Editorial Commentary/Summary

Specificity and Accuracy Data for Ligand-binding Assays for Macromolecules Should be Interpreted with Caution

John W. A. Findlay^{1,2}

Received 1 May 2008; accepted 17 June 2008; published online 17 July 2008

KEY WORDS: catabolism; cross-reactivity; metabolism; pharmacokinetics; protein; validation.

Over the past several years, there has been a marked increase in the number of biological macromolecules under development as potential therapeutics by pharmaceutical and biotechnology companies (1). This interest has been driven by factors such as the biological specificity and tolerability of some of these agents, the clinical and commercial successes of some recently approved biotechnology products, and the perceived high future revenue growth rate for such products. Concomitantly, there has been a marked upsurge in interest in the development and marketing of “generic protein” products, also referred to as “similar biological medicinal products”, “biosimilar protein products” or “follow-on protein products” in Europe and the USA. The latter development is highlighted by recent European and US approvals of such recombinant proteins as human growth hormone as a follow-on product, since patent protection for the innovator products had expired.

The preferred bioanalytical method for determination of these macromolecules in biological matrices for support of pharmacokinetic or bioequivalence studies is ligand-binding assay (LBA), typically immunoassay in one of a number of possible formats. Given the importance of these products, realistic validation of LBAs for their analysis in biological matrices is important. Several workshops and publications have addressed the development and validation of bioanalytical methods in general (2–4), and of LBAs for biological macromolecules specifically (5–7). The FDA has also issued a guidance on bioanalytical method validation (8). Validation of assays for biomarkers, many of which are ligand-binding assays, has also been the subject of recent workshops and publications (9,10), which have advanced the notion of “fit-for-purpose” as a consideration in deciding on the extent of validation needed for support of a particular assay application—exploratory studies requiring less assay validation support and definitive, late-stage studies needing more extensive validation support.

LBAs are essentially the only current bioanalytical option with sufficient sensitivity for the measurement of biologic macromolecules in such complex matrices as serum or plasma, and considerable energy has gone into defining appropriate experi-

ments for their validation. One of the parameters receiving emphasis in these validation discussions is accuracy. However, caution needs to be exercised in the final interpretation of the accuracy of concentration data for incurred (study) samples for macromolecules, as determined by LBA. While assay accuracy should address the issue of “closeness of the measured value to the true value” and sometimes is referred to as “guaranteeing” this agreement (11), closeness of measured and true values cannot easily be evaluated or verified for the typical biological macromolecule in a sample from a dosed animal or human. This is because LBAs are typically run without prior separation of the molecule of interest, and the intermediary anabolism or metabolism products, particularly of proteins, are largely unknown. Thus, the interference from cross-reactivity of such products of metabolism with the capture reagent (and also their relative biological activity compared to that of the parent macromolecule) is unknown. In other situations in which the macromolecule elicits an immune response to itself, where soluble receptors for the macromolecule may exist in circulation or where a candidate therapeutic antibody to a soluble antigen may lead to the possibility of “free” and “total” antibody concentrations, difficult-to-define interferences with the assay for the macromolecule of interest may also occur, with unknown effects on reported concentrations. Another potential effect on the reported LBA concentrations which may vary greatly between subjects is interference from endogenous components in the sample, such as an endogenous protein that is also given exogenously as a therapeutic, rheumatoid factor, which occurs in some disease populations, or the presence of anti-macromolecule antibodies. Advance knowledge of the presence of such factors, their variability between subjects/patients and their effects on apparent concentrations of the macromolecule is generally not available. However, appropriate screening of pre-dose samples from each individual will help detect the presence of such factors and determine their effects on assay performance, perhaps leading to a more meaningful interpretation of assay results from subjects following administration of the macromolecule. A recent report (12) showed that use of different capture reagents in an assay for rituximab (capture by immobilized target peptide vs. capture by an anti-idiotypic antibody) gave discrepant plasma concentrations and derived pharmacokinetics, again illustrating the inherent difficulty

¹ Gilead Sciences Inc., 4 University Place, 4611 University Drive, Durham, NC 27707-3458, USA.

² To whom correspondence should be addressed. (e-mail: john.w.findlay@gilead.com)

in deriving “true” values from LBA analysis of macromolecules in incurred samples. LC-MS/MS assays and LBAs for small molecules are more fortunate in this regard; in the case of the former, the combination of chromatographic separation and specific fragmentation pattern and, in the latter case, the ability to compare LBA results for study samples with a chromatographic assay as part of the validation exercise increase confidence that the assay reports data specific for the analyte of interest.

While use of calibration standards, validation samples and quality controls permit evaluation of the LBA performance using samples to which the macromolecule of interest has been added *in vitro* (“spiked” samples), it may not make good sense for analysts to spend extensive time and resource developing assays with better and better accuracy and precision for spiked samples, only to report what may be inherently approximate data for incurred study samples. This potential inaccuracy in LBA data for study samples and the potential sources of such inaccuracy need to be clearly acknowledged during interpretation of study data and derivation of conclusions about calculated pharmacokinetic parameters. In some cases (consistent with the fit-for-purpose approach at a Discovery or early Development stage), this approximation of the study sample concentrations will be acceptable, while in other experiments intended to derive pure pharmacokinetic parameters for a macromolecule, additional work should be done to select assay reagents most likely to reflect values closest to true parent macromolecule concentrations, or to demonstrate correlation of immunoreactivity data (and subsequently calculated pharmacokinetic data) with biological (pharmacodynamic) activity. If a biological activity assay is available, the relationship between LBA concentrations and biological activity should be evaluated; in some cases, considerable divergence between these two parameters has been noted over increasing time since *in vivo* administration of the macromolecule (13), indicating that LBA-measured concentrations result from more than just the binding of parent molecule in the assay. Extents of *in vivo* degradation or metabolism of the macromolecule to mixtures of differentially cross-reacting compounds may also vary with route of administration. A thorough evaluation of methods and results should be conducted on a case-by-case basis. Pharmacokinetic data for biological macromolecules should generally be viewed with circumspection.

Given this uncertainty regarding actual concentrations of macromolecules in incurred samples, more emphasis should be placed on the evaluation of incurred samples in LBA applications (14). Incurred samples should be analyzed on two separate occasions in two separate assay runs, to provide data for assessment, regardless of whether the sample has been analyzed at more than one dilution in the first assay. While acknowledging the current practical limits on our ability to assess specificity and accuracy of LBAs for macromolecules, additional energy should be devoted to evaluation of new approaches that may elucidate or confirm true macromolecule concentrations in study samples, as determined by LBA analyses. Although solutions to this challenge are not readily apparent, applications of advances in automated, high-throughput LBAs coupled with some specificity-enhancing

technique, such as prior immunoaffinity or chromatographic separation or subsequent mass spectral characterization, should be pursued more aggressively than they have been to date...

Finally, although the principles of the “fit-for-purpose” approach make excellent logical sense, analysts should realize that, since LBA specificity for the macromolecule of interest can rarely be defined even with the most highly developed and validated version of the assay, the “fitness” of the assay should be honestly assessed in studies intended to provide definitive pharmacokinetic data for the parent macromolecule.

REFERENCES

1. PhRMA report. *Medicines in Development, Biotechnology*, 2006. Available at <http://www.phrma.org/files/biotech%202006.pdf>. Accessed April 24, 2008.
2. V. P. Shah, K. K. Midha, S. Dighe, *et al.* Analytical methods validation: bioavailability, bioequivalence and pharmacokinetics studies. *Pharm. Res.* **9**:588–592 (1992).
3. V. P. Shah, K. K. Midha, J. W. A. Findlay, *et al.* Bioanalytical method validation—a revisit with a decade of progress. *Pharm. Res.* **17**:1551–1557 (2000).
4. C. T. Viswanathan, S. Bansal, B. Booth, *et al.* Quantitative bioanalytical methods validation and implementation: best practices for chromatographic and ligand-binding assays. *AAPS J.* **9**:E30–E42 (2007).
5. J. W. A. Findlay, W. C. Smith, J. W. Lee, *et al.* Validation of immunoassays for bioanalysis: a pharmaceutical industry perspective. *J. Pharm. Biomed. Anal.* **21**:1249–1273 (2000).
6. K. J. Miller, R. R. Bowsher, A. Celniker, *et al.* Workshop on bioanalytical methods validation for macromolecules: summary report. *Pharm. Res.* **18**:1373–1383 (2001).
7. B. DeSilva, W. Smith, R. Weiner, *et al.* Recommendations for the bioanalytical method validation of ligand-binding assays to support pharmacokinetic assessments of macromolecules. *Pharm. Res.* **20**:1885–1900 (2003).
8. Food and Drug Administration *Guidance for Industry: Bioanalytical Method Validation*, US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Rockville, MD, 2001.
9. J. W. Lee, R. S. Weiner, J. M. Sailstad, *et al.* Method validation and measurement of biomarkers in nonclinical and clinical samples in drug development. A conference report. *Pharm. Res.* **22**:499–511 (2005).
10. J. W. Lee, V. Devanarayan, Y. C. Barrett, *et al.* Fit-for-purpose method development and validation for successful biomarker measurement. *Pharm. Res.* **23**:312–328 (2006).
11. B. Boulanger. Results of pilot dataset—and how this led to the design of a larger data set. In *Variability of Ligand Binding Assays Used for PK Protein Drug Bioanalysis—Database Collected as a Follow-up to the 2006 AAPS Crystal City III Workshop*, AAPS Annual Meeting, San Diego, CA, November, 2007.
12. H. Blasco, G. Lalmanach, E. Godat, *et al.* Evaluation of a peptide ELISA for the detection of rituximab in serum. *J. Immunol. Methods.* **325**:127–139 (2007).
13. R. J. Wills and B. L. Ferraiolo. The role of pharmacokinetics in the development of biotechnologically derived agents. *Clin. Pharmacokinet.* **23**:406–414 (1992).
14. M. Rocci, V. Devanarayan, D. B. Haughey, and P. Jardieu. Confirmatory reanalysis of incurred bioanalytical samples. *AAPS J.* **9**:E336–E343 (2007).