exchange), GC (GC-MS, GC-FID), TLC, etc.
 - Electron scatter (ESI-LS/MS)
 - Circular Dichroism
 - Spectroscopy: MS, MALDI-TOF, EI, CI, FAB, ESI, NMR, FT-NIR,
 - Collisionally Induced Dissociation (CID)
- **Peptide Sequences:**
 - Amino acid analysis (e.g., AA sequencing (entire sequence or amino- and carboxy-terminal sequences))
 - Peptide mapping via restriction enzymatic digestion/ fragment analysis

See **Attachment 1** for a complete listing of analytical methods. Electronic version of that table is also in The Library/Analytical Methods/Test Method Table.

Bioassay Equivalence: Points to Consider

- **Binding is linked to signal transduction:** Need to use bioassays that assess an effect via signal transduction - not just binding alone
- **Compendial assays vs. non-compendial or proprietary assays:** Need to assess if compendial methods (if applicable) are sensitive enough for distinguishing slight variations in critical product parameters; if compendial methods exist, do crossover studies for comparison
- **Establishing critical product parameters:** Need to assess/ correlate with functional activity/ stability in bioassays (e.g., antigen content vs. immunogenicity)
- **Relative Standard Deviation (RSD):** What is noise-to-signal ratio for bioassay? > 25% RSD? Need to use statistical modeling to ensure adequate sample size for assessing wide variability (see Juran's *Handbook of Quality*, Chapter 25 - Acceptance Sampling).
- **Use "gold standards" in assays:** Compare product to compendial reference standards or lots of innovator product. Establish what physico-chemical differences relate to bioassay activity.
- **Process Development:** Using neural nets to (1) help establish critical process parameters with impact on product quality and (2) develop an "edge of failure" for any given process and establishing ranges within those critical areas. Don't depend on early stage material for market needs.
- **Statistical Process Control (SPC):** Sampling: continued analysis of product quality against long-term manufacturing history
- **Combining Tests into a Panel:** Complex moieties may require several tests combined into a conglomerate panel to assess potency (e.g., RP-HPLC, SDS PAGE, and bioassay)
- **Express Potency as Quantifiable Units:** Need to express potency in some quantifiable measure - units [of activity]/ mg of compound (anhydrous weight)

Links: Need to assess impact of CMC changes to -

- Analysis of Product Characteristics: Comparability testing
- Surrogate Equivalence: Impact on activity
- Stability: Emergence of undetected impurities
- Analytical Methods: Consistency of methodology/ data
- Setting Rational Specifications

Analytical Equivalence: Comparability Protocols - Characterization Testing vs. Release Testing

- **Characterization Testing:**
 - Establishing a "Gold" reference standard of internal product - comparison to external standards (if available)
- **Establishing critical product parameters:**
 - assessing correlation with functional activity/ stability
- **Release Testing:**
 - refinement of characterization testing required to assess product identity, safety, and purity.
- **Scale of Manufacturing:**
 - Pilot vs. Commercial scale changes - what to assess?
- **Process Development:**
 - Using neural nets to help establish critical process parameters with impact on product quality
- **Process Development:**
 - Using neural net analysis to develop an "edge of failure" for any given process and establishing ranges within those critical areas.
- **Process Validation:**
 - confirmation of product vs. process with release testing + stability data to meet SUPAC/ BIOSUPAC
- **Statistical Process Control (SPC):**
 - Sampling: continued analysis of product quality against long-term manufacturing history

Analytical equivalence - shown with multiple layers of testing and analogous to comparability testing currently done for innovator biotech products - must show differences do not impact safety, efficacy, potency. Often includes assessments of microheterogeneity for impact on **safety, purity, and strength**.

Identity confirmed through MS-NMR, circular dichroism (CD), MALDI-TOF, SDS-PAGE, immunological profiles. Impurities confirmed through chromatographic methods, SDS-PAGE, TLC, but are not necessarily limited to the following: amino acid analysis, amino acid sequencing, entire sequence or amino- and carboxy-terminal sequences, peptide mapping, determination of disulfide linkage, SDS-PAGE (reduced and non-reduced), isoelectric focusing, Conventional and High Pressure Liquid Chromatography (HPLC) e.g., reverse-phase, size exclusion, ion-exchange, etc., mass spectroscopy, assays to detect product-related proteins including deamidated, oxidized, cleaved, and aggregated forms and other variants e.g., amino acid substitutions, adducts/derivatives, assays to detect residual host proteins, DNA, reagents, immunochemical analyses, assays to quantitate bioburden, endotoxin, etc.

Be intimately familiar with analytical method applications and limitations. Search the WEB for innovative technologies to apply to your product.

Additional physicochemical characterization may be required for products undergoing post-translational modifications, for example, glycosylation, sulfation, phosphorylation, or formylation. Additional physicochemical characterization may also be required for products derivatized with other agents, including other proteins, toxins, drugs, radionuclides, or chemicals. The information submitted should include the degree of derivatization or conjugation, the amount of unmodified product, removal of free materials (e.g., toxins, radionuclides, linkers, etc.), and the stability of the modified product.

Bioequivalence (PK/PD) testing may be waived for most parenteral, otic, or ophthalmic **drug products** since they are made identical to innovator, but **biotech products** have microheterogeneity issues and altered PK/PD relationships versus classic drugs - different study designs required.

With some well characterized biologic product classes, bioassay comparisons may substitute partially for this, but with life-threatening conditions, there may be additional need for clinical endpoints (e.g., BIO-IND as currently done for topical anti-fungal creams).

Surrogate endpoints may be incorporated into a PK/PD study to assess dynamics on receptor population or comparability of impact versus innovator. Some biologics may not require classic Phase III studies to assess efficacy via surrogate endpoints. Opens options to allow studies to be done without IND.

Viral Validation Methods & Limitations

- Selection of Model Viruses
 - No single virus alone good enough; select panel from both suspected contaminants and model viruses (e.g., lipid-enveloped vs. non-enveloped, resistant strains, etc.)
 - Some early studies may be done with surrogates (e.g., bacteriophages in filter validation)
- Comparability of Scaled-down Process
 - Not necessary to validate every step - just those likely to remove or inactivate viruses
 - Product, flow, time, temperature, purity, potency, yield, etc.: can use IPC data to validate challenge process
 - Impact on product parameters: validation processes can impact material via increased aggregation, thrombogenicity, etc.
 - Use worst-case conditions (e.g., minimum column wash to elute virus with product)
- Justification for Viral Challenge Dose
 - Generally start with high challenge dose to maximize potential clearance claim
 - Careful that techniques used to create high titer stocks don't contribute to aggregation
 - Should not be more than 10% of final volume used to keep feedstream representative of manufacturing process
 - FDA-ICH guidelines recommend replicate challenge doses to increase confidence in data but there's no agreement on how to handle wide variation in data results. Some conservatively use lowest clearance ratio when variation is more than the assay variability.

See the following articles in the section on Assessing Risk and Uncertainty in Biotech Development - Attachment 1: Adventitious Agent Removal/Inactivation Validation:

- Viral Clearance Strategies for Biopharmaceutical Safety - Part I: *General Considerations*, *Pharmaceutical Technology* April 2001
- Viral Clearance Strategies for Biopharmaceutical Safety - Part II: *A Multifaceted Approach to Process Validation*, *Pharmaceutical Technology* June 2001
- Virus Inactivation in the 1990s - and into the 21st Century - Part I: *Skin, Bone, and Cells*, *BioPharm* July 2002

Viral Validation Methods & Limitations

- Calculating Viral Inactivation/Removal Efficiencies
 - Clearance efficiency (\log_{10} titer reduction [LTR]) usually is 12-15 LTR for a process
 - Viral kinetics rarely linear
 - Additive vs. Cumulative: only data for the same virus can be cumulative across many steps since different viruses will have varying profiles at the same step **and** only different mechanisms of virus inactivation can be cumulative
- Limits of Viral Detection Method
 - Sensitivity & specificity: not all viruses detected equally - requires assay selection & validation. Clearance of less than 1 log unit may be due to assay variability.
 - Sample size relationship to sensitivity: PCR with a LOD of 100 viral particles/mL would be negative even with a total of 10^6 viral particles in a 10 L batch (given a 100 mL aliquot used for testing)
 - Product Interference: neutralizing antibodies in plasma can mask infectious virus; extra cellular debris during a column chromatography phase can bind virus and compete with resin binding sites; product must be shown to have no inhibition
 - Virus Lots: vendor formulations of virus stocks can interfere with data validity such as BSA used as a stabilizer; lot-to-lot differences in activity; pre-filtration to eliminate cell debris can reduce activity
 - PCR may be a false positive due to inactivated virus, underestimating the LTR efficiency

Viral inactivation methods include pH, solvent/detergent, heat, column chromatography, etc. Kinetics of inactivation usually not a simple first order reaction; often has a fast initial phase followed by slower phase. A dramatic drop off or reduction in viral inactivation efficiency may indicate a loss of activity by the inactivating agent or that a residual fraction of the virus population is resistant - which implies the step is neither highly effective or robust.

In terms of assay sensitivity, the following examples are relevant in that they are neither definitive or all encompassing:

- When a process is challenged with a 6-log dose but only a 4-log dose is recovered, the step cannot be claimed effective - although it may contribute to overall removal.
- Where a process is challenged with a 6-log dose, but cytotoxicity of the product limits assay sensitivity to a 4-log dose, only 2-logs removal have been demonstrated, but the step cannot be claimed as effective. However, the process might be more effective - as shown by a different experimental design.
- Where a process is challenged with a 6-log dose and 2-logs are recovered, the product is not virologically sterile but ample amounts have been removed. If the reduction is reproducible and not influenced by process variables, one could claim it is of some efficacy and contributes overall to reduction of virus load (and can be counted as such in the calculations).
- Where a process is challenged with a 6-log dose and no virus is detected with a limit of sensitivity (about 2-logs), approximately 4-logs removal have been demonstrated. This is a substantial step where in fact more virus may be inactivated than quantitated or claimed.
- Where a virus is inactivated, the kinetics of loss of infectivity are important. If a process step involves prolonged incubation (e.g., heating for 10 hours) - and infectivity drops below the limit of detection rapidly - the process is likely to be more efficient than demonstrated. Conversely, if infectivity dwindles slowly and drops below the limit of detection towards the end of the process step, the step is less likely to assure viral safety. For instance, solvent/detergent steps often inactivate virus below the LOD within the first hour - although most processes involve stirring up to 4 - 4.5 hours.

Integration of Analytical Testing Profile to Manufacturing and Stability

- SAR Links to Manufacturing & Stability
 - What is SAR for binding to receptor site or antigen epitope (e.g., specific activity or K_d (dissociation constant) to receptor)? How does this change over time, over certain forced degradation conditions?
 - What is SAR link to process parameters and how close to "edge of failure" is the material? Is the process robust or brittle?
 - What is the SAR to preclinical testing?
 - What is the SAR of your compound compared to other products in the same class?
 - What is the SAR and analytical profile of your compound over stability compared to innovator products from the same class?
 - With SAR correlation from databases, can you construct a QSAR that will be useful in comparability protocols?
 - How relevant are impurities to bioassay activity? That is, what sensitivity does the bioassay have to detect impurity levels? This is particularly true for aggregated, deamidated, oxidized, or other forms of impurities.
 - In the end, can you define the product characteristics in such a discrete physico-chemical manner as to obviate the need for potency testing?

This is perhaps the most critical aspect of the program. You need to integrate the physico-chemical characteristics of product with preclinical, process parameters, clinical, and stability areas to correlate the key SAR aspects for comparability studies later on.

Summary of Key Points

- Analytical methods have strengths and limitations.
 - The more you can correlate your product purity, potency, and safety with physico-chemical SAR - and ultimately to clinical data - the better the comparability studies later on.
- Keeping the analytical 'engine' in pace with manufacturing and preclinical 'engines'
 - It's tough and it's critical to not let one part get too far astray. This will avoid having to repeat a portion of the program later.
- Analytical equivalence must be established using the most current and holistic approaches.
 - Be careful not to delve too far into 'scientific issues' that don't bear on the product quality. For example, detailed binding kinetics over a variety of forced degradation conditions may be useful to a point, but don't carry this out other than to support comparability assessments.
- Analytical data must be extensive to support bioassay variability, manufacturing flexibility, microheterogeneity, stability changes, etc.
 - For example, some products will be heterogeneous (e.g., glycosylation patterns) so it's important to monitor trends vs. absolute quantification.
- Comparability (or therapeutic equivalence) criteria will probably be a mixture of your own data plus comparisons to other products from the same class.