Meeting Report

Antidrug Antibody Assay Validation: Industry Survey Results

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Abstract. Immunogenicity of biopharmaceutical products has attracted considerable attention from the industrial, academia, and regulatory organizations. Many methods exist to detect and characterize level of antidrug antibody response in patients. Still, additional work is required to harmonize various approaches used throughout the industry. This review presents results of a survey sponsored by the American Association of Pharmaceutical Scientists that was designed to collect relevant information and to understand various methods used throughout the bioanalytical field for the detection and evaluation of antidrug antibody responses.

KEY WORDS: antidrug antibody; antiprodukt antibody; biopharmaceutics; immunogenicity.

INTRODUCTION

Immunogenicity is generally recognized as an important safety concern for the development of biopharmaceuticals. In addition, immunogenicity can affect the efficacy of biopharmaceutical products. Methods used to evaluate and predict antidrug immunogenicity have been rapidly advancing, but to date, it is still not possible to fully predict whether the generation of antidrug antibodies will be inconsequential or have serious clinical outcomes. For this reason and because currently the immunogenic potential of a biopharmaceutical drug can only be definitively assessed in human studies, emphasis has been placed on optimizing assays designed to detect antidrug antibody (ADA) response. Multiple regulatory authorities issued position statements in which they require antidrug antibody assays to be thoroughly evaluated and aligned with the current understanding of the issue (1–3). A recently published “white paper” and several review articles (4–13) present an overview of the important considerations for the development and validation of antidrug antibody assays. This ultimately should lead to greater harmonization of approaches used when developing and validating appropriate assays within the pharmaceutical and biotechnology industries. In spite of the progress in the understanding of the required assay parameters, the analytical performance characteristics that should be assessed during method validation are less clear.

The quasiquantitative nature of the antidrug antibody assay formats means the analytical response variable is determined without interpolation against a calibration curve of a reference standard, as it is commonly done for drug component testing. These characteristics and unique features of ADA assay formats have given rise to questions about the appropriate method validation and statistical analysis procedures for antidrug antibody assays. Some important analytical performance characteristics for method validation include characterization of the sources of variability, estimation of imprecision, and reproducibility of assay controls. Particularly noteworthy are the issues pertaining to statistical estimation of assay “cut point” value. Determining and understanding such assay characteristics as drug tolerance, sensitivity, assay control, and critical reagent stability have been subjects of extensive discussions. Some of the steps involved in ADA assay validation have been identified as desirable, but not necessarily generating values that can be utilized during sample analysis.

Related discussions occurred at several roundtable sessions held at the National Biotechnology Conference (American Association of Pharmaceutical Scientists, AAPS) and multiple other conferences and meetings. The growing level of understanding of the issue leads to better understanding of the steps required during assay validation as well as better understanding of the magnitude of the validation process needed. Yet, the overall industry-wide position remains diverse. A survey was designed to gather information and to better understand various methods used throughout the bioanalytical field regarding detection and evaluation of antidrug antibody responses.

The survey results allow the comparison of opinions on the processes followed to assess antidrug antibody development in nonclinical and clinical studies. The survey was distributed with the help of the AAPS organization between February and March of 2008. A total of 51 completed responses were received during this time period. The results of the survey were presented at the 2008 National Biotechnology Conference in Toronto,

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ABBREVIATIONS: AAPS, American Association of Pharmaceutical Scientists; ADA, antidrug antibody; ECL, electrochemiluminescence; ELISA, enzyme-linked immunosorbent assay; NC, negative control; OD, optical density; SD, standard deviation; SPR, surface plasmon resonance.

Canada at the roundtable session “Data Analysis for Anti-Product Antibody Assessment”.

SURVEY RESULTS

Almost half of the responses (47%) were received from larger pharmaceutical companies. Responders from midsize and small biotechnology companies amounted to 31% and 12% of responses, respectively. Nonindustrial organizations provided approximately 10% of the responses. A vast majority of the responders indicated involvement in supporting both clinical and nonclinical studies (80%). Therefore, this survey represents a wide group of scientists and companies with experience in supporting clinical as well as nonclinical immunogenicity evaluations.

Enzyme-linked immunosorbent assay (ELISA), a well-established methodology verified by time and countless applications, remains the main platform used during the evaluation of the immunogenicity of biopharmaceutical products. Several reasons can be suggested for moving away from the routine use of ELISA. These include assay sensitivity, long assay time, narrow dynamic range, and limited assay drug tolerance potential. Multiple incubation and wash steps used in the ELISA approach result in generally low sensitivity of the method toward low affinity antidrug immunoglobulins (14). Other methods (e.g., surface plasmon resonance-based Biacore) can offer better ability to detect low affinity antibody but suffer from generally lower sensitivity when higher affinity antibody used as the assay positive control. Several other platforms, including electrochemiluminescence-based protocols, have been recently introduced as alternatives (15) promising better assay drug tolerance and ability to detect immunoglobulins with a wide range of affinities to the drug molecule. The survey results indicate that at this time, such issues have not provided significant pressure to displace the well-validated and understood ELISA platform that is supported by multiple vendors. The method continues to dominate the market. All responders identified that ELISA-based protocols are used within their laboratories. At the same time, survey indicated that majority of the laboratories (90% of responses) are implementing other assay platforms. High as well as low affinity antidrug antibodies have a potential to affect both clearance and activity of biotherapeutic agent. It may be critical therefore to detect antibodies with a wide range of affinities. Evaluation of new technologies should be considered if low affinity responses are viewed as important for a particular drug development program.

Assays generally used to determine the presence of immune response to a protein drug molecule include screening and specificity confirmation assay platforms. In addition, one can perform assays designed to further characterize ADA activity by determining sample antidrug antibody titer value and presence of neutralizing antibody activity (7,10,11). In general, samples are initially tested in a screening assay format and are classified as potentially ADA positive or ADA negative. Such yes or no classification requires a criterion for distinguishing positive and negative samples, generally called a screening cut-point. Vast majority of survey responders (88%) indicated the use of a screening cut-point when assessing antidrug antibody assay results. Some responders (12%)

indicated use of alternative approaches. For example, a wide range of preexisting antidrug antibody responses may greatly complicate statistical analysis of the data collected to calculate assay cut-point parameter. Here, a single negative control sample may not be representative of the range of pretreatment responses obtained in the clinic. A direct comparison of the pretreatment and corresponding posttreatment sample results for a given patient may be required.

As a part of the assay screen cut-point analysis, one needs to evaluate assay background signal. The importance of using statistical approaches when determining an assay screening cut-point has been emphasized previously. Normally, such analysis is conducted during the prestudy assay validation step. Generally, three assay screening cut-points have been suggested (11): fixed, floating, and dynamic, listed here in the order of flexibility allowed during sample analysis. Briefly, a fixed cut-point is defined as an absolute assay response value (e.g., optical density) determined during assay prestudy validation and used at the time of in-study sample testing. A floating cut-point allows for a readjustment of the actual cut-point value used on each plate based on the performance of the assay background sample (e.g., assay negative control). Normally, a floating cut-point is determined by multiplying a specific normalization factor, determined during prestudy assay validation, by the averaged biological background signal produced in the assay. As the biological background, the assay negative control, assay diluent, or, in some cases, predose sample collected from the same patient can be used. Other approaches where the normalization factor is directly added to the background value have been described (7,10,11). Finally, a dynamic cut-point value is determined for each plate individually and is not based on the estimations made during prestudy validation. Dynamic cut-point approach may be appropriate when a high degree of run to run variability is observed. Here, cut-point value is calculated based on data analysis generated on a particular plate (run). The approach requires a considerable amount of space on the assay plate to be dedicated for the cut-point determination and therefore limits number of unknown samples tested. More details on the application and calculation of the various cut-points described above can be found in (11). The survey requested participants to identify the type of cut-point used in each of the responding laboratories. Possible answers included the use of fixed, floating, dynamic cut-points, or none at all. Since the entire field of biopharmaceutical bioanalysis has been transitioning and the industry position on methods designed to analyze the data is being formulated, it is easy to imagine a situation where a given laboratory is utilizing more than one type of cut-point. The survey therefore allowed for multiple responses and many participants used this feature.

The distribution of the responses is shown in Fig. 1. Fixed cut-point approach is used by 35% laboratories that responded to the survey. About 30% of this group use fixed cut-point as the main method of data analysis. Almost half of the responders (47%) identified the use of a floating cut-point. Of these, 40% use floating cut-point exclusively. Floating cut-point platform has been largely viewed as the most flexible, robust, and allowing for greater control over the experimental variability in the assay. Relative to the dynamic cut-point approach, analysis of the negative control, required for calculation of the floating cut-point value, necessitates limited

Fig. 1. Type of screening cut-point used

amount of space on the assay plate allowing for more unknown samples to be tested. Dynamic cut-point was identified by 14% of the labs with 18% of the group indicating dynamic cut-point approach as the main method used. Due to the ease of use and robustness of the generated value, floating cut-point can be generally recommended (11).

It is important to understand the range and level of antidrug antibody responses in the patient (subject) population to be used in the future clinical or nonclinical studies. Therefore, both drug naïve normal and disease state individual samples should be tested during prestudy assay validation phase (7,10,11). Generated data are used to determine assay cut-point type and cut-point value as well as to test for the presence of any preexisting antidrug antibodies. Yet, obtaining disease-state subject samples may be challenging and initial evaluation could be limited to the analysis of healthy normal drug naïve individual samples. In the survey, vast majority of the responders (94%) confirmed the use of the drug naïve normal individual matrix samples. Evaluation of the assay response (biological background) in the drug naïve disease population samples is particularly important when developing clinical ADA assays. Accordingly, the majority of the responders (total 88%) indicated that their laboratories either routinely (46%) or on a case-by-case basis (42%) utilize drug naïve disease state individual matrix samples during prestudy assay validation.

Further analysis of the matrix reactivity data may include identification of highly reactive samples that could be deemed as outliers and excluded from further statistical data analysis. Generally, at this point, investigators do not have well-defined screen and specificity assay cut-points and therefore, the assessment has to be based on a well-defined scientific rationale. According to the survey results, the majority (80%) of the laboratories identifies and excludes statistical outliers during analysis of the matrix reactivity data on a regular (42% of the responses) or a case-by-case (38%) basis.

After any relevant outliers are identified and removed from the data pool, individual sample matrix results should be analyzed using an appropriate statistical method to determine optimal screening cut-point value. Several methods have been identified and described in detail (11), including parametric (mean negative control + 1.65 standard deviation) and nonparametric (based on 95% tile) approach. When using parametric approach, the distribution of matrix sample results should be initially analyzed for normality. Data transformation could be required. The use of nonparametric data analysis generally does not require transformation of the data. At this point, data set should be analyzed to determine the most appropriate type of the screening cut-point, as presented above.

In the survey, the majority of responders (72%) indicated the use of a parametric approach for both clinical and nonclinical assays. Smaller percent of laboratories indicated using either nonparametric approach or other methods (about 15% in each case) when calculating assay cut-point values.

Analysis of matrix interference is generally conducted to determine possibility of any matrix component to affect assay ability to detect presence of antidrug-specific antibodies. Well-developed selective ADA assay is expected to detect positive response in a sample in the presence of other matrix elements. One of the main interfering factors in ADA assays is the drug molecule itself. The ability of the drug component in the matrix sample to affect ADA assay performance is often referred to as assay drug tolerance and is discussed below. Matrix interference experiment can be conducted by analysis of either unspiked (neat) matrix samples and/or matrix samples spiked with some concentration of ADA positive control. Often the positive control antibody originates from species different from the matrix of the analyzed sample. In this case, xenogenic antibodies may often greatly reduce positive control response. This can be easily observed by comparing results for matrix and assay buffer spiked positive control samples. It is important to understand that analysis of the assay selectivity information obtained based on the performance of assay positive control will not be predictable of the assay selectivity when testing real study samples. Yet, when interference is expected, potential issues should be investigated during assay development and validation. Survey responders indicated that preferred approach (80% of responses) when assessing potential matrix interference includes a combination of analysis of spiked and unspiked (neat) samples. Other survey responders indicated exclusive use of either ADA positive control spiked (12%) or unspiked matrix samples (8%) only.

The final reportable value for a positive sample has to be presented using appropriate units. ADA assays are generally regarded as quasiquantitative due to the lack of an actual reference standard and the polyclonal and heterogeneous nature of the ADA analyte (16). Historically, both mass-based and titer units have been used. If reporting of the positive sample results using mass units is intended, an appropriate parallelism analysis of the sample and the assay positive control has to be demonstrated in order to determine ADA sample concentration with an acceptable accuracy. When parallelism between the sample and calibrator cannot be shown, which is often the case, the accuracy of ADA determination in mass units is questionable. Additionally, mass unit-based approach requires requalification in cases when the assay reference material antibody is changed. Alternatively, a recommended approach to measure ADA levels in a sample is to determine sample titer value. Although sample titer does not provide quantitative evaluation and is also susceptible to a potential lack of dilutional parallelism between assay control and study samples, this approach has been used extensively for many years and is generally recommended (11). In the survey, approximately half of the responders indicated the use of titer units (46%) while 38% of responders reported use of mass units in supported assays. (Fig. 2).

Based on the approach used in calculating assay cut-point value, approximately 5% of tested samples are expected to generate false-positive ADA response during

Fig. 2. Type of units used to report positive sample results

initial screening analysis. Therefore, further confirmation of the specificity of the ADA activity in the samples, identified as potentially positive, is recommended. This is particularly important for the assays designed to analyze clinical study samples. Specificity analysis for nonhuman samples should be considered on a case-by-case basis.

According to the survey results, the most common approach (80% of the responses), when confirming specificity of the ADA signal, is based on a competitive drug inhibition test. In this test, samples are spiked with certain amount of drug, as determined during assay prevalidation procedure. The reduction in the assay signal is generally expressed as relative percentage drop in the raw assay value generated by the sample and is analyzed to determine sample specificity. In the past, a general 50% reduction in the assay signal was used. The true value of the assay specificity cut-point is assay specific and has to be determined objectively based on analysis of matrix sample information (11). Similar to the procedure used when determining assay screening cut-point, drug naïve normal and disease population samples should be used. Ideally, naïve samples with low positive ADA reactivity should be applied for the test. Approximately half of the responders (51%) indicated the use of both normal and disease matrix samples. In some cases, obtaining disease-state samples could be challenging. About a quarter of the responders (24%) indicated the use of normal matrix samples only (Fig. 3).

Alternative methods of confirming ADA signal specificity exist. For example, in the case of plate based assays, where samples are tested on a plate coated with a specific capturing agent (generally drug molecule), additional analysis can be conducted using a plate which was not coated with the capturing agent, but was blocked. Results from both plates for a particular sample are compared to determine whether the difference between signals is significant (as compared to a parameter identified during assay validation). Use of alternative approaches was indicated by 16% of the survey responders.

Due to the semiquantitative nature of the ADA assays, some assay characteristics may be considered less than critical when conducting prestudy assay validation. For example, due to the lack of an assay specific reference standard and therefore the inability to generate a true assay reference standard curve, it is impossible to calculate true ADA assay sensitivity that can be later applied for each individual study sample. It has been shown that depending on the methodology used, assay sensitivity to high vs. low affinity antibody will differ (14). The assay format as well as the assay procedures should be carefully considered to allow for successful detection of a wide range of immunoglobulins. This is particularly relevant to ELISA-based protocols that have a relatively

reduced sensitivity to low affinity antibodies due to high number of incubation and wash steps. Generally, sensitivity of the ADA assays is calculated as the lowest concentrations of the assay positive control that can consistently generate a positive signal (11). Therefore, it should be expected that assay sensitivity will be highly dependent on the properties of a particular positive control used in the assay, for example on the positive control affinity. Yet, sensitivity assessment is commonly expected as a standard part of an ADA assay validation process. In the survey, approximately 93% of the responders indicated that assay sensitivity assessment test is conducted either always (71%) or on a case-by-case basis (22%).

Similarly to the assay sensitivity, assay drug tolerance may be regarded as an assay characteristic that is of concern, but one that cannot be defined as a parameter in the assay validation procedure. Due to the nature of the ADA assays in general, it is understood and expected that the presence of the drug molecule in the sample will result in some or complete reduction of the assay signal. Assay tolerance is generally determined based on the ability of the drug molecule to inhibit positive control activity. At some drug concentration, a positive sample is expected to generate assay signal similar to that generated by the negative control sample and therefore to be scored as “negative”. As a result, assay tolerance can be defined as a drug concentration that is capable of a complete inhibition of the assay positive control signal. This value cannot accurately predict a drug concentration that will interfere with ADA detection in actual study samples. Similar to what was said about assay sensitivity, assay drug tolerance value determined as indicated above is highly dependent on the particular properties of the assay positive control antibody, including positive control concentration, affinity, and other binding characteristics. Due to expected heterogeneity of the immune response, the drug tolerance value determined during assay prestudy validation cannot be directly applied during in-study sample analysis (10,11). The assay drug tolerance value should be considered as a guide. Drug tolerance could be used as an assay performance parameter during assay development when various assay platforms, including protocols that involve sample pre-treatment step, are considered (14,17,18). As a mitigation factor, steps should be taken to ensure that appropriate wash-out period was used in the study to allow for drug molecule to clear completely from circulation and enable sensitive detection of antidrug immunoglobulins. Majority (93%) of the survey responders indicated that drug tolerance is often or always conducted. More than half of the responders (63%) indicated that drug tolerance value is used during the sample analysis phase but only as an indication of possible interference in the

Fig. 3. Type of matrix samples used when determining assay specificity drug inhibition based cut-point value

assay. Though predictive power of the drug tolerance value determined based on the performance of the positive control is limited, about a quarter of the responders (24%) indicated that it is directly applied during analysis of study samples. Other responders (22%) indicated that the value is not used at the time of analysis of the study data.

Various methods promoting antibody–antigen complex dissociation can be used to improve assay drug tolerance. A method that includes sample treatment with a low pH buffer is referred to as “acid dissociation” protocol (17,18). Details of sample pretreatment protocols that can improve assay tolerance characteristic differ but the number of groups utilizing such technique is growing. In the survey, more than half (56%) of the responders indicated using some approach that helps to improve assay tolerance parameters either always or on a case-by-case basis. Still, a considerable number of responders (44%) indicated that acid sample pretreatment or similar procedures are not conducted. At the same time, many survey responders (79%) indicated that plans to initiate application of acid dissociation or similar protocol exist or will be considered in the near future.

Similar to what is generally done for the validation of the assays designed to determine drug concentration in biological matrices, it could be expected that the stability of ADA samples should become a part of the ADA assay prestudy validation protocol. Based on what is said above regarding the expected sample heterogeneity, positive control used in the assay is not expected to be representative of a real study sample. Therefore, the assay positive control is not expected to fully predict the stability of all study samples as well. It has to be noted though that immunoglobulins are generally considered to be very stable protein molecules, particularly when stored frozen in serum or plasma matrix. Hence, assessment of the ADA sample stability is not generally expected. Therefore, the survey focused on the question of assessment of the critical assay reagent stability only. For an ELISA-based method, critical assay reagents not only may include assay controls, capturing reagent, coated assay plate, and detector reagent conjugates, but may also involve blocking reagents and various assay buffers. Such determination has to be done on a case-by-case basis as a result of a careful analysis of the assay performance during assay validation phase. The majority of the survey responders indicated that some assay reagent stability is often tested during ADA assay validation (86%) but may be conducted on a case-by-case basis (approximately 37%). Generally, ADA assays should be considered stability indicating where loss of stability of any of the critical reagents will result in poor assay performance, or assay failure, monitored via the performance of the assay controls. Establishing appropriate objective range for the assay control acceptance criteria is therefore an important step in monitoring assay performance in general and a key factor in monitoring assay reagent performance. Stability characterization of assay-critical reagents can be viewed as a business decision.

Assay performance monitoring should continue as the assay is applied for analysis of study samples, or during in-study assay monitoring (in-study validation phase). In-study assay validation should be viewed as a critical component of any bioanalytical method used in nonclinical and clinical testing. Method validation therefore does not end until the

method is ultimately retired from the analytical use. Generally, performance of the assay controls, including the positive and negative control, is considered for the in-study monitoring. Also, assay prestudy validation is often supported by a limited number of assay runs conducted within a short period of time with a relatively small number of matrix samples. Therefore, in-study validation may be a very helpful tool in better understanding the performance of the assay screening and assay specificity cut-points, performance of assay reagents and assay robustness in general. Based on the results of in-study monitoring, assays may be revalidated either periodically or on an as-needed basis to control assay performance. As an example, a change in the assay critical reagent performance (e.g., drift in the positive control titer value) may dictate assay revalidation.

In the survey, majority of the responders (80%) indicated that in-study assay monitoring is either done routinely (52%) or on a case-by-case basis (28%). Many laboratories (66%) conduct assay revalidation based on the data obtained during in-study monitoring.

CONCLUSION

Survey responders represented a wide group of scientists and companies. The survey results demonstrate that many of the methods and approaches used in the field are in general agreement with the recommendations presented in the recently published “white papers” (7,10,11). The industry continues to evaluate its position toward potential generation and detection of the antidrug immune responses and it is not surprising that some variability in the assay data analysis exists.

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