AN ELISA FOR EVALUATION OF THE RELATIVE AFFINITY OF AN ADA RESPONSE

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INTRODUCTION

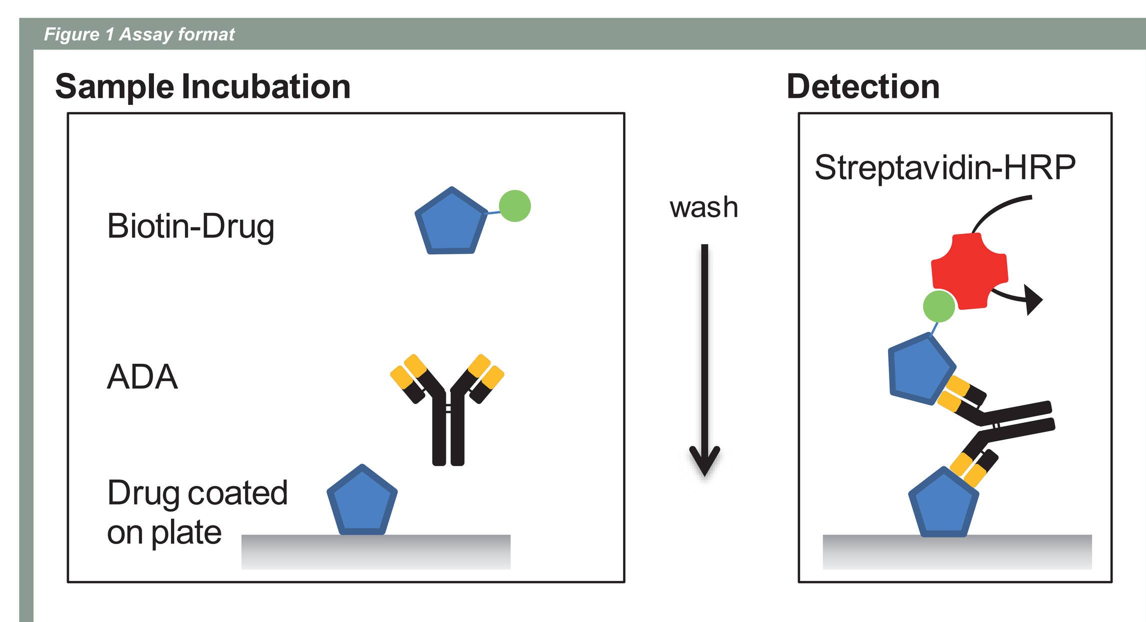
Immunogenicity of biopharmaceutical products is a major concern in both pre-clinical and clinical studies. In addition to the specific detection of anti-drug antibodies (ADA), regulatory bodies are increasingly expecting detailed characterization of any drug elicited immune response, including isotype determination and affinity evaluation. There are several approaches available to measure the affinity of an antibody molecule for its antigen, but most of these require either access to expensive equipment (e.g. Surface Plasmon Resonance[1]) or are not generally applicable (e.g. dialysis[2]). An ELISA based approach to determine the relative affinity of ADAs against a therapeutic serine protease was developed and validated in our laboratory.

ANALYTICAL METHODS

Evaluation of ADA response against a therapeutic serine protease in human plasma was conducted using ELISA based techniques in our laboratory as follows:

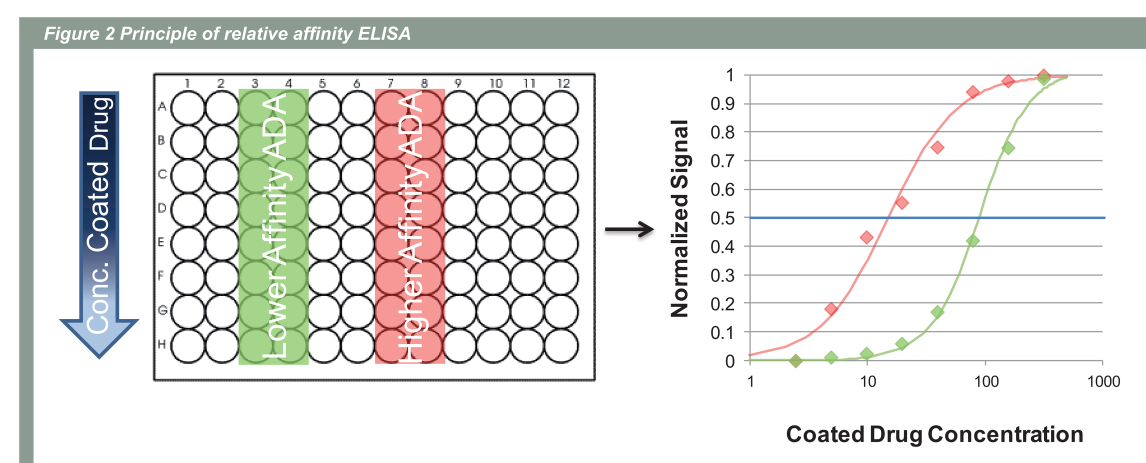
- Screening for ADA positive samples, then confirmation of specificity for the drug of ADA response followed by sample titration
- Determination and titration of immunoglobulin species (IgG, IgM, and IgE) present in confirmed ADA positive samples
- Determination of relative affinity on confirmed ADA positive samples

Figure 1 Assay format



All assays (excepted those for isotype determination) use the bridging format (Fig. 1). ADA present in the samples forms a bridge between the drug adsorbed onto the plate (used as capture reagent) and the labeled drug in solution (used as detection reagent). The complex is detected by specific binding of the enzyme horseradish peroxidase (HRP) via the biotin-streptavidin interaction and revealed using a chromogenic substrate for quantification by absorbance readout (Optical Density, OD).

Figure 2 Principle of relative affinity ELISA

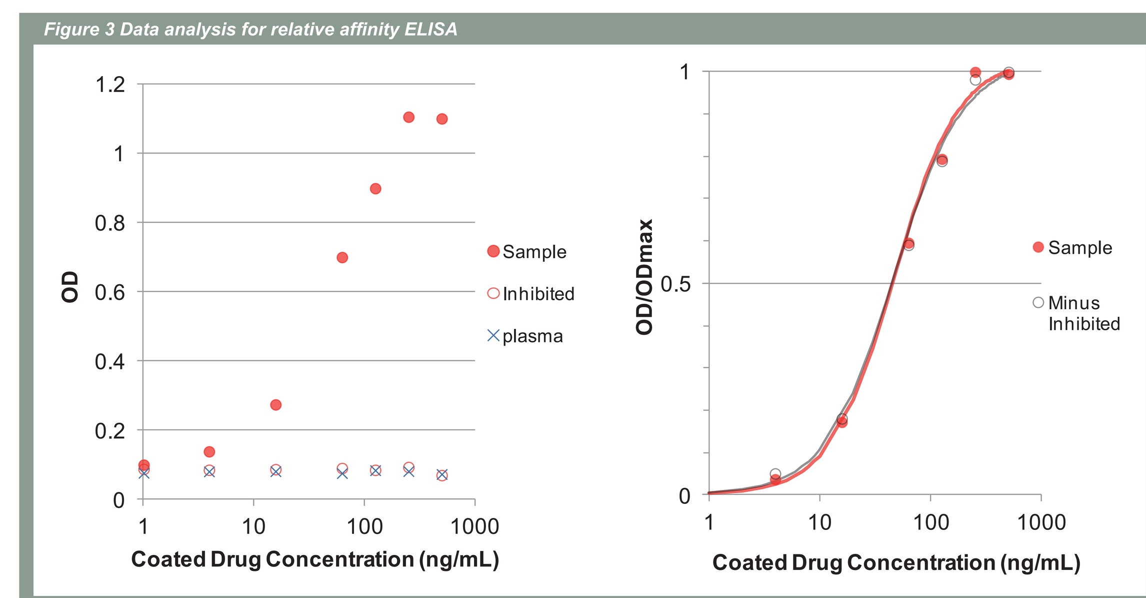


The hallmark of the relative affinity ELISA is the use of a gradient of coated drug concentration row wise across the plate (Fig. 2, left panel).

Affinity as evaluated in this assay denotes the net binding force of the ADAs present in the sample for the antigen. Lower affinity ADAs will detach from the plate "first" (i.e. the signal will be lost at relatively high coated drug concentration) whereas higher affinity ADAs will detach from the plate "last" (i.e. the signal will be lost at relatively low coated drug concentration). The coated drug concentration at which half of the signal is lost is used to report the relative affinity (rA) of the ADAs in the sample (Fig. 2, right panel).

Practically, a suitable dilution factor (at constant MRD) is first determined for each sample so that they all yield a similar level of signal at the highest coated drug concentration; they are then processed at this dilution in the relative affinity ELISA.

Figure 3 Data analysis for relative affinity ELISA



A typical response obtained in the affinity ELISA from an ADA positive clinical sample can be completely inhibited in presence of high concentration of free drug to the level of an ADA naïve plasma sample at all coated drug concentrations used (Fig. 3, Left).

This background level (ODmin) corresponds to the signal obtained with the sample at the lowest coated drug concentration and is used for data normalization using the formula (OD-ODmin)/(ODmax-ODmin) (Fig. 3, Right, "Sample"). The relative affinity rA is then calculated from the normalized data using the dose response equation $1-(1+((\text{Conc. Coated Drug})/rA)^{slope})^{-1}$.

Results from this simple data treatment are similar to a point by point subtraction of the free drug Inhibited data from the Sample data (Fig3, Right, "Minus Inhibited") and allows for the "extraction" of the specific ADA signal at all coated drug concentration. This is useful as it avoids the need to also evaluate samples in presence of free drug and thus increase assay throughput.

DEVELOPMENT OF A RELATIVE AFFINITY ELISA

Availability of at least 2 reference ADA preparations of different affinity is critical to establish a suitable range of coated drug concentration. 3 reference ADA preparations against a therapeutic serine protease were used for development of the ELISA affinity assay: 2 monoclonal antibodies of respectively high affinity (mAb1) and low affinity (mAb2) to the drug, and 1 polyclonal rabbit purified IgG fraction (rAb).

Figure 4 Evaluation of reference ADA preparations in relative affinity ELISA

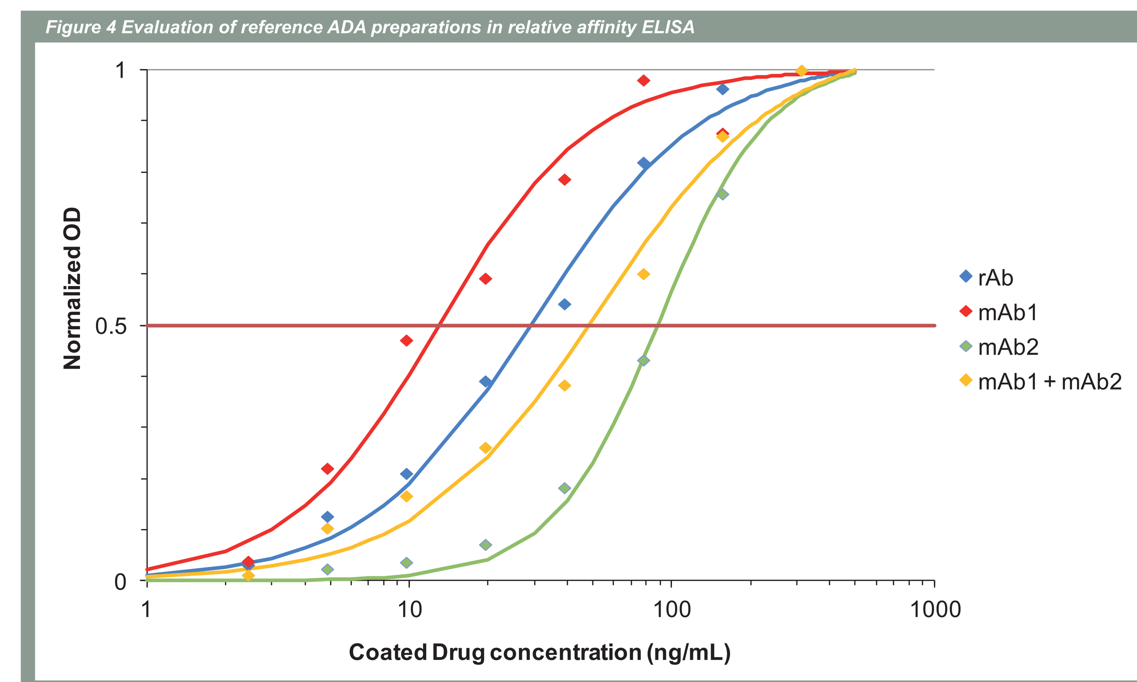


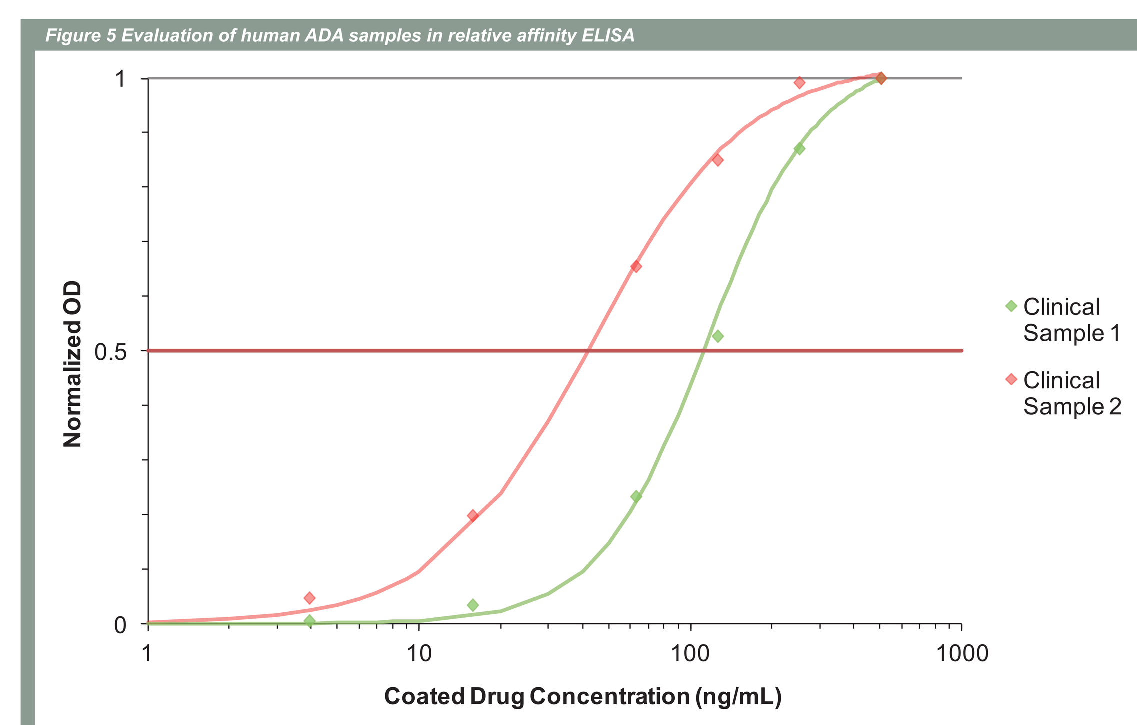
Table 1 Relative affinity of reference ADA preparations

	polyclonal rAb	mAb1	mAb2	mAb1 + mAb2
rA (ng/mL)	30 ± 3*	14 ± 3*	95 ± 10*	48 N.D.

*n≥3 determinations

Following optimization, the ELISA assay is able to discriminate all 3 preparations in terms of affinity; mAb1 and mAb2 are correctly ranked as respectively (relative) higher and lower affinity antibodies, and a mixture of both (mAb1+mAb2) yields a "polyclonal" preparation of intermediate affinity between that of mAb1 and mAb2. The true polyclonal rabbit ADA (rAb) was determined as having a relative affinity closer to the higher affinity monoclonal antibody mAb1 (Fig. 4).

Figure 5 Evaluation of human ADA samples in relative affinity ELISA



ADA positive clinical samples were evaluated as part of method development. The range of relative affinity that can be measured in the assay is confirmed to be adapted for determination of the relative affinity of ADA present in clinical samples (Fig. 5, representative results obtained for clinical samples)

VALIDATION OF A RELATIVE AFFINITY ELISA

The following parameters were evaluated as part of the validation study for the ELISA affinity assay

- inter and intra assay precision for rA determination using a positive control (PC, plasma pool spiked with one level of reference ADA preparation) and ADA positive clinical samples (CS1 and CS2)
- selectivity in individual ADA naïve plasma spiked at PC level with reference ADA preparation
- range of acceptable OD at highest coated drug concentration used to establish the sample dilution factor in the assay
- stability of control samples and ADA positive clinical samples

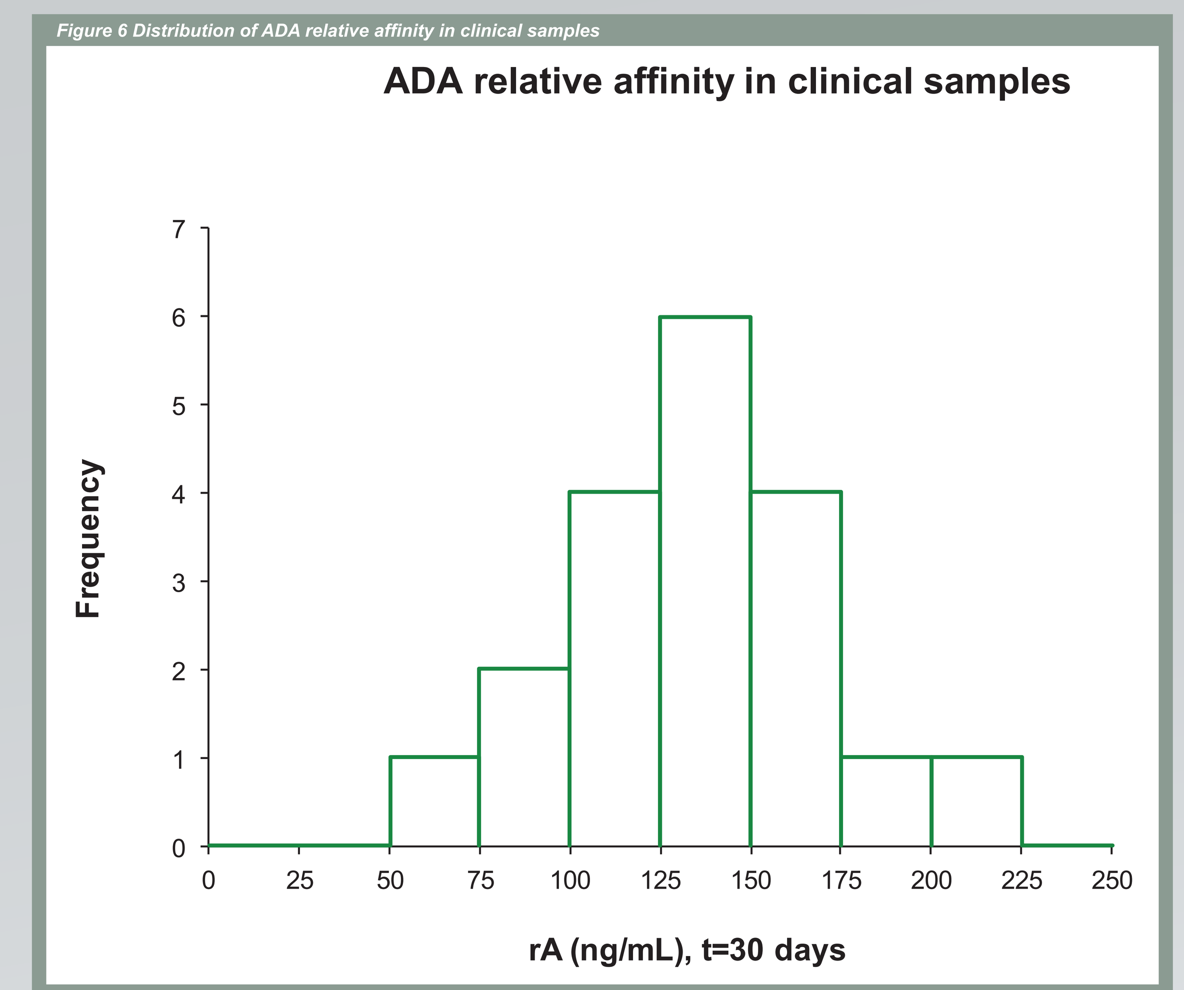
Table 2 Validation results of Relative Affinity ELISA

	PC		CS1		CS2	
	rA (ng/mL)	%CV$_{rA}$	rA (ng/mL)	%CV$_{rA}$	rA (ng/mL)	%CV$_{rA}$
Precision Intra Run (n=11)	16-29	0.2-15.4	ND		ND	
Precision Intra Run (n=11)	23	20	116	25	36	19
Selectivity	10/10 acceptable		N/AP			
Starting OD	0.2 to 1.6		N/AP			
Stability	minimum 6 Freeze/Thaw minimum 3 hours at RT minimum 2 hours in process					

CASE STUDY

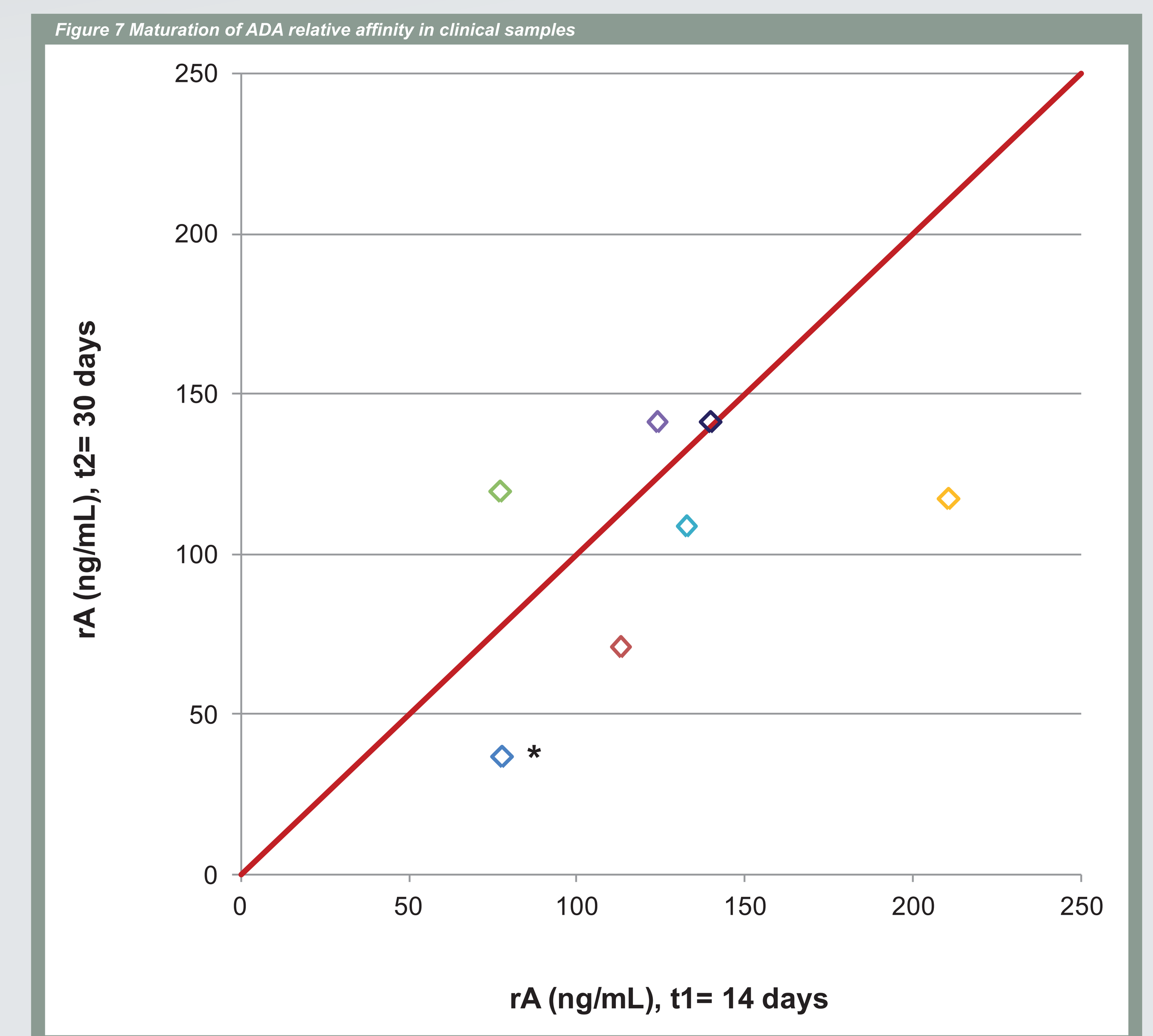
Thirty six healthy individuals were submitted to a single dose of serine protease drug with sampling time of Day -1, Day 7, Day 14, and Day 30 for a total of 98 study samples. Following screening and confirmatory assays, 27 samples were confirmed to be positive for specific ADAs and analyzed in the relative affinity ELISA.

Figure 6 Distribution of ADA relative affinity in clinical samples



Individual samples at a given time point can be ranked in terms of relative affinity. Eighty three percent of the samples measured showed an ADA response of low relative affinity (rA>100 ng/mL) when measured 30 days after exposure to the drug. The highest affinity response measured was of rA ca. 70 ng/mL, which indicates a binding strength of the corresponding ADA response for the drug that is still well below the one measured for the high affinity polyclonal rAb control preparation (Fig. 6).

Figure 7 Maturation of ADA relative affinity in clinical samples



* t1=30 days, t2=unscheduled visit

Six individuals that displayed a detectable ADA response as early as 14 days after exposure to the drug could be evaluated at both time points in the relative affinity ELISA. Three of those demonstrated an increase in relative affinity of the ADAs at 30 days (data points below the red line, Fig. 7). A seventh individual (Fig. 7, starred) tested at t1=30 days and at a later unscheduled visit showed a similar increase in rA between the 2 time points. Taken together these data demonstrate that the assay is able to identify individuals that display an apparent maturation of their immune response against the drug.

CONCLUSION

The screening and confirmatory assays for detection of the specific ADA response against the serine protease drug use the common bridging ELISA format. The same bridging format was used in an ELISA for evaluation of ADA relative affinity to ensure that all previously confirmed positive samples could be evaluated in the assay.

Critical assay parameters such as the range of coated drug concentration were optimized in the relative affinity ELISA using purified ADA preparations typically available as part of a regular immunogenicity assay development. Adapted data treatment decreased overall assay complexity and increased sample throughput.

The resulting ELISA was validated with acceptable precision for determination of relative affinity. Assay suitability for analysis of clinical samples was determined during development and validation, and then confirmed in a case study. ADA positive samples could be ranked in terms of relative affinity at a given time point, and individuals were identified that showed a shift of their ADA response to higher affinity antibodies over time that would be indicative of an apparent maturation of the immune response.

REFERENCES

1 Karlsson R. and Larsson A., Meth Mol Biol, 2003, 248, 389-415

2 Stanley C et al., J Immunol Meth, 1983, 64, 119-132

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