Research paper

# Improvement of drug tolerance in immunogenicity testing by acid treatment on Biacore

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## Abstract

Detection of anti-drug antibodies (ADA) can be difficult, if not impossible, in the presence of drug in the sample. This is a particular concern with therapeutic monoclonal antibodies (mAbs), which have typically longer half-lives than other proteins. For detection of ADA in presence of high drug concentrations, assay choice is limited to ELISA-like methods, capable of incorporating acid dissociation procedures to separate drug-ADA immune complexes. To our knowledge, Biacore assays have not been shown to be directly compatible with acid dissociation procedures, until now. As a consequence, steps to ensure adequate clearance of the drug are prerequisite to enable sensitive detection of ADA. Here we describe the development of a novel, rapid and highly drug tolerant Biacore method that uses an acid dissociation step to detect ADA in the presence of excess drug in human serum. Removal of drug after acid treatment is not required.
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## 1. Introduction

As more therapeutic proteins become available on the market, the incidence of unwanted anti-drug antibody (ADA) responses in treated patients is rising (Hermeling et al., 2004). Although, in the majority of patients, ADA do not cause serious side-effects, they can neutralize the biological activity of the drug, alter pharmacokinetics and in rare cases have been responsible for life threatening conditions (Casadevall et al., 2002). In pre-clinical studies, ADA can seriously impact interpretation of pharmacokinetic, pharmacodynamic and biological activity; therefore, it is important to confirm that interpretation of toxicology data is not compromised by ADA (Bugelski and Treacy, 2004; Shankar et al., 2006). Thus, it is important to monitor and evaluate ADA responses during both clinical and pre-clinical studies. (Patton et al., 2005; Wadhwa et al., 2005; Koren et al., 2002).

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In presence of residual drug in a sample, ADA may be present as part of an ADA-drug complex. Therefore, assay sensitivity to ADA may be low while drug levels are high thus causing a requirement for washouts of several weeks. This is a particular concern with therapeutic monoclonal antibodies (mAbs), which have typically longer half-lives than other proteins.

To improve assay sensitivity in presence of drug, approaches to dissociate ADA-drug complexes with acid and consecutive detection by ELISA based methods have been applied successfully (Shankar et al., 2006). It has been shown that such methods reduce drug interference and tolerate the presence of ~100-fold molar excess of drug (Patton et al., 2005; Lofgren et al., 2007; Lofgren et al., 2006). Recently, assay variations, based on affinity capture of ADA from drug containing samples followed by removal of excess free drug, have also been described. (Smith et al., 2007; Bourdage et al., 2007).

Our aim was to develop an assay that is highly tolerant to drug and rapid at the same time. We have therefore developed a Biacore method that is independent of labeled compounds and does not require removal of excess drug. Like the above mentioned ELISA assays, we used acid pre-treatment of samples followed by a neutralization step. ADA were then detected in real time when binding to the drug on the sensor chip surface.

The method was tested for a chimeric monoclonal antibody (drug Y) using rabbit anti-drug antibodies and immunogenicity positive patient samples as control. 87-110 % ADA were recovered in the presence of 1 mg/ml drug Y, which corresponded to a ADA:DRUG Y ratio of 1:200 for the lowest ADA concentration tested (5 ug/ml).

We believe that this method will be generally applicable to the detection of ADA to biotherapeutics. Acid treatment conditions may need to be adapted to the respective species and compounds.

## 2. Materials and methods

### 2.1. Reagents

Blank human sera from healthy and untreated individuals, were obtained from Novartis Pharma AG (Basel, Switzerland). Therapeutic chimeric monoclonal antibody (drug Y) was supplied by Novartis. A rabbit polyclonal antibody to drug Y, depleted of anti-human IgG antibodies and affinity purified on a drug Y column was generated by Novartis together with Eurogentec (Seraing, Belgium). The resulting rabbit positive control

(rADA) recognized mainly the CDR regions and only weakly the mouse variable regions of drug Y. It did not recognize a non-specific human monoclonal antibody or full human IgG by ELISA or Biacore methods. Immunogenicity positive clinical serum samples (hADA) were obtained from 2 patients treated with either 0.3 mg/kg drug Y every 3 weeks or with 15 mg/kg drug Y every three weeks in a clinical study.

### 2.2. Preparation and acid dissociation of immune complexes with rabbit positive control (rADA)

Immune complexes were prepared by spiking rADA and drug into neat pooled human serum. Samples were prepared at rADA concentrations of 0, 5, 7.5, 10, 25, and 100 µg/ml. Drug was spiked at a concentration of 1 mg/ml and samples were gently vortexed followed by an incubation time of 1 h at 37 °C in a thermomixer at 300 rpm. The rADA-drug containing serum was adjusted with 1 M hydrochloric acid (HCl, Fluka, Buchs, Switzerland) to a final pH of 2.5. After incubation at 37 °C for another 30 min the samples were neutralized by adding 1 × PBS (Roche, Mannheim, Gemany), followed by 1 × NSB reducer (BIAcore AB, Uppsala Sweden) and 1 × running buffer containing 1 × PBS-EDTA 1 mM (Fluka) and 0.05% Tween 20 (Fluka). The final sample pH of acid treated samples was 6.0 and of non-treated acid treated samples 6.5. Samples were then measured in the Biacore assay.

### 2.3. Acid dissociation of immune complexes in immunogenicity positive clinical samples (hADA)

The hADA-drug containing serum was adjusted with 1 M hydrochloric acid to a final pH of 3.0. Consecutive steps were performed as described above.

### 2.4. Biacore assay

The ADA responses were assessed by Biacore 2000 and T100 instruments by Biomolecular Interaction Analysis (BIA). Protein G was used to immobilize drug Y on the dextran matrix of a CM5 sensor chip, ensuring exposure of potentially immunogenicity Fabv and CDR regions. Drug Y was injected at a concentration of 1 mg/ml to ensure saturation of protein G. Samples were injected and binding of ADA measured in real time. Since drug Y was bound to protein G reversibly, it was removed from the sensor surface after every regeneration cycle and injected newly before the next sample was applied. This procedure ensured that integrity of drug Y was not affected by the chip regeneration procedure with
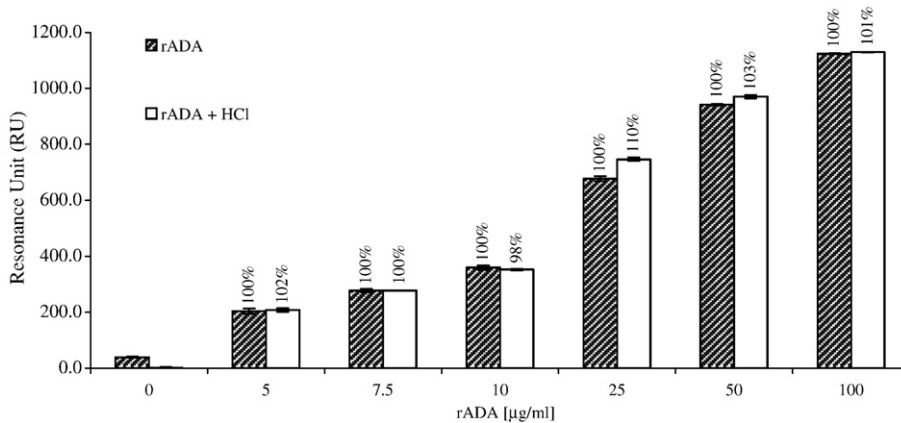
Fig. 1. Comparison of non-treated and acid-treated rabbit anti-drug antibodies (rADA) spiked in human serum samples. After acid treatment (pH 2.5, 37 °C, 30 min), followed by dilution in running buffer to reach a pH of around 6, the rADA recovery was 98- 110 % as compared to the corresponding non-treated sample. Each calibrator was assayed in duplicate.

100 mM HCl. A free chip sensor surface (reference surface) for background subtraction was generated by activating with EDC/NHS and deactivated with EtOH-NH only. The final signal was given as RU (Response Unit)=$RU_{drugY} – RU_{ref}$.

## 3. Results

### 3.1. Impact of acid treatment on rabbit anti-drug antibodies

In a first step, the impact of acid treatment on stability of the rabbit positive control (rADA) was tested. Affinity purified rADA were spiked into neat pooled human serum to generate a concentration curve ranging from 5 to 100 µg/ml. An aliquot of each sample was measured in the Biacore assay. A second aliquot was adjusted with hydrochloric acid to a final pH of 2.5 before injection onto the Biacore chip. All samples were diluted with running buffer containing NSB reducer. A final pH of around 6.0 was obtained for acid treated and of around pH 6.5 for non-treated samples. Final dilution factors of non-treated and acid-treated samples were identical for all samples. Recovery of rADA in acid treated samples was 98 – 110 % as compared to non-treated samples (Fig. 1). These results suggest that acidification did not impact rADA stability nor their capability to bind drug Y.
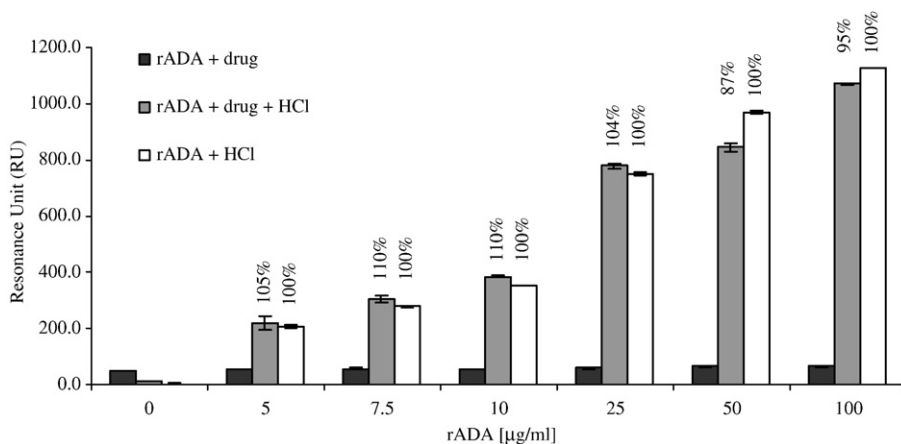


Fig. 2. Recovery of rabbit anti-drug antibodies (rADA) from rADA-drug Y immune complexes in human serum by acid dissociation. In untreated serum samples spiked with rADA and drug Y, the detection of rADA was inhibited in the Biacore assay. When treated with acid (pH 2.5, 37 °C, 30 min), followed by adjustment to pH 6.0, the rADA recovery was 87- 110 % as compared to the corresponding control sample without drug. n=2 for each sample with standard deviation bars.

### 3.2. Recovery of rADA from rADA-drug immune complexes by acid dissociation

Immune complexes were prepared by spiking drug Y at a concentration of 1 mg/ml together with rADA, ranging from 5 – 100 μg/ml, into neat pooled human serum. The inhibition of rADA binding to drug Y on the sensor chip surface evidenced that the immune complex formation was successful (Fig. 2). After forming immune complexes, serum samples were acidified to pH 2.5 and further treated as described above. Samples containing rADA but no drug served as reference. These samples were also treated with acid and diluted in the same way as the rADA containing samples with drug. Using the acid treated rADA spiked samples without drug as a reference, 87 –110.0 % rADA were recovered from the drug inhibited samples by this procedure (Fig. 2). The molar excess of drug tolerated in the Biacore assay ranged from 10-to 200-fold. Higher drug concentrations or lower rADA concentrations were not investigated.

### 3.3. Recovery of hADA from clinical samples with spiked drug

To compare rabbit positive control rADA and human derived hADA with respect to acid stability and treatment conditions, clinical samples with known immunogenicity and minimal to no residual drug levels were used. The respective patient had been treated every three weeks over 18 weeks with 0.3 mg/kg drug Y. Sample were collected pre-dose on day 1, day 42, day 63, day 84, day 105, day 126. Drug Y was spiked at 1 mg/ml into the respective serum samples to form

immune complexes. In samples without acid treatment, the spiked drug interfered with hADA detection. Applying acid treatment conditions as used in experiments with the rabbit surrogate rADA, only around 25 % hADA were recovered. However, by changing the acid treatment conditions from pH 2.5 to pH 3.0, a recovery of 92- 100 % for all time points was observed using acid treated samples without drug as a reference. To assess acid stability of these reference samples, an aliquot spiked with 1 × PBS was compared to acid treated samples in the Biacore assay. A reduction in non-specific binding of 40 % was observed for the acid treated pre-dose sample. The decrease for specific signals of the post-dose samples ranged from 2 – 17 % and was considered non-significant (Fig. 3).

### 3.4. Recovery of hADA from clinical samples containing residual drug

To investigate, whether hADA detection in clinical samples containing residual drug can be improved, a patient, who had been treated every three weeks with drug Y at 15 mg/kg, was selected. Pre-dose samples on day 21, 42, 63 and 84 and one sample on day 27 were analyzed.

An aliquot of each sample was adjusted with 1 M HCl to pH 3. A second aliquot was spiked with the same volume 1 × PBS. Both samples for each time point were measured in the Biacore assay after dilution in running buffer. All acid treated post-dose samples showed higher screening signals (13 – 55%) as compared to the non-treated samples (Fig. 4). The obtained signals were subsequently confirmed as drug specific hADA (Table 1
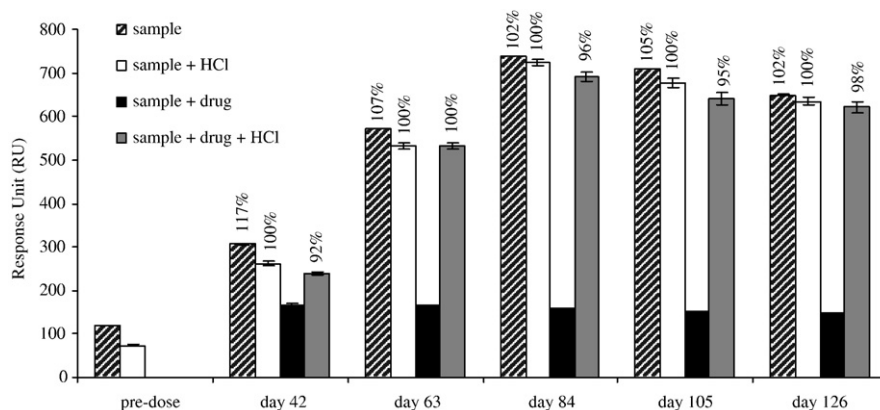


Fig. 3. Recovery of hADA from clinical samples. Serum samples were obtained from a patient treated every three weeks with 0.3 mg/kg drug Y. Pre-dose samples were collected on day 1, 42, 63, 84, 105 and 126. After acid pretreatment (pH 3, 37 °C, 30 min), a slight loss in specific signals was observed (2-17 %). Samples were spiked with 1 mg/ml drug Y. Acid treatment lead to 92 – 100 % recovery of hADA from drug Y containing samples as compared to acid treated samples without drug. Samples were assessed in duplicate.
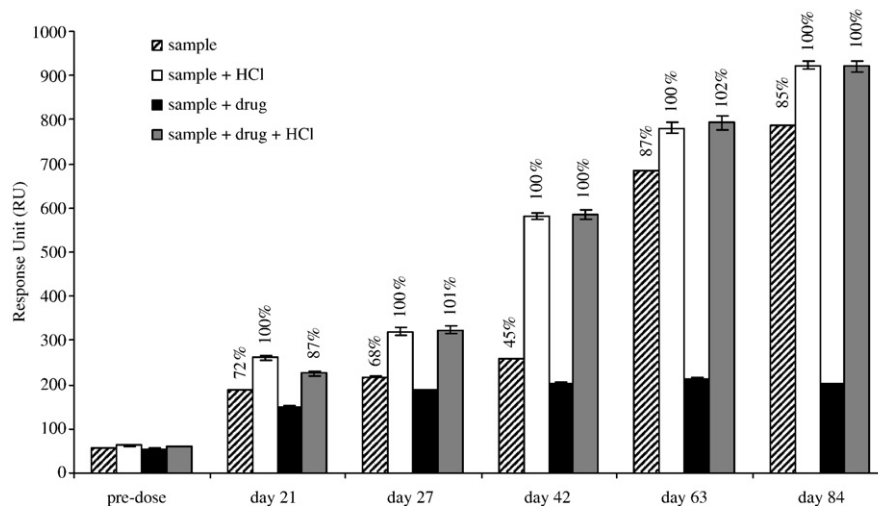
Fig. 4. Recovery of hADA from clinical samples containing residual drug Y. Serum samples were obtained from a patient dosed with 15 mg/kg every three weeks. Pre-dose samples were collected on day 1, 21, 42, 63 and 84. An additional sample was taken on day 27. After acid treatment (pH 3, 37 °C, 30 min) of residual drug Y containing samples, up to 55 % higher screening signals were measured as compared to the non-treated samples. These signals were subsequently confirmed as drug specific hADA (see Tables 1 and 2). When samples were spiked with additional 1 mg/kg drug Y, 87 – 102 % of patient hADA were recovered as compared to acid treated samples without spiked drug Y. All samples were assessed in duplicates.

and 2). To investigate the recovery of hADA from samples with higher drug levels, 1 mg/ml drug was spiked into all serum samples. Without acid treatment, the spiked drug interfered significantly with hADA detection. Applying the acid treatment procedure, 92 – 100% of hADA were recovered using acid treated samples without spiked drug as a reference (Fig. 4).

Positive immunogenicity results were based on the comparative analysis to the negative cut-off (NCO) and subsequent confirmation of drug specificity by incubating the sample with 1 mg/ml drug Y. Only sample values inhibited >30 % were qualified immunogenicity positive. For calculation of the NCO, see next section.

Without acid treatment, although all post-treatment samples were above the NCO, only samples on day 63 and day 84 were determined drug specific and therefore immunogenicity positive (Table 1). After acid treatment, also samples on day 21, day 27 and day 42 were determined immunogenicity positive (Table 2).

### 3.5. Negative cut-off (NCO) calculation

To determine a sample as immunogenicity positive, the negative cut-off (NCO) was assessed. The NCO

Table 1
Evaluation of drug Y specific hADA in non-treated samples with residual drug Y

| Sampling date (day) | NCO value (RU) | PBS | | Drug Y at 1000 µg/ml | | |
|---|---|---|---|---|---|---|
| | | Mean (RU) | Above NCO | Mean (RU) | Inhibition (%) | Specific ADA |
| pre-dose | 64.2 | 55.5 | No | 54.8 | 1.3 | NO |
| 21 | 64.2 | 186.6 | YES | 150.6 | 19.3 | NO |
| 27 | 64.2 | 217.6 | YES | 186.4 | 14.3 | NO |
| 42 | 64.2 | 258.9 | YES | 203.7 | 21.3 | NO |
| 63 | 64.2 | 683.9 | YES | 214.1 | 68.7 | YES |
| 84 | 64.2 | 786.9 | YES | 200.9 | 74.5 | YES |

Samples above a certain threshold value (NCO) were considered screening-positive. To prove, whether the binding obtained was specific to the drug Y, samples were pre-incubated in a confirmatory assay with drug Y. Immunogenicity was defined as specific to the drug Y, when the signal was inhibited more than 30 % using PBS spiked samples as reference.

Table 2
Evaluation of drug Y specific hADA in acid treated samples with residual drug Y

| Sampling date (day) | NCO value (RU) | PBS (acid treated) | | Drug Y at 1000 µg/ml | | |
|---|---|---|---|---|---|---|
| | | Mean (RU) | Above NCO | Mean (RU) | Inhibition (%) | Specific ADA |
| pre-dose | 84.8 | 62.5 | No | 54.8 | 12.3 | NO |
| 21 | 84.8 | 260.8 | YES | 150.6 | 42.3 | YES |
| 27 | 84.8 | 319.9 | YES | 186.4 | 41.7 | YES |
| 42 | 84.8 | 582.4 | YES | 203.7 | 65.0 | YES |
| 63 | 84.8 | 781.2 | YES | 214.1 | 72.6 | YES |
| 84 | 84.8 | 923.3 | YES | 200.9 | 78.2 | YES |

After acid treatment, all post-dose samples were assessed immunogenicity positive, since sample signals were inhibited >30 % with drug Y.

defines the threshold between a negative and a positive screening result. The NCO was assessed by analyzing 25 individual naive human sera with and without acid treatment in the Biacore assay. The NCO was defined as the mean of 25 blank sera+ 2 standard deviations (SD), which represents the 95th percentile of a normal distribution. Acid-treated and non-treated samples showed a difference in non-specific binding, which resulted in different NCOs. In this particular study, this difference in NCO did not change the final immunogeniciy results. The NCO was normalized between runs by a cut-point factor (CPF), which was the difference between the NCOs (acid treated vs non-treated) and a negative control blank serum. To obtain the NCO in a specific run, the CPF was added to the mean value of the negative control serum.

### 3.6. Investigation of the influence of different drug concentrations on hADA recovery

The impact of drug concentration on hADA recovery from drug-hADA complexes was evaluated, by spiking increasing amounts of drug (0.2, 1 and 2 mg/ml) into a clinical serum sample with known immunogenicity. A sample after repeated drug Y treatment was chosen, assuming that the hADA affinity to drug Y had matured with each drug Y administration. It has been shown that Biacore assays are less sensitive for detection of high affinity antibodies as compared to low affinity antibodies in presence of drug (8). None of the three different drug concentrations were tolerated in the assay

and detection of hADA in the Biacore assay was not possible. The acid dissociation step resulted in a recovery of 92 % ADA for 0.2 mg/ml drug Y, 96 % for 1 mg/ml drug Y and 99 % for 2 mg/ml drug Y (Fig. 5).

## 4. Discussion

Immunogenicity of therapeutic antibodies is an important topic, since it can affect safety, pharmacokinetics and efficacy. Thus, it has been suggested that during development of biotherapeutics, immunogenicity should always be monitored and potential clinical consequences evaluated (Patton et al., 2005; Wadhwa et al., 2005; Koren et al., 2002). Anti-drug antibody (ADA) responses have also been reported in patients treated with fully human antibodies (Klitgaard et al., 2006). It can be argued that, despite the effort to reduce immunogenicity of therapeutic antibodies, because of the variation in antibody repertoires, TCR usage and tissue-type haplotypes of the human population, the risk of immunogenicity cannot be completely removed.

A hurdle for immunogenicity assays has been the detection of ADA in the presence of high drug concentrations. Residual drug levels may interfere with immunoassays by competitive inhibition or by forming immune complexes (Nygren et al., 1985). Under such conditions, ADA are not detectable by the ADA screening assay anymore. Our aim was to develop a rapid screening assay that is highly tolerant to drug.

It is well known that immune complexes even antibody aggregates can completely dissociate at low
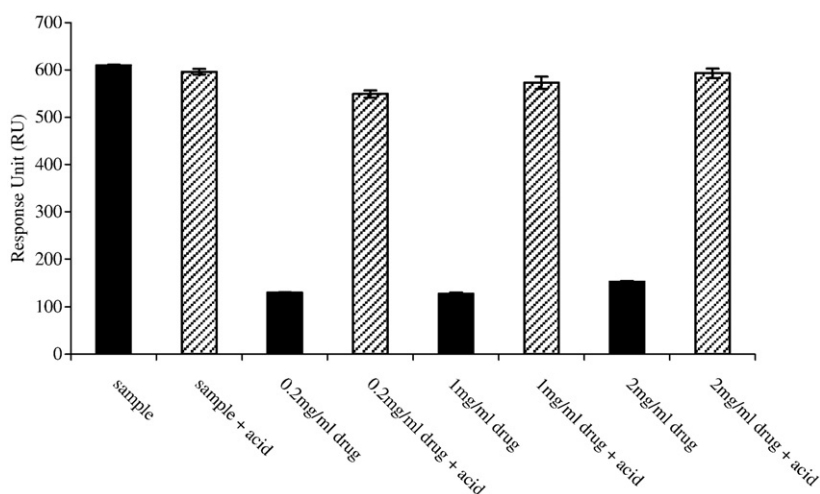


Fig. 5. Investigation of drug Y interference in clinical samples containing different drug Y levels. To assess the impact of drug Y level on the recovery of hADA, different concentrations of drug Y (0.2, 1, 2 mg/ml) were spiked in an immunogenicity positive patient sample. All three drug levels inhibited binding of hADA to the drug Y on the Biacore chip. However, acid dissociation resulted in a 92 – 99 % recovery of ADA. All samples were assessed in duplicates.

pH enabled by non-covalent bonding. In support of immunogenicity assessment in clinical samples with high residual drug levels, the introduction of acid treatment was evaluated for a Biacore assay. We first evaluated the acid stability of drug specific rADA, which served as a positive control. Our data (Fig. 1) suggest that acidification did not have a major impact on rADA and that the antibodies remained their capability to bind their antigen.

We then investigated the potential of sample acidification to recover ADA from drug-ADA complexes using the rabbit positive control as well as immunogenicity positive patient samples with negligible drug concentrations. Drug was added at a final concentration of 1 mg/ml into human serum samples spiked with different concentrations of rADA (5 – 100 µg/ml) corresponding to a 10-200- fold drug excess. Drug was also spiked into patient samples. Since the ADA concentration of patient samples was unknown, the drug excess could not be calculated. ADA-drug complex formation was performed in serum at 37 °C to mimic physiological conditions. The drug significantly inhibited the detection of rADA and hADA in untreated samples (Figs. 2, 3). Applying acid treatment 87- 110 % of rADA and 92 – 100 % of hADA were recovered, including all patient samples and rabbit ADA samples tested (Figs. 2, 3). In a next step, we analyzed clinical samples from a patient with remaining drug levels. After acid treatment of these samples, 13 % to 55 % higher ADA levels were measured compared to untreated samples (Fig. 4). As a consequence, samples on days 21, 27 and 42 determined immunogenicity positive after acid treatment, whereas without acid treatment these samples were determined as immunogenicity negative. The results suggest that Biacore assays are capable of incorporating acid dissociation procedures to separate drug-antibody immune complexes before analysis.

The high recovery was irrespective of ADA concentration or drug excess. It seems that antibodies directed against therapeutic proteins can be reliably detected in presence of a stable drug surface to sequester and bind antibodies. A number of studies have demonstrated that antibodies bound to immobilized antigens in general form more stable complexes compared to the soluble antigen-antibody complexes (Mason and Williams, 1980; Nygren et al., 1985; Nygren et al., 1987). Furthermore, avidity effects come into play, when ADA bind to immobilized antibodies, which might favor ADA binding to the sensor chip surface. Partial unfolding of the drug in the sample by acid treatment might be another reason. The combination of acid dissociation and solid-phase binding may also be facilitated by the nature of the flow system or unfavorable

conditions (pH, salt) in the sample for rebinding in solution.

Although the recovery of rADA and hADA from the ADA-drug complex was successful, slight differences in the acid treatment conditions were noted. For rADA, pH 2.5 was required, whereas for patient samples pH 3.0 was used to obtain the maximum ADA recovery. The discrepancy in pH may be explained by different sensitivity of antibodies to acid between species. The affinity of ADA-drug complexes could be another source of different behavior to acid treatment. Due to affinity maturation of hADA after repeated drug applications, we assume that the affinity of hADA was increasing with repeated treatment (Agur et al., 1991). We found no significant difference in the recovery of hADA for different sampling time points of repeatedly treated patients after acid treatment. This suggests that the immune complex dissociation at low pH is probably not affected by the antibody affinity.

Finally, acid treatment may have additional positive effects on ADA determination. It may be reduce non-specific binding but also undesired specific binding, e.g. by denaturation of soluble target in samples, in case of antibody drugs.

Based on the obtained data, we believe that acid treatment of clinical samples followed by the here described Biacore or similar assays, should allow for a more reliable analysis of immunogenicity at high drug concentrations. Using such approaches, immunogenicity sampling time points should not be limited to low drug levels anymore, but should only be guided by clinical considerations.

## Acknowledgement

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