Peptides and proteins are emerging as an increasingly important chemical class of drugs as they become more readily available through improvements in recombinant DNA technology and approaches to their chemical synthesis.

Their development and production as drugs present daunting scientific challenges. The rigor of the analytical methods that are adopted to characterize and evaluate this group of substances will not only help define our understanding of their chemical and biological properties, but will likely impact on their regulatory approval and the success and safety of the resulting products.

In a recent review (de Vlaminck, 1984), it was suggested that the level of analysis necessary to support regulatory filing of recombinant DNA products should include: in-process monitoring of the DNA sequence of the micro-organism producing the protein; HPLC tryptic mapping of in-process materials and final products; sensitive techniques to evaluate purity; and biological properties evaluated through clinical trials. The vagueness and simplicity of this list reflects the current uncertainty within the Industry regarding the subtleties and complexities involved in developing peptide/protein drug substances into safe and efficacious products. Indeed, it appears that regulatory approval of Protropin (human growth hormone) was based on analytical methods that gave relatively little information on absolute identity and purity of the drug substance. Other than routine tests for color, appearance, pH, sterility, pyrogenicity and general safety, the only tests performed for batch release were determination of protein concentrations (using Lowry's method), identity confirmed by silver stained SDS PAG electrophoretograms, amino acid analysis and HPLC of the tryptic digest, and therapeutic potency evaluated in terms of relative rates of growth of treated and untreated hypophysectomized rats. Similar approaches to analysis were found in the NDA approval for human insulin - Humanlin. The need for analytical methodology that can provide more information regarding drug identity and purity is clear.

Several properties of peptides and proteins present particular challenges to their analysis. The biological potency characteristic of
these molecules requires methods that are exquisitely sensitive. Additionally, the striking similarity in structure between analyte, degradates, impurities and matrix components places tremendous demands for selectivity on analytical methodology. Such methods must also consider the relationship between the purity and activity of the analyte. Whereas for small molecules, physical chemical identity and purity assessment is sufficient to guarantee therapeutic potency; peptides and proteins, due to their complexity, also require an independent evaluation of activity. Purity must reflect not only chemical composition, but also chirality, conformation and physical states of aggregation of the analyte. Consideration must be given to the fact that, in some cases, loss of purity measured in terms of these criteria may not impact on biological activity, while in other cases, deficiencies in the scope of available analytical methodology cause impurities that do influence biological activity not to be recognized.

Peptides and proteins may exist in several active forms (i.e., they exhibit microheterogeneity). Portions of the backbone termini may be lost through proteolytic degradation, without compromising drug potency. For example, variants ("clipped products") of tissue plasminogen activator (in which the N-terminus is serine [1]) have been reported where the N-terminus amino acid is glycine [-3] (Wallen et al., 1983), valine [4] (Jornvall et al., 1983) or a more dramatic loss of about 3000 daltons (Ranby et al., 1982), which represents the loss of about 27 amino acids from the N-terminus. All of these species have apparently identical thrombolytic activity. Similarly, the extent and nature of post-translational modifications (e.g., glycosylation) may vary over a range among the naturally occurring active molecules without affecting activity. It is likely that peptide and protein samples will contain a number of active compositions and forms that will have to be individually understood before a precise assessment of drug purity can be made.

The activity of larger polypeptides (≥10,000 daltons) usually resides in their tertiary and quaternary structure and thus, depends on maintaining certain conformation(s). This fragile property may be the only feature that distinguishes an active from an inactive material. Thus, it is important to recognize factors that can alter conformation and have analytical methods at hand to sense such subtle changes in form.

Analytical methods for the determination of peptides and proteins can be divided into four types: bioassays, immunoassays, enzyme assays and physical/chemical assays. The distinction is somewhat artificial, since many methods are hybrids of two or more of these categories. However, within a category, the methods share common properties making these divisions useful for discussion.

Bioassay

Historically, most proteinaceous materials are evaluated by bioassay. The inactivation of such molecules is frequently chemically invisible and samples appear by all chemical/instrumental methods to be completely homogenous. Thus, activity measured in terms of biological response divided by total protein concentration (i.e., specific activity) has proven to be a useful measure of purity.

Most bioassays are difficult to use in an analytically rigorous program of drug substance evaluation. They are generally highly variable, very time consuming, economically unsound and not suitable for automation. They are, therefore, impractical for formulation optimization tasks in which a variety of excipients and experimental conditions must be evaluated. Assay reproducibility is generally on the