Journal of Pharmaceutical & Biomedical Analysis
Vol. 7, No. 9, pp. 1071–1076, 1989
Printed in Great Britain

0731–7085/89 $3.00 + 0.00

Analytical challenges to the pharmaceutical industry in developing products of biotechnology*

C. MICHAEL DICKEN and LARRY A. STERNSON†

Sterling Research Group, 9 Great Valley Parkway, Great Valley, PA 19355, USA

Abstract: Problems associated with the analysis of peptides and proteins in pharmaceutical products produced by biotechnology are discussed. Analytical techniques for the determination of peptides and proteins in such products are classified into four categories: bioassays, binding assays, enzyme assays and physical/chemical methods.

Keywords: *Biotechnology; proteins; peptides; bioassays; immunoassays; enzyme assays; physical/chemical assays.*

Introduction

The development of peptides and proteins as drugs has been made possible by improvements in their availability through advances in recombinant and synthetic technologies. Many of these putative drugs are characterized by high potency and limited stability (undergoing both enzymatic and non-enzymatic breakdown) with degradation products that structurally resemble the parent drug and endogenous materials. Their short biological half-lives have prompted the use of delivery strategies that sustain drug release over a prolonged period of time, contributing further to the observed low plasma levels of drug. Accordingly, analytical methods suitable for monitoring peptidergic drugs in various complex matrices (e.g. fermentation broths, pharmaceutical formulations, biological fluids) require a high level of selectivity and sensitivity. Such methods must also consider the relationship between the purity and activity of the analyte. Whereas for small molecules, physical and chemical identity and purity assessment is sufficient to guarantee therapeutic potency, peptides and proteins also require an independent evaluation of activity. Consideration must be given to the fact that, in some instances, loss of purity measured in terms of chirality, conformation and physical states of aggregation of the analyte may not reflect loss of biological activity. The terminology used to describe this phenomenon is microheterogeneity, where either a portion of the backbone has been "clipped" by proteolysis or some post-translational modification has occurred, without reducing the drug potency.

*Presented at the "International Symposium on Biomedical Applications of Liquid Chromatography", 23–25 March 1988, Bradford, UK.

†To whom correspondence should be addressed.

An example where limited proteolytic degradation has no effect on the biological activity of a protein is tissue plasminogen activator. Variants ("clipped products") have been reported where the N-terminus, instead of the usual serine, is glycine [1] (which corresponds to the addition of three amino-acids to the N-terminus), valine [2] (loss of the first three amino-acids from the N-terminus) or a more dramatic loss of about 3000 dalton [3], which represents the loss of about 27 amino-acids from the N-terminus. In each instance the resulting species has thrombolytic activity that is nearly identical to that of native tissue plasminogen activator.

Alternatively, an apparently homogeneous sample may be a mixture of active and inactive species that have not been recognized because of the deficiencies in the scope of available analytical methodology. Two common examples are conformational alterations and physical aggregation. The activity of larger polypeptides (>10,000 dalton) usually resides in their tertiary and quaternary structures and thus depends on maintenance of a certain conformation. This fragile property may be the only feature that distinguishes an active substance from an inactive material. Therefore, it is important to be able to recognize the factors that can alter conformation and to use analytical techniques that are capable of detecting such subtle differences in form.

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

Analytical Methods

Bioassays

Currently, the most useful method for measuring the purity of proteinaceous materials is the bioassay since this technique can determine the activity in terms of biological response as a function of total protein concentration (referred to as specific activity). The advantages of employing such an assay in establishing purity are the therapeutic relevance of the methodology and the fact that it is supportive of chemical analysis.

There are numerous disadvantages, however, that make it difficult to implement a bioassay in an analytically rigorous programme of drug substance evaluation. If structural changes occur that do not alter the biological activity, the bioassay will not be responsive to those changes. If a co-analyte is present that influences the biological response, once again the bioassay cannot distinguish the contributions of the two analytes. Bioassays also suffer from poor reproducibility due to biological variability, the need for multiple controls and standards, extensive time consumption, an inability to automate and from being economically unsound. These problems make bioassays impractical for formulation optimization tasks or for the assignment of shelf-life of the formulated product. Because of these limitations, assay reproducibility is generally accepted to be in the range of ±20–50%. With the advent of recombinant technology and improvements in peptide synthesis, however, more stringent criteria for acceptance, approaching the criteria for "small, conventional" molecules (usually ±10% of label claim when establishing the shelf-life with assay variability of ±1–3%), are anticipated.

Thus, more convenient, economically sound analytical approaches are needed to supplement bioassays which will remain essential for validating the new methodology. It is likely that correlations between assays based on chemical and biological endpoints will

be required to define "protein concentration" in terms of drug purity, stability and pharmacokinetics. The confidence gained by such correlations is illustrated in the evaluation of the vasopressin antagonist, SK&F 101926, when administered intranasally [4]. Bioavailability determined independently by HPLC analysis of drug concentration in plasma and in terms of increased urine output and urine osmolality were both approximately 20% at doses of 100 $\mu g\ kg^{-1}$ in the dog.

Immunoassays

The specific and tight association of antibody with drug substance antigens can be exploited to determine very low concentrations of peptide and protein drugs in a variety of complex matrices. Two basic strategies have been adopted in the design of immunoassays — competitive and direct. Competitive immunoassays allow a small amount of labelled antigen (otherwise identical to the analyte) to equilibrate with the antibody and the analyte. The greater the concentration of unlabelled analyte in the sample being assayed, the less the amount of labelled antigen that will be bound to antibody. Experimental determination of the ratio of free/bound forms of the labelled analyte compared with a standard curve will allow the analyte concentration to be determined. Some of the more common reporter groups that are used to label analytes are radioisotopes, fluorescent moieties, enzyme conjugates and spin labels.

The sensitivity of the immunoassay is determined not only by the instrumental sensitivity of the selected reporter group but is also a function of both the binding constant for the analyte and the effect of the reporter group on the binding affinity of the antigen. It is implicitly assumed that the incorporation of the reporter group into the antigen does not alter its binding affinity for the antibody. However, this assumption is most likely to be valid only for radioisotope labels.

The specificity of immunoassays is also crucial in determining the analytical confidence that can be placed in such a method. Two of the more prevalent factors that play a role in specificity are: the cross-reactivity of the antibody used in the assay, i.e. its binding constant for analyte relative to its association constant toward other species present in the sample; and the fact that the antibody recognizes only a small portion of a macromolecular antigen and, therefore, may not be truly indicative of either purity or activity. Thus, immunoassays may be of little value in establishing drug substance and product release criteria unless: (a) antibodies can be made to recognize conformationally dependent epitopes that correlate with drug potency; (b) panels of antibodies are employed to recognize the entire structure of the drug substance (in assessment of purity); or (c) immunoassays are combined with other analytical techniques that provide added assurance of specificity (e.g. SDS–PAGE or HPLC).

All the immunoassay methods described so far require physical separation of the free and bound forms of the analyte. The most common approaches to achieve such separation include adsorption of the free fraction on Dextran-coated charcoal or precipitation of the bound fraction using solvent, salt, immunochemical (second antibody) or Protein A from *Staphylococcus aureus* [5]. Thus, although more convenient than bioassays, such heterogeneous immunoassays require significant manual manipulation and the use of fresh standard curves for each analytical run owing to poor reproducibility of reagents, conditions and quality of separation. A number of homogeneous competitive immunoassays have been developed which involve a change in the antigen that is induced in the label on binding to the antibody [6–8]. However,

these methods employ fluorescent, spin label or enzyme reporter groups which have the problem of most likely altering the antigen binding affinity.

More recently, direct immunoassays (also referred to as immunoradiometric assays), which are not based on a competitive binding principle but require that the antibody be labelled with a reporter group, have been utilized. The ELISA (enzyme-linked immunosorbent assay) technique is the most common example of such a method [9]. Direct analysis is achieved by allowing the analyte to first associate with a stationary support phase and then adding a labelled antibody that associates with a complimentary antigen. Excess antibody is washed away before the enzyme activity is measured. A comparison of the radioimmunoassay (RIA) against the immunoradiometric assay has been made for human growth hormone and a common specific antibody to the antigen [10]. The immunoradiometric assay showed a 13-fold increase in sensitivity and a 6-fold increase in assay range over that of the RIA procedure.

The value of immunoassays can be greatly enhanced by use in conjunction with separation techniques such as electrophoresis or HPLC. Furthermore, the development of monoclonal antibodies now offers the opportunity for obtaining antibodies which adhere to the same rigorous standards of purity and homogeneity that have been established for more traditional analytical reagents. Such species, potentially specific for a single epitope, offer a level of discrimination that allow detection of conformational changes in an antigen (although the antibody is still responsive to only a portion of the antigen [11–12]).

Enzyme assays

If the drug substance is an enzyme or modulates the performance of an enzyme, the biomacromolecule may be quantified by methodology based on a kinetic measurement of enzyme activity. For those very few drug substances that currently fall into one of these two categories, there are distinct advantages to this type of analysis. Enzyme assays can be made extremely sensitive, highly specific to the point where the assay can include chiral and conformational recognition and can be easily automated. Furthermore, the biochemical action of catalytic molecules or molecules that specifically inhibit or accelerate catalysis usually allows for an inherent correlation to be made between *in vitro* enzyme activity and therapeutic potency.

The generation of many molecules of product per molecule of analyte amplifies the analytical signal and has the potential to provide the basis for achieving very low detection limits. An example where this assay characteristic has been utilized, is in "enzyme cycling" [13]. Claims for such procedures include amplification factors of 4.0×10^8 and the ability to measure 10^{-18} mol of certain cellular metabolites. The possibility of creating specific and sensitive geometrically amplified enzyme cycles has been proposed but has not been fully developed [14].

The most serious concern about enzyme assays lies with the possible influence of co-analytes in the sample on enzyme activity and viability. Even slight modulations in enzyme kinetics produced by impurities, degradation products or matrix components could result in large analytical inaccuracies.

A number of proteinaceous drugs are proteases or affect proteases and have been analysed by observing the rate of degradation of synthetic substrates that are specifically designed, based on the amino sequence at the protease cleavage sites and that produce a chromophoric or fluorophoric product on hydrolysis [15–23]. Among these drugs are plasma proteases responsible for haemostasis which normally exist in their zymogenic

form and can be activated to promote thrombolysis or fibrinolysis. Enzyme assays exploiting this property have been developed for drugs such as streptokinase, urokinase and tissue plasminogen activator (tPA). The chromogenic substrate D-val-L-leu-L-lys-*p*-nitroanilide (S-2251) has been used in a coupled assay to measure the concentration of plasminogen activators. For every mole of plasminogen activator present in the sample, many molecules of plasmin are formed; for every mole of plasmin formed, many molecules of chromophoric *p*-nitroaniline are subsequently produced. The method provides high sensitivity by geometric amplification of the mass of analyte that is ultimately monitored spectrophotometrically as *p*-nitroaniline at 405 nm.

$$\text{Plasminogen} \xrightarrow{\text{activator protease}} \text{plasmin}$$

$$\text{S-2251} \xrightarrow{\text{plasmin}} p\text{-nitroaniline.}$$

Physical/chemical assays

In order to fully characterize proteinaceous pharmaceuticals, a few milestones must be achieved. The amino-acid sequence must be verified, no occurrence of miscoding must be demonstrated, trace impurities must be identified and quantified and the presence and structural identity of blocking groups and other post-translational modifications must be established. One of the most useful techniques in dealing with these issues is fast atom bombardment mass spectrometry (FAB-MS) [24] of tryptic digests of peptides and proteins. FAB mass spectra of trypsin-generated peptide fragments and FAB-MS of the Edmann degradation of these fragments can be compared with a library of anticipated fragments to make unambiguous assignments of the observed peaks to portions of the anticipated sequence. Other enzymes and reagents that allow predictable and reproducible fragmentation (with specificities different than those of trypsin) can be used to generate fragments that will overlap the tryptic fragments and extend the portion mapped. Peptide fragments containing residues that have been post-translationally modified will not generally appear in the FAB-MS analysis. The absence of such peaks is only an indication of post-translational modification. Verification can be gained following further treatment of the tryptic digest [25]. For example, a sample containing a putative glycopeptide can be treated with peptide:*N*-glycosidase F to cleave asparagine-linked glycosyl adducts across the linking amide bond. This cleavage gives an aspartic acid in place of the original asparagine and results in the appearance of a spectral signal at one mass unit greater than that of the anticipated fragment. This spectrum is conclusive proof of the occurrence of asparagine-linked glycosylation within the peptide. New peaks in the spectrum can provide additional information on the composition of the original carbohydrate adduct.

Conclusions

Some of the inadequacies that currently exist in the methodology available to the pharmaceutical industry for the analysis of products of biotechnology are poor sensitivity and selectivity, lack of acceptable reproducibility and the high cost required to obtain meaningful data. Because of these factors, hybrid techniques have had to be employed, and the specifications and expectations of quality have been lessened when compared with those for small organic molecules. Therefore, it is imperative that analytical research efforts be focussed on overcoming these problems.

References

[1] P. Wallen, G. Pohl, M. Ranby and H. Jornvall, *Eur. J. Biochem.* **132**, 681–686 (1983).
[2] H. Jornvall, G. Pohl, W. Bergsdorf and P. Wallen, *FEBS Lett.* **156**, 47–50 (1983).
[3] M. Ranby, N. Bergsdorf, G. Pohl and P. Wallen, *FEBS Lett.* **146**, 289–292 (1982).
[4] G. G. Liversidge, C. G. Wilson, L. Sternson and L. B. Kinter, *J. Appl. Physiol.* **64**, 377–383 (1988).
[5] S. Y. Ying, in *Immunochemical Techniques, Methods in Enzymology* (J. J. Langone and H. VanVunakis, Eds), pp. 245–253. Academic Press, New York (1981).
[6] L. J. Blecka and G. J. Jackson, *Clin. Lab. Med.* **7**, 357–370 (1987).
[7] M. E. Jolley, *J. Analyt. Toxicol.* **5**, 236–240 (1981).
[8] M. E. Jolley, S. D. Stroupe, K. S. Schwenzer, C. J. Wang, M. Lu-Steffes, H. D. Hill, S. R. Popelka, J. T. Holen and D. M. Kelso, *Clin. Chem.* **27**, 1575–1579 (1981).
[9] E. Engvall and P. Perlman, *Immunochemistry* **8**, 871–874 (1971).
[10] M. A. Wilson, Y. Kao and L. Miles, *Nucl. Med. Commun.* **2**, 68–73 (1981).
[11] T. Bachi, W. Gernard and J. W. Yewdell, *J. Virol.* **55**, 307–313 (1985).
[12] J. W. Yewdell, W. Gernard and T. Bachi, *J. Virol.* **48**, 239–248 (1983).
[13] O. H. Lowry and J. V. Passonneau, *A Flexible System of Enzymatic Analysis*. Academic Press, New York (1972).
[14] L. A. Sternson and T. R. Malefyt, in *Directed Drug Delivery*, pp. 291–307. Humana Press, Clifton (1985).
[15] I. Kiss, L. Aurell, M. Pozsgay and P. Elodi, *Biochem. Biophys. Res. Commun.* **131**, 928–934 (1985).
[16] L. G. Svendsen, J. Fareed, J. M. Walenga and D. Hoppensteadt, *Seminars in Thrombosis and Hemostasis* **9**, 250–262 (1983).
[17] J. Tang, S. Li, P. McGray and A. Vecchio, *Ann. N.Y. Acad. Sci.* **434**, 536–540 (1984).
[18] J. Fareed, H. L. Walenga, E. W. Messmore and E. W. Bermes, *CRC Critical Review in Laboratory Sciences*. CRC Press, Boca Raton (1983).
[19] H. L. Messmore, J. Fareed, J. Kniffin, G. Squillaci and J. M. Walenga, *Ann. N.Y. Acad. Sci.* **370**, 785–797 (1981).
[20] Y. Ohno, H. Kato, S. Iwanaga, K. Takada, S. Sakakibara and J. Steuflo, *J. Biochem.* **90**, 1387–1395 (1981).
[21] I. Witt and W. Tritschler, *J. Clin. Chem. Clin. Biochem.* **20**, 587–591 (1982).
[22] I. Witt, *J. Clin. Chem. Clin. Biochem.* **15**, 239–244 (1977).
[23] I. Witt and W. Tritschler, *J. Clin. Chem. Clin. Biochem.* **21**, 429–436 (1983).
[24] M. Barber, R. S. Bordoli, G. V. Garner, D. B. Gordon, R. D. Sedgwick, L. W. Tetler and A. N. Tyler, *Biochem. J.* **197**, 401–404 (1981).
[25] S. A. Carr and G. D. Roberts, *Analyt. Biochem.* **157**, 396–406 (1986).

[*Received for review 16 May 1988*]