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Research paper

Novel data analysis methods to overcome cut point challenges and enable comprehensive assessment of antidrug binding activity in confirmatory assays

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ABSTRACT

Immunogenicity assessments in response to drug treatment are commonly performed using a tiered approach strategy. All samples are initially tested in a screening assay followed by the evaluation of the screened positive samples in a confirmatory assay. Percent inhibition of signal intensity by the competing unlabeled drug in a confirmatory assay is typically used to measure the specificity of antidrug binding activity in samples, and has been successfully applied to most immunogenicity assays. However, the percent inhibition approach may not be suitable in cases where broadly distributed and high percent inhibition values are observed in drug-naïve subjects or when persistent operator-dependent differences in assay performance are encountered. Herein, we present the case studies faced with such challenges and provide appropriate solutions by introducing two novel data analysis methods: (1) Reference Delta, and (2) Reference Percent Inhibition, — in which relative-to-baseline signal inhibition is calculated for each sample. These novel methods significantly improve the confirmatory assay's ability to detect the samples positive for antidrug antibodies (ADA), especially when challenges are encountered using the traditional percent inhibition approach. Furthermore, both methods can be implemented in parallel with the percent inhibition method, enabling not only confirmation of ADA specificity, but also providing additional insights about the relevance of this antidrug binding activity to drug treatment.

1. Introduction

Clinical assessments of immunogenicity responses to protein therapeutics are often performed using a multi-tiered approach, where samples are screened for the presence of antidrug antibodies, followed by a confirmation of ADA specificity to a drug in samples which screened positive, and the semi-quantitative assessment of ADA titers in the confirmed-positive samples. Other assessments such as neutralizing antibody activity, antibody isotyping, etc. may also be performed depending on the study type, clinical development phase, immunogenicity risk factors and other considerations (Koren et al., 2008). Confirmation of the ADA-positive samples is typically performed using a competition assay format in which the binding of ADA to a labeled drug is competed by the addition of a large excess of unlabeled drug. Other confirmatory assay formats were also reported (Shankar et al., 2008; Wakshull and Coleman, 2011). For simplicity, the more commonly used competition assay format will be discussed in this manuscript. Regardless of the confirmatory assay format, the measured outputs are often calculated as percent inhibition or depletion of the measured signal intensity (*%I*). The magnitude of signal inhibition is then evaluated by comparing the *%I* values to a confirmatory cut point derived by modeling the percent inhibition values in a target population of drug-naïve samples (Shankar et al., 2008; Smith et al., 2011). The ratio (*R*) of signal intensities with and without drug competition is functionally related to percent inhibition

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($\%I = 100\% \times (1 - R)$) and can also be used for the modeling of confirmatory data and setting a cut point (Shankar et al., 2008). Unlike the percent inhibition values, these ratios are always positive and may be more suitable for data modeling, especially when certain data transformations (e.g., log transformation) are required. T-test comparison of signal intensities with and without drug competition has also been reported (Neyer et al., 2006) and is based on the assumption that competed and non-competed samples should have the same signal intensity in the absence of ADA (or null hypothesis is not rejected). Such assumption, however, is hard to satisfy across the entire population of samples. By far, setting a confirmatory cut point based on the percent inhibition or ratio values is among the most commonly used analysis method by the bioanalytical community and has been described in several recent publications (Shankar et al., 2008; Smith et al., 2011).

In some cases, however, the resulting distribution of percent inhibition values or ratios in drug-naïve samples may not be suitable for reliable data modeling. For example, an operator-dependent distribution of percent inhibition values or broad distribution of these values beyond the 50% level may pose significant challenges in setting a meaningful confirmatory cut point without potentially increasing the risk of false negatives. Selected examples of such challenges will be discussed in this manuscript and are rather common based on our experience in other assays across several biologic drug development programs (data not shown). Also, recent publication by Peng et al. (2011) showed examples of high percent inhibition values in negative control samples and cited other examples where such problems have occurred with considerable frequency across multiple assays. The first choice in solving these problems is always to perform a root-cause analysis and mitigate the encountered challenges by troubleshooting the assay. In some cases, however, problems associated with frequently occurring high percent inhibitions in drug-naïve samples may be innate to the samples and cannot be readily fixed at an assay level.

In this manuscript, we describe two novel data analysis methods of confirmatory assay results: (1) Reference Delta ($R\Delta$), and (2) Reference Percent Inhibition ($R\%I$), — in which relative-to-baseline, instead of absolute signal inhibition, is calculated for each sample. The relative-to-baseline values may be more suitable for setting a confirmatory cut point than the traditional $\%I$ values in cases where significant challenges are encountered either due to the pre-existing drug-specific interactions in drug-naïve samples, or due to persistent operator-dependent differences in confirmatory assay results. Both $R\Delta$ and $R\%I$ methods were successfully applied to several case studies in which significant improvement in confirmatory assay results were obtained compared to the $\%I$ method. We also discuss how the proposed $R\Delta$ or $R\%I$ methods could potentially be used as an extension of the percent inhibition approach and be helpful in confirming not only the specificity of antidrug binding activity in samples but also the relevance of this antidrug binding activity to drug treatment. This could be accomplished even in cases when the traditional $\%I$ method works but occasional pre-existing antidrug binding activity in drug-naïve samples is encountered.

2. Materials and methods

2.1. Reagents, materials and serum samples

The humabA and humabB drugs (immunotherapeutic human IgGs) and anti-target blocking mouse monoclonal antibody were developed at Biogen Idec, Inc. (Cambridge, MA). Antidrug idiotype antibodies were custom generated at Maine Biotechnology, Inc. (Portland, ME). The NUNC Streptavidin coated plates, PIERCE Streptavidin coated plates, 96-well Round-bottom polypropylene plates, PBS Casein and plate sealers were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA). The TMB (3,3′,5,5′-Tetramethylbenzidine) assay substrate was purchased from Surmodics, Inc., MD. The PIERCE SuperSignal ELISA femto Maximum Sensitivity Substrate (Luminol) was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA).

The individual normal human sera and RA (rheumatoid arthritis) sera were purchased from Bioreclamation, Inc. (Westbury, NY). The RA sera for longitudinal studies were collected from RA patients at 2 different time points separated by at least 5 months. Time difference between the two longitudinal time points ranged between approximately 5 months and 14.5 months.

2.2. Labeling of antibodies

The 10 mM stock solution of digoxigenin-3-0-methyl-carbonyl-aminocaproic acid-N-hydroxysuccinimide (DIG-NHS ester from Roche Diagnostics, Mannheim, Germany) was prepared by dissolving the powder material in anhydrous dimethyl sulfoxide (DMSO supplied by Sigma, St Louis, MO). The drug was prepared as 2 mg/mL solution in phosphate buffer saline (PBS) without added Ca^{2+} and Mg^{2+}. The drug was labeled with digoxigenin for 1 h with constant agitation at ambient temperature using the molar drug-to-label challenge ratios of 1 to 10 and 1 to 15. Uncoupled digoxigenin was removed by Zeba Desalt Spin columns (Thermo Fisher Scientific, Inc. Waltham, MA) equilibrated in PBS with 0.1% sodium azide and the buffer exchanged product was aliquoted and stored at −70 °C.

The biotin-labeled drug was prepared using EZ Link Sulfo NHS LC Biotin (Thermo Fisher Scientific, Inc.) dissolved in PBS and added to a 2 mg/mL drug solution in PBS at 1 to 10 drug-to-label molar challenge ratio. Drug labeling and purification of the labeled product was performed in a similar way as for DIG labeling described above.

2.3. Solution ELISA methods

The method for detection of ADAs was based on a solution phase bridging ELISA (Solution ELISA) format with chemiluminescent or chromogenic detection. The anti-humabA Solution ELISA with acid pretreatment of samples and chemiluminescent detection was performed as described elsewhere (Mikulskis et al., 2011). The anti-humabA and anti-humabB Solution ELISAs with chromogenic detection were performed in a similar way but without acid pretreatment of samples. Briefly, samples were diluted 1/100 in PBS-casein buffer containing 2 μg/mL of each biotin- and DIG-labeled drug. Competition with unlabeled drug in a confirmatory assay was performed using 100 μg/mL of