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Heat pre-treatment can abolish anti-drug antibody interference in ligand binding pharmacokinetic assays

Svend Poulsen*[ID], Louise Jørgensen and Pia Søndergaard Galle

Abstract

Anti-drug antibodies (ADAs) can interfere with ligand binding assays (LBAs) by binding to epitopes recognized by the assay antibodies or by preventing assay antibody binding through steric hindrance. This can lead to underestimation of total drug concentration in pharmacokinetic (PK) samples which can confound decisions during drug development. We hypothesized that ADA interference in LBAs can be removed by sample heat pre-treatment. We heat pre-treated ADA-spiked samples by incubating them in a shallow water bath at 56–100 °C for 5–30 min prior to measuring the samples by a traditional electrochemiluminescence (ECL) assay. Heat pre-treatment at minimum 85 °C for 5 min completely removed the ADA interference. We then compared the analyte concentrations measured with and without heat pre-treatment of blood samples from toxicology studies performed for two different analytes in 60 cynomolgus monkeys and 29 minipigs, respectively. The overall difference in measured concentration of ADA-positive samples was significantly different from the overall difference in measured concentration of ADA-negative samples. For the cynomolgus monkey study samples, the ADA titer was determined, and the difference in measured concentration, when comparing heat pre-treatment to no heat pre-treatment, was significantly correlated to the ADA titer. Additionally, heat pre-treatment removed parallelism issues observed in a subset of study samples. Our data suggest that sample heat pre-treatment can abolish ADA interference in an LBA and could serve as a tool to assess the degree of ADA interference and the total drug concentration in a PK sample. Of note, before utilizing this strategy on a new analyte, it is necessary to assess whether heat pre-treatment negatively affects the detection of the analyte by the assay antibodies.

Keywords: Anti-drug antibody, Ligand binding assay, Pharmacokinetics, Assay interference, Heat pre-treatment

Introduction

Pharmacokinetic (PK) assays are crucial in drug development as they provide critical data for the assessment of the safety and effectiveness of therapeutics (Roskos et al. 2011; European Medicines Agency 2012; U.S. Food and Drug Administration 2018). As biologics from a market as well as a drug discovery point of view have become a thriving field, immunogenicity, including the development of anti-drug antibodies (ADAs), in patients has become an increasing concern (Sethu et al. 2012; Wierda

et al. 2001). Several reviews acknowledge the potential impact of ADA development on PK assessments (Rosenberg 2003; Sailstad et al. 2014; Shankar et al. 2006; Smith et al. 2016), and this is also recognized by the authorities who recommend immunogenicity assessments during non-clinical development when unexpected findings in drug exposure or pharmacodynamics are observed (U.S. Food and Drug Administration 2011).

ADA development can pose many issues besides the impact on PK assessments, but the focus of this paper is on the impact of ADAs on ligand binding assay (LBA) performance. Here, an LBA is defined as an assay where a ligand of interest is quantified by the binding of tagged

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assay antibodies. ADAs can interfere with LBAs by binding to epitopes recognized by the assay antibodies or by preventing assay antibody binding through steric hindrance (Sailstad et al. 2014; Thway et al. 2013; Wang et al. 2012). This phenomenon is defined as ADA interference and leads to an approximate determination of free rather than total drug concentration. Free drug is in this case and going forward defined as a drug that is not bound to one or more interfering ADAs. Wang et al. (Wang et al. 2012) and Thway et al. (Thway et al. 2013) have shown practical examples of this phenomenon as they applied assay formats with different tolerances to ADA interference on identical ADA-positive PK study samples and obtained different results.

In the review by Sailstad et al. (Sailstad et al. 2014), it becomes clear that ADA binding does not necessarily neutralize drug activity in vivo and that the active drug concentration may therefore be underestimated by an LBA that measures only free drug. They state that ADA-drug complexing can lead to an accumulation of the drug by protecting the drug from degradation, and an underestimation of the active drug concentration could therefore potentially be exacerbated. Lastly, given various affinities and avidities of ADAs, dilution of ADA-positive samples may skew the ratio of bound and total drug (Sailstad et al. 2014). This would lead to parallelism issues which complicate assay validation and increase the risk of misinterpreting the measured analyte concentration in samples that must be diluted to fit the dynamic range of the assay. Taken together, an assay that measures only free drug concentration due to ADA interference may provide incomplete or potentially misleading information. A completely ADA-tolerant approach could therefore provide useful additional information and help decision-making during development.

Several methods to dissociate drug and ADAs prior to analysis of PK study samples have been suggested and are reviewed by Kelley et al. (Kelley et al. 2013). Of these, an acid pre-treatment step appears to be the most popular, and several studies show reduced interference from either drug or ADA when acid pre-treatment is applied (Ahene 2011; Kavita et al. 2017); however, the reported amount of interference reduction varies. This makes such an assay unable to reliably assess the total drug concentration in the presence of ADAs.

We propose heat pre-treatment as a simple approach to abolish ADA interference in LBAs as temperatures above approximately 70 °C generally denature antibodies (Akazawa-Ogawa et al. 2017). Sample heat pre-treatment has previously been utilized to denature immune complexes in order to improve the sensitivity of various pathogen antigen tests (Lima Mda et al. 2014; Little et al. 2014; Swartzentruber et al. 2009; Starkey et al. 2020;

Schüpbach et al. 1996). It is hypothesized that ADAs can be irreversibly denatured by heat exposure and that this will completely prevent them from interfering in an LBA. For this to be useful, the analyte should not be an antibody itself and it should be recognizable by the assay antibodies after heat pre-treatment. This may not be the case for larger molecules that for instance require assistance for refolding tertiary structure essential for epitope recognition.

Here, we show that the introduction of a heat pre-treatment step in an LBA is effective at abolishing ADA interference in ADA-spiked samples and in ADA-positive PK study samples.

Materials and methods
Traditional electrochemiluminescence (ECL) assay
Two different monoclonal antibodies against the analyte were tagged with *N*-hydroxysuccinimide variants of either biotin or a ruthenium complex (MSD Gold Sulfo Tag, Meso Scale Diagnostics, LLC) and stored in 50% glycerol at −18 °C. Calibration standard (Cal) and quality control (QC) samples were prepared by spiking concentrated analyte into pooled mixed gender cynomolgus monkey K_2-EDTA plasma (BioIVT) or pooled mixed gender minipig serum (BioIVT) and were stored at −18 °C. Initially, the tagged antibodies were diluted in dilution buffer (25 mM HEPES, 50 mM NaCl, 10 mM Na-EDTA, 0.2% dextran, 0.5% ovalbumin, 0.05% bovine γ-globulin, 0.02% HBR-1, 0.1% Tween 20, 0.01% ProClin 300, 0.01% gentamycin, pH 7.4), and the solutions were mixed (AB mix). The dilution buffer is commonly used in our lab for historic reasons, and the main purpose of its components is to keep dissolved peptides or proteins in solution as well as prevent microbial contamination. Antibody concentrations that resulted in the highest signal-to-noise ratio across the entire standard curve were selected. Samples to be analyzed were then diluted in the pooled matrix if necessary, so that the expected measured concentration would fall within the dynamic range of the assay. Ten microliters of each Cal, QC, and (diluted) sample was mixed with 90 μL dilution buffer in 0.75 mL tubes (Non-coded push cap V-bottom, Micronic). Thereafter, 200 μL AB mix was added to each tube, and each tube was vortexed and incubated on a plate shaker (400 rpm) at room temperature (RT) for 60 min. Meanwhile, a 96-well ECL plate (MSD GOLD Small Spot Streptavidin Plate, Meso Scale Diagnostics, LLC) was blocked by the addition of 50 μL/well blocking buffer (Synthetic Blocking Buffer – ELISA ECO-TEK, Kem-En-Tec Diagnostics) and incubated on a plate shaker (400 rpm) at RT for 10 min. Following incubation, blocking buffer was aspirated, and each well was washed three times with 150 μL washing buffer (PBS ×1, 0.05% Tween 20). The washing buffer

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was aspirated, and the remaining buffer was removed by inverting the plate and blotting it against a clean paper towel. When the incubation of the Cal/QC/sample-AB-mix was concluded, each tube was vortexed and 50 µL from each tube was transferred in duplicates to the blocked and washed plate. The plate was then incubated on a plate shaker (400 rpm) at RT for 60 min. Following incubation, the plate was washed as described earlier, and 150 µL 2× read buffer (diluted in type 1 water from MSD Read Buffer T (4×) with Surfactant, Meso Scale Diagnostics, LLC) was added to each well. Within 10 min, the plate was read on a SECTOR Imager 6000 (Meso Scale Diagnostics, LLC). Watson LIMS (Thermo Fisher Scientific) was used to fit the calibration standards and back-calculate concentrations.

ECL assay with heat pre-treatment

The procedure for this assay was identical to the traditional ECL assay except that a sample heat pre-treatment step was added. Following the mix of 10 µL of each Cal, QC, and diluted sample with 90 µL dilution buffer in tubes, the tubes were capped with Thermo Plastic Elastomer push caps (Micronic) and incubated in a shallow water bath at 85 °C for 5 min. For producing the data presented in Fig. 1, samples were incubated in the water bath at 56–100 °C for 5–30 min. The water level was low enough so that a full 96-1 rack (Micronic) would not float. Following the water bath incubation, tubes were decapped and the procedure was continued as in the traditional ECL assay.
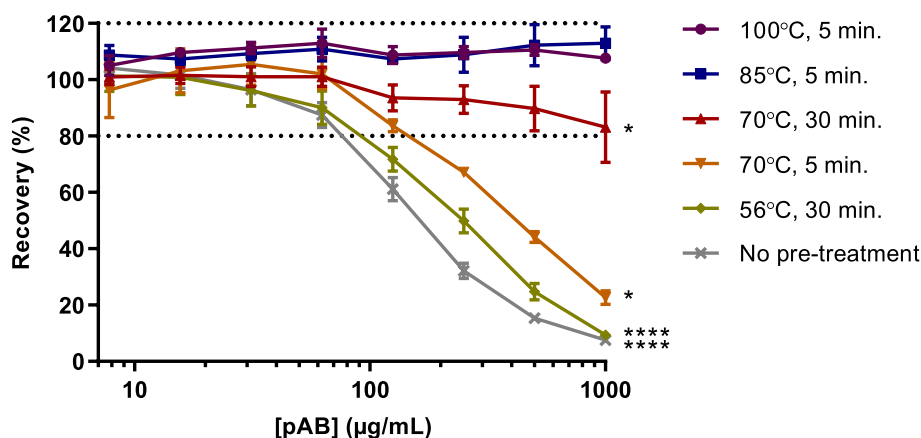
Analytes

Two different analytes, NNC9204-1177 and NNC0247-0829, were utilized throughout this study. NNC9204-1177 is the analyte in all experiments except for the experiment leading to Fig. 2b where NNC0247-0829 is the analyte. NNC9204-1177 is a peptide with a molecular weight of 4570 Da while NNC0247-0829 is a protein with a molecular weight of 32 kDa. Both compounds are undergoing development at Novo Nordisk, and further details on their properties can therefore not be disclosed. For NNC9204-1177, the lower level of quantification was 1 nM, and Cal samples ranging from 0.3 to 1200 nM and QC samples at 3 nM, 30 nM, and 320 nM were utilized. For NNC0247-0829, the lower level of quantification was 0.01 nM, and Cal samples ranging from 0.005 nM to 8 nM and QC samples at 0.03 nM, 0.45 nM, and 6.4 nM were utilized.
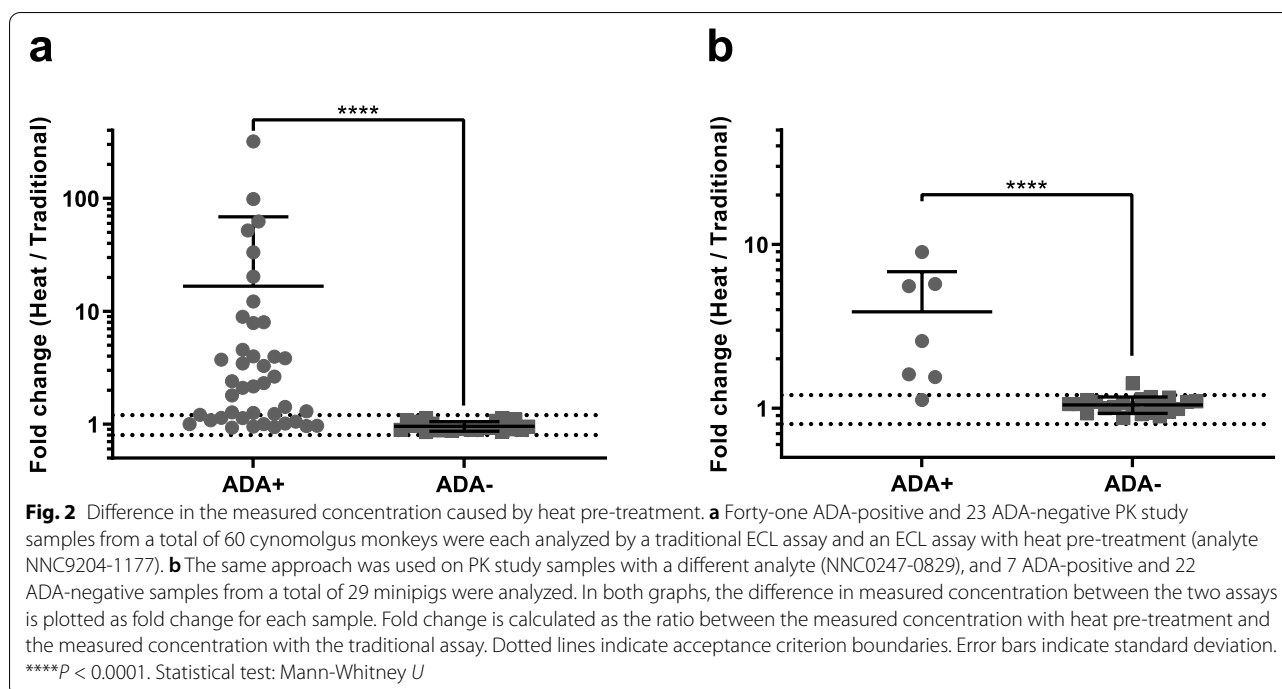
Acceptance criteria
Recovery
Recovery is defined as the accuracy of the measurement of spiked samples. It is calculated as the ratio between the measured concentration and the nominal concentration in percent. As in the current guidelines for bioanalytical method validation regarding the accuracy, the recovery acceptance criterion was set to 100% ± 20.0% (European Medicines Agency 2012; U.S. Food and Drug Administration 2018).

Fig. 1 Effect of heat pre-treatments on recovery of pAB-treated spiked samples. pAB towards the analyte (NNC9204-1177) was added to the spiked samples to mimic ADA interference. Samples were analyzed by a traditional ECL assay or an ECL assay with one of five different heat pre-treatments. Recovery was calculated as the ratio between the measured concentration and the nominal concentration in percent and was plotted against the pAB concentration. The results from three independent experiments are included. Dotted lines indicate acceptance criterion boundaries. Error bars indicate standard deviation. For each assay, the correlation between recovery and pAB concentration was calculated by the Spearman correlation test. *$P < 0.05$, ****$P < 0.0001$

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Fig. 2 Difference in the measured concentration caused by heat pre-treatment. **a** Forty-one ADA-positive and 23 ADA-negative PK study samples from a total of 60 cynomolgus monkeys were each analyzed by a traditional ECL assay and an ECL assay with heat pre-treatment (analyte NNC9204-1177). **b** The same approach was used on PK study samples with a different analyte (NNC0247-0829), and 7 ADA-positive and 22 ADA-negative samples from a total of 29 minipigs were analyzed. In both graphs, the difference in measured concentration between the two assays is plotted as fold change for each sample. Fold change is calculated as the ratio between the measured concentration with heat pre-treatment and the measured concentration with the traditional assay. Dotted lines indicate acceptance criterion boundaries. Error bars indicate standard deviation. ****$P < 0.0001$. Statistical test: Mann-Whitney U

Inter-assay difference

Inter-assay difference denotes the difference between measured concentrations in the same sample by two different assays. Here, it is calculated as the ratio between the measured concentration by the assay with heat pre-treatment and the measured concentration by the traditional assay. To reflect the recovery criterion, the inter-assay difference acceptance criterion was set to 1 ± 0.2.

Parallelism

To evaluate parallelism, the coefficient of variation (CV) between the measured concentrations of samples in a dilution series was calculated. As in the current guidelines, the parallelism acceptance criterion was set to no more than 30.0% (European Medicines Agency 2012).

Qualification of assays

The traditional ECL assay and the ECL assay with heat pre-treatment were both qualified as the validation parameters calibration curve, quality controls, selectivity, accuracy, precision, and dilution linearity were each addressed and approved according to the current guidelines for LBAs (European Medicines Agency 2012; U.S. Food and Drug Administration 2018) (data not shown).

ADA-spiked samples

To mimic ADA interference, polyclonal antibody (pAB) with a level of analyte-specific immunoglobulin G (IgG) around 0.5–5% was acquired from rabbits. ADA-spiked samples were produced by spiking 7.8–1000 μg/mL pAB and a fixed concentration of analyte into pooled cynomolgus monkey K_2-EDTA plasma (BioIVT). These samples were freshly produced and allowed to incubate on a plate shaker (400 rpm) at RT for 60 min prior to analysis.

ADA assay

The ADA assay was a radioimmunoassay (RIA) for serum samples. The principle is that radioactively labeled drug binds to ADA present in the sample. The formed antibody-antigen complexes are then precipitated by the addition of polyethylene glycol 6000 (PEG-6000) and measured in a gamma counter. To increase assay sensitivity in the presence of the drug in samples, all samples were subjected to a PEG-6000 pre-precipitation step prior to incubation with the radiolabeled drug.

All samples were screened in the assay together with negative and positive quality control (QC) samples, and assay-specific cut points were calculated based on negative controls and a normalization factor determined during the assay validation (Shankar et al. 2008). Samples

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with a %B/T result (percent bound over total added radiolabeled drug) below or equal to the assay-specific cut point were re-analyzed in a confirmatory assay based on competition with the excess unlabeled drug to confirm the specificity of the antibody result. Samples confirmed positive for ADA were titrated by analyzing 3-fold dilution series of the samples in the screening assay. Each sample was assigned with a titer corresponding to the reciprocal of the dilution factor for the first sample in the dilution series with a result in %B/T below the assay-specific cut point.

To perform the screening assay, 10 µL sample or assay QC sample was mixed with 90 µL of assay buffer (0.040 M phosphate, 0.15 M sodium chloride, 0.5% w/v BSA, 0.25% γ-globulin, 0.010 M EDTA, pH 7.4) and 650 µL PEG-6000 solution (14.4% PEG-6000 in 0.010 M TRIS, 0.15 M NaCl, 0.1% v/v Tween 20, pH 8.6). The mixture was centrifugated at 1600 rcf for 15 min, and the precipitate resuspended in a total of 150 µL of assay buffer containing 40 nCi/mL of radiolabelled drug and incubated overnight at 4 °C. Samples were mixed with 650 µL of PEG-6000 (16% PEG-6000 in 0.010 M TRIS, 0.15 M NaCl, 0.1% v/v Tween 20, pH 8.6) and centrifuged at 1600 rcf for 30 min, and the precipitate was washed with 650 µL of PEG-6000 (16% PEG-6000 in 0.010 M TRIS, 0.15 M NaCl, 0.1% v/v Tween 20, pH 8.6) and finally counted on a Gamma Counter Wizard 3470 (Perkin Elmer). In each assay, run samples not subjected to any precipitation steps (totals) were included and used for the calculation of the assay result in %B/T. The confirmation assay was performed in the same way as the screening assay with the only exception that the assay buffer with the radiolabelled drug also contained excess (6.7 µg/mL) of the unlabelled drug for competition.

Results
Heat pre-treatment completely reverses induction of antibody interference in spiked samples
To simulate ADA interference, polyclonal antibody (pAB) towards the analyte (NNC9204-1177) was added to spiked samples. Analysis of these samples with a traditional ECL assay (see the "Materials and methods" section) with no heat pre-treatment showed a decrease in recovery dependent on the concentration of pAB (total IgG concentration) in each sample. Samples with a pAB concentration of 125 µg/mL or above revealed a recovery outside the acceptance criterion (see Fig. 1). In identical samples, this decrease in recovery was limited by mild heat pre-treatments (56 °C for 30 min or 70 °C for 5 or 30 min). A heat pre-treatment step of 85 °C or 100 °C for 5 min completely reversed the interference of pAB, as the recovery of all samples met the acceptance criterion (see Fig. 1). Additionally, when the correlation between

recovery and pAB concentration was calculated for each assay, the assays with no pre-treatment or mild heat pre-treatment (56 °C for 30 min, 70 °C for 5 min, and 70 °C for 30 min) showed a significant correlation. However, the assays with more intense heat pre-treatment (85 °C for 5 min or 100 °C for 5 min) did not show any significant correlation (see Fig. 1).

Since 85 °C for 5 min was deemed sufficient to completely reverse antibody interference, this heat pre-treatment configuration was used for the remaining experiments.

Heat pre-treatment alters the measured analyte concentration in ADA-positive but not in ADA-negative PK study samples
As concentration, affinity, and avidity of ADAs can differ from subject to subject and sample to sample it is very difficult to truly replicate the behavior of ADA-positive PK study samples. Therefore, to confirm the efficacy of heat pre-treatment on ADA interference in a real-world setting, blood plasma samples from two concluded toxicology studies were obtained. These studies included samples taken at various time points from a total of 60 cynomolgus monkeys that had been injected with the same peptide drug (NNC9204-1177). Samples were categorized into ADA-positive or ADA-negative based on %B/T results from the ADA assay (see the "Materials and methods" section, ADA-negative: %B/T < assay-specific cut point, ADA-positive: %B/T > 10, data not shown). An animal was considered to be ADA-positive if at least one of its samples was determined to be ADA-positive. From each ADA-positive animal, the sample with the highest %B/T result was picked. A maximal number of ADA-negative samples were picked at random under the condition that only one sample from each animal could be included. The resulting 41 ADA-positive and 23 ADA-negative samples were each analyzed by a traditional ECL assay and an ECL assay with heat pre-treatment (see the "Materials and methods" section). Since several samples for the same animal were taken at different time points, it was possible for an animal to contribute with both an ADA-positive and an ADA-negative sample. Hence, the total number of included samples was higher than the total number of animals. For each sample, the difference between the measured concentrations by the two assays was expressed as fold change which was calculated as the ratio between the measured concentration with heat pre-treatment and the measured concentration with the traditional assay. The overall fold change of the ADA-positive samples was significantly different from the overall fold change of the ADA-negative samples (see Fig. 2a). All differences in measured concentration of the 23 ADA-negative samples met the inter-assay difference

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acceptance criterion. This was the case for only 29% (12 out of 41) of the ADA-positive (see Fig. 2a). To substantiate these results, blood serum samples from a concluded toxicology study with a different analyte (NNC0247-0829) were obtained. This study included samples from 29 minipigs, and 7 ADA-positive and 22 ADA-negative samples were analyzed using a similar approach to the one described above. Again, the overall fold change of the ADA-positive samples was significantly different from the overall fold change of the ADA-negative samples (see Fig. 2b). Ninety-five percent (21 out of 22) of the ADA-negative samples met the inter-assay difference acceptance criterion while this was the case for only 14% (1 out of 7) of the ADA-positive samples (see Fig. 2b).

Measured concentration fold change caused by heat pre-treatment correlates to ADA titer

To assess whether the difference in measured concentration caused by heat treatment correlated to the amount of ADA present in the samples, ADA titer was determined for the cynomolgus monkey PK study samples. ADA titer was defined as the minimal dilution factor where the measured %B/T of the sample was below the assay-specific cut point (see ADA titer assay). For each sample, the ADA titer was plotted against the difference in measured concentration when analyzed by a traditional ECL assay and an ECL assay with heat pre-treatment (see Fig. 3). Linear regression revealed a significant linear correlation ($P = 0.007$) although the effect size was relatively small ($r = 0.334$).

Heat pre-treatment can abolish parallelism issues in study samples
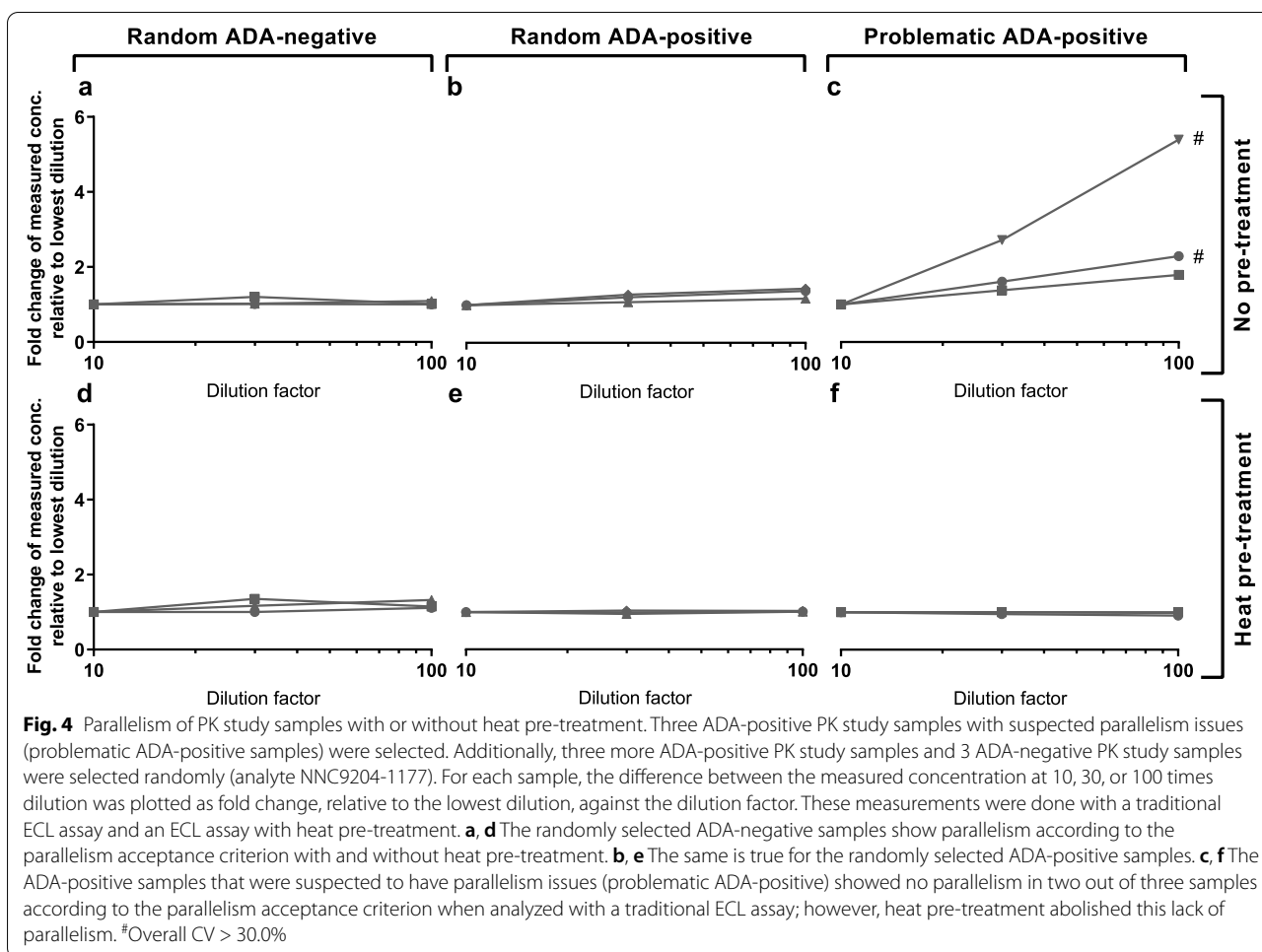
To investigate the effect of heat pre-treatment in relation to parallelism issues, three ADA-positive cynomolgus monkey PK study samples with suspected parallelism issues (problematic ADA-positive samples) identified from preliminary tests were selected. Additionally, three more ADA-positive cynomolgus monkey PK study samples with no parallelism issues and 3 ADA-negative cynomolgus monkey PK study samples were selected randomly. When analyzed by a traditional ECL assay, two of the three samples with suspected parallelism issues did not meet the parallelism acceptance criterion while the rest of the samples did (see Fig. 4a–c). When analyzed by an ECL assay with heat pre-treatment, any parallelism issues were abolished as all samples met the parallelism acceptance criterion (see Fig. 4d–f).

Discussion

Firstly, we have shown that ADAs can interfere with analyte concentration measurements in a traditional LBA and that this interference can be completely reversed by heating the samples at a minimum of 85 °C for 5 min prior to assay antibody application. This finding was substantiated as we showed an increase in the measured analyte concentration that correlated to the ADA titer when heat pre-treatment was applied to PK study samples. Secondly, we have demonstrated that heat pre-treatment can mitigate the lack of parallelism. Importantly, the heat pre-treatment had little to no effect on the measured concentration of ADA-negative samples. We speculate
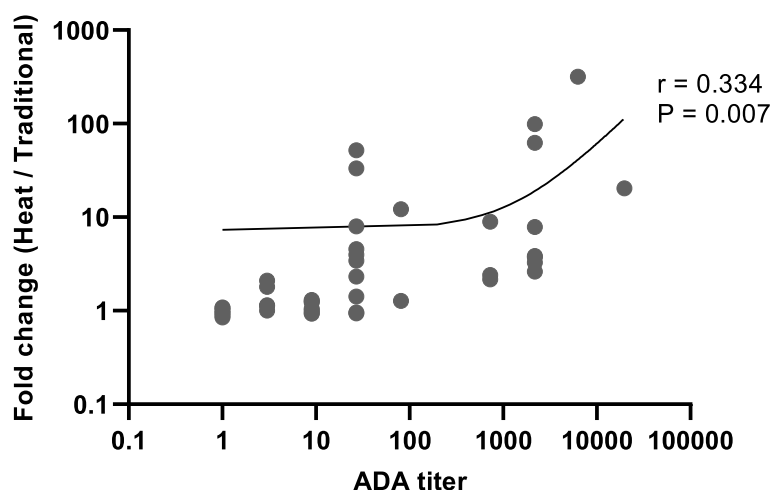
Fig. 3 Correlation between the difference in measured concentration caused by heat treatment and the ADA titer. ADA titer, defined as the minimal dilution factor where % B/T was below the assay-specific cut point, was determined for each cynomolgus monkey PK study sample and plotted against the difference in measured concentration when analyzed by a traditional ECL assay and an ECL assay with heat pre-treatment (analyte NNC9204-1177). Linear regression was performed, and the regression line is indicated by the solid line

Fig. 4 Parallelism of PK study samples with or without heat pre-treatment. Three ADA-positive PK study samples with suspected parallelism issues (problematic ADA-positive samples) were selected. Additionally, three more ADA-positive PK study samples and 3 ADA-negative PK study samples were selected randomly (analyte NNC9204-1177). For each sample, the difference between the measured concentration at 10, 30, or 100 times dilution was plotted as fold change, relative to the lowest dilution, against the dilution factor. These measurements were done with a traditional ECL assay and an ECL assay with heat pre-treatment. **a**, **d** The randomly selected ADA-negative samples show parallelism according to the parallelism acceptance criterion with and without heat pre-treatment. **b**, **e** The same is true for the randomly selected ADA-positive samples. **c**, **f** The ADA-positive samples that were suspected to have parallelism issues (problematic ADA-positive) showed no parallelism in two out of three samples according to the parallelism acceptance criterion when analyzed with a traditional ECL assay; however, heat pre-treatment abolished this lack of parallelism. [#]Overall CV > 30.0%

that high heat denatures ADAs irreversibly which prevents them from interfering with the subsequent binding of the assay antibodies.

We did not directly show that heat pre-treatment completely removes ADA interference in PK study samples. However, the antibody interference in ADA-spiked samples was completely removed and the increase in measured analyte concentration in heat pre-treated PK study samples was shown to correlate to the ADA titer. We see this as compelling evidence that sample heat pre-treatment can abolish ADA interference in an LBA. Therefore, the change in measured concentration due to heat treatment can be seen as a measure of ADA interference. Using this logic, it becomes clear from Fig. 2 that ADA-positive samples did not necessarily suffer from ADA interference. This is expected because ADAs can theoretically associate to the drug in a way that does not influence the binding of the assay antibodies. This is supported by the fact that larger ADA titers were more likely to result in interference as is apparent in Fig. 3. While the ADA titer was shown to correlate with the degree of

assay interference, the variance was large. This indicates that the amount of ADA in a sample is just one of several factors that contribute to assay interference. Other factors likely include the affinity and avidity as well as the particular epitopes of the ADAs, all of which can vary from sample to sample. Additionally, drug concentration in the sample likely plays a role, as low amounts of drug can be expected to be more sensitive to the presence of ADAs. Also, the drug can cause interference in the ADA analysis in a similar way that ADAs can cause assay interference and higher drug concentrations may therefore lower the accuracy of the ADA analysis. Taken together, immunogenicity assessment alone appears to be insufficient for the prediction of ADA interference in LBAs, while a difference in measured concentration upon introduction of sample heat pre-treatment seems to be a good indicator of ADA presence.

As to what extent a heat pre-treatment approach to mitigate ADA interference can be applied with success remains uncertain as this study includes only two analytes and a single assay platform. Our preliminary

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investigations suggest that other LBA platforms such as luminescent oxygen channeling assay (LOCI) and enzyme-linked immunosorbent assay (ELISA) are compatible with heat pre-treatment of samples (data not shown). Multiple papers describe the use of heat pre-treatment as a way to increase the sensitivity of pathogen antigen detection in antibody-based diagnostic assays (Lima Mda et al. 2014; Little et al. 2014; Swartzentruber et al. 2009; Starkey et al. 2020; Schüpbach et al. 1996). Most likely, the cause of the increased sensitivity is a decrease in interference by antibodies binding to the antigen, and the approach is therefore very similar to the one applied in this paper. In the aforementioned studies. the ELISA platform was utilized, and it can therefore be confirmed that this platform can be compatible with heat pre-treated samples. One study revealed an increase in the detection sensitivity to a level that was on par with a PCR-based diagnostic assay, supporting the notion that antibody interference in LBAs can be completely removed by heat pre-treatment (Schüpbach et al. 1996). Collectively, the mentioned studies (Lima Mda et al. 2014; Little et al. 2014; Swartzentruber et al. 2009; Starkey et al. 2020; Schüpbach et al. 1996) were done on various antigens, and they therefore confirm that heat pre-treatment can be feasible for a range of different analytes. It should be noted that the dynamic range of the assay may be affected by heat pre-treatment and that either raising or lowering of the lower limit of quantification can occur. This could be due to the changes in the accessibility of the assay antibody epitopes, which can either be improved or reduced as the heat treatment affects the structure of the analyte. It naturally follows that loss of analyte tertiary structure in some cases can be devastating for the binding of the assay antibodies and therefore devastating for the assay performance. Preliminary data that we have collected indicate that especially analytes of larger size such as therapeutic antibodies are less likely to be compatible with heat pre-treatment (data not shown). The analytes that were measured in this study have sizes of approximately 5 and 32 kDa. More thorough investigations of heat pre-treatment tolerance of a variety of compounds and assay platforms may be beneficial. It is straightforward to test whether an analyte is compatible with heat pre-treatment, if a traditional LBA for that compound already has been developed. The heat pre-treatment step is simply added to the assay, and a single run of a calibration curve will usually reveal whether an assay with heat pre-treatment is feasible for that particular analyte.

Conclusion

Our data suggests that an LBA with heat pre-treatment can tolerate ADAs completely and could serve as a tool to assess the degree of ADA interference and the total drug concentration in a PK study sample. A heat pre-treatment step consisting of 5-min incubation in a shallow 85 °C water bath is deemed sufficient. Before utilizing this strategy on a new analyte, it is necessary to assess whether such a heat pre-treatment negatively affects the detection of the analyte by the assay antibodies.

Declarations

Ethics approval and consent to participate
As animal samples were used retrospectively, no approval by an ethics committee was required.

Consent for publication
Not applicable.

Competing interests
All authors were employed by Novo Nordisk A/S within the duration of this research project. No financial impact of the publication is anticipated.

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