Increased need for ADA assays with enhanced drug tolerance and suggested strategies
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- Methods for improvement of drug tolerance
  - Acid dissociation approaches
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- Conclusions
All biotherapeutics have the potential to induce an immune response

Possible adverse effects on safety or efficacy

Safety
Neutralizing activity of ADA to endogenous proteins
Hypersensitivity reactions

Efficacy
Neutralizing activity of ADA to drug causing low drug levels and reduced efficacy

Anti-drug antibody (ADA) assessments essential part of filling package
Regulatory guidance documents (EMA and FDA) stress the importance of sensitive and drug tolerant ADA immunoassays.

Drug interference causing poor drug tolerance

= The biggest technical challenge in immunogenicity assays

Risk of poor drug tolerance: false-negative screening of low-positive samples and therefore underestimation of the immunogenicity potential of a drug.
Bridging immunogenicity assays susceptible to endogenous drug interference
The need for high Drug Tolerance

Drug interference is especially challenging in case of therapeutic biologicals due to:

• Long half-lives
• High dosage

Ideally ADA sampling after drug clearance, however

• In multi-dose trials
• Requested early ADA sampling by regulators

Waiting for drug clearance is not an acceptable solution
• AIM: separate the analyte (ADA) from interfering factors
• Acid dissociation
  – Standard protocol
  – Enhanced dissociation protocol
• Extraction methods
  – Affinity Capture Elution (ACE) assay
  – Solid-Phase Extraction with Acid Dissociation (SPEAD)
  – Precipitation and Acid dissociation (PandA)
  – Biotin-drug extraction and acid dissociation (BEAD) procedure
Standard Acid dissociation approach (300mM Acetic Acid)

1. Possible drug-ADA complexes
2. Dissociation of complexes with 300mM Acetic Acid
3. Neutralization with Master Mix with Trizma Base
Improvement of Drug Tolerance

Acid dissociation to improve drug tolerance

**Standard Acid dissociation approach**

*(300mM Acetic Acid pH 3.00)*

**Drug tolerance (100 ng/mL PC)**

- **DT 4.00 µg/mL**
- **DT 78.0 µg/mL**

**Drug tolerance (100 ng/mL PC)**

- **DT 10.0 µg/mL**
- **DT 100 µg/mL**
In case Drug Tolerance is not improved sufficiently by Standard Acid Dissociation *(300mM Acetic Acid pH 3.00)*

**Options:**

- Increased dilution in the assay MRD (decreased sensitivity)
- Increased Master Mix concentration
- Increased Acidity (HCL/Glycine instead of Acetic Acid)
- Increased incubation time with Master Mix (overnight)

- Use independently or in combination
Increased Acidity (90mM HCL, pH 1.90)
Increased dilution in the assay MRD (decreased sensitivity)
Increased Master Mix concentration
Increased incubation time with Master Mix (overnight)

Standard acid dissociation
(300mM Acetic Acid, pH 3.00)
• **AIM:** separate the analyte (ADA) from interfering factors

• **Acid dissociation**
  - Standard protocol
  - Enhanced dissociation protocol

• **Extraction methods**
  - Affinity Capture Elution (ACE) assay
  - Solid-Phase Extraction with Acid Dissociation (SPEAD)
  - Precipitation and Acid dissociation (PandA)
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Affinity Capture Elution (ACE) assay
Extraction methods to improve drug tolerance

- Dissociation of ADA-drug complexes by acid treatment
  (300mM acetic acid, 5 min, RT)

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Affinity Capture Elution (ACE) assay
Extraction methods to improve drug tolerance

- Dissociation of ADA-drug complexes by acid treatment (300mM acetic acid, 5 min, RT)
- Neutralization in presence of solid-phase drug (overnight incubation) ➔ Affinity capture of ADA on solid-phase
- ADA eluted off with acid (300mM acetic acid, 5 min, RT) and bound to a fresh buffered solid phase

Affinity Capture Elution (ACE) assay

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- Neutralization in presence of solid-phase drug (overnight incubation) ➔ Affinity capture of ADA on solid-phase
- ADA eluted off with acid (300mM acetic acid, 5 min, RT) and bound to a fresh buffered solid phase
- Detection with biotinylated drug and Strep-HRP and substrate (amplification of signal to increase sensitivity)

Affinity Capture Elution (ACE) assay
Extraction methods to improve drug tolerance

- Sensitive and high tolerant to free drug (500 ng/mL ADA detected in presence of 500 µg/mL free drug)
- Advantage: Multivalent binding of the ADA: detection of low affinity antibodies
- Disadvantage: possible re-association of drug and ADA upon pH neutralization

Solid-Phase Extraction with Acid Dissociation (SPEAD)

Extraction methods to improve drug tolerance

**Day 1:** Sample Pretreatment

- Step 1 - Biotin-Drug Complexation
  - **Overnight Incubation ~12-18 hrs**

**Day 2:** Sample Pretreatment

- Step 2 - Streptavidin: Biotin-Drug Complex Capture
  - **Incubate ~2 hrs**
  - **Wash**

- Step 3 - Acid Dissociation
  - **Incubate ~5 min**

- Step 4 - Sample Neutralization
  - **Incubate ~1 min**
  - **Transfer to ELISA plate**

**Day 2:** Sample Analysis

- Step 5 - Coat Neutralized sample onto ELISA plate
  - **Incubate ~1 hr and wash**

- Step 6 - Block
  - **Incubate ~1 hr and wash**

- Step 7 - Enzyme HRP-Drug Addition
  - **Incubate ~1 hr and wash**

- Step 8 - Substrate Addition & Absorbance Reading

Detection of antibodies against therapeutic proteins in the presence of residual therapeutic protein using a solid-phase extraction with acid dissociation (SPEAD) sample treatment prior to ELISA. Smith HW et al. Regul Toxicol Pharmacol. (2007)
Solid-Phase Extraction with Acid Dissociation (SPEAD)

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  - Step 1 - Biotin-Drug Complexation
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Day 2:
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Solid-Phase Extraction with Acid Dissociation (SPEAD)

Extraction methods to improve drug tolerance

- >10–100-fold increase in residual drug tolerance
- Disadvantage: possible re-association of drug and ADA upon pH neutralization

Detection of antibodies against therapeutic proteins in the presence of residual therapeutic protein using a solid-phase extraction with acid dissociation (SPEAD) sample treatment prior to ELISA. Smith HW et al. Regul Toxicol Pharmacol. (2007)
Precipitation and Acid dissociation (PandA)

Extraction methods to improve drug tolerance

Critical Step 1: Saturation of ADA with Excess Drug (Complex Formation)
1. Add 40 µL of 10-50 µg/mL of drug to 10 µL of sample (to form ADA/drug complexes). Incubation: 1 hr @ 24°C, 450 rpm

Critical Step 2: Precipitation of Complexes
2. Add 50 µL of PEG (3-6%). Incubation: Overnight @ 4°C
3. Spin samples, aspirate then resuspend pellets with 200 µL of PEG; spin and aspirate supernatant (repeal 2x).

Critical Step 3: Acid Dissociation and Coating in Acid on a Large Capacity Surface
4. Add 250 µL of 300 mM acetic acid to reconstitute pellet and dissociate ADA/drug complexes.
5. Coat samples in acetic acid on high bind plate. Incubation: 1 hr @ 24°C, 450 rpm

Critical Step 4: Specific Detection
6. Wash and then block plate with 3% nonfat dry milk in PBS. Incubation: 1 hr @ 24°C, 450 rpm
7. Wash and add sulfitog-drug. Incubation: 1 hr @ 24°C, 450 rpm
8. Wash, add 2x Read Buffer T, and read plate.

Table:

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<th>Drug A present µg/mL</th>
<th>Assay sensitivity ng/mL</th>
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Precipitation and Acid dissociation (PandA)

Extraction methods to improve drug tolerance

- Assays sensitivity was maintained while drug tolerance was improved to 100 - 250 µg/mL free drug
- Advantage: acid dissociation on solid phase to prevent the binding partners from re-binding
- Disadvantage: Optimization of the concentration of PEG is critical and might change between different lots of PEG

A breakthrough novel method to resolve the drug and target interference problem in immunogenicity assays.
Zoghbi J¹, Xu Y², Grabert R², Theobald V², Richards S².
Biotin-drug extraction and acid dissociation (BEAD) procedure

Extraction methods to improve drug tolerance

1. Acid dissociation of ADA:drug complexes
2. Neutralization with excess biotinylated drug
3. Streptavidin-coated magnetic beads added
   Formation of ADA:biotin-drug:streptavidin magnetic bead complexes
4. Separation of bead complexes from sample

Biotin-drug extraction and acid dissociation (BEAD) procedure

Extraction methods to improve drug tolerance

5. Acid treatment to free the ADA from bead-complexes

6. Detection ADA using direct assay format

ADA complexed with soluble biotinylated drug
Captured on streptavidin plate
Detected using a specific sulfo-tag labelled IgG antibody

Biotin-drug extraction and acid dissociation (BEAD) procedure

Extraction methods to improve drug tolerance

- High drug tolerance: > 200 µg/mL drug for measurements of low level ADA (20 - 30 ng/mL)
- Acceptable inter- and intra-assay precision
- Disadvantage: pre-existing drug will compete biotin-labelled drug in binding to ADA

Risks that should be considered
When using methods to improve drug tolerance

Pre-treatment steps might lead to:

• Decrease of assay sensitivity
• Affected assay precision
• Altering analyte binding affinity
• Analyte loss
• Target dimerization due to acid dissociation causing false positive results
• Time consuming and labor intensive
1. Determine required drug tolerance

2. Find the assay in which the drug tolerance meets the requirements
   - Standard assay without pre-treatment
   - Include acid dissociation to the protocol
   - Test extraction method

3. Keep an eye on sensitivity and precision of the assay and compare results with “standard” assay

FIND THE RIGHT BALANCE!
WE ARE DEDICATED TO THE FUTURE OF CLINICAL DEVELOPMENT AND TO EVERY LIFE IT SAVES.