ANTI-BODY DRUG CONJUGATE BIOANALYSIS

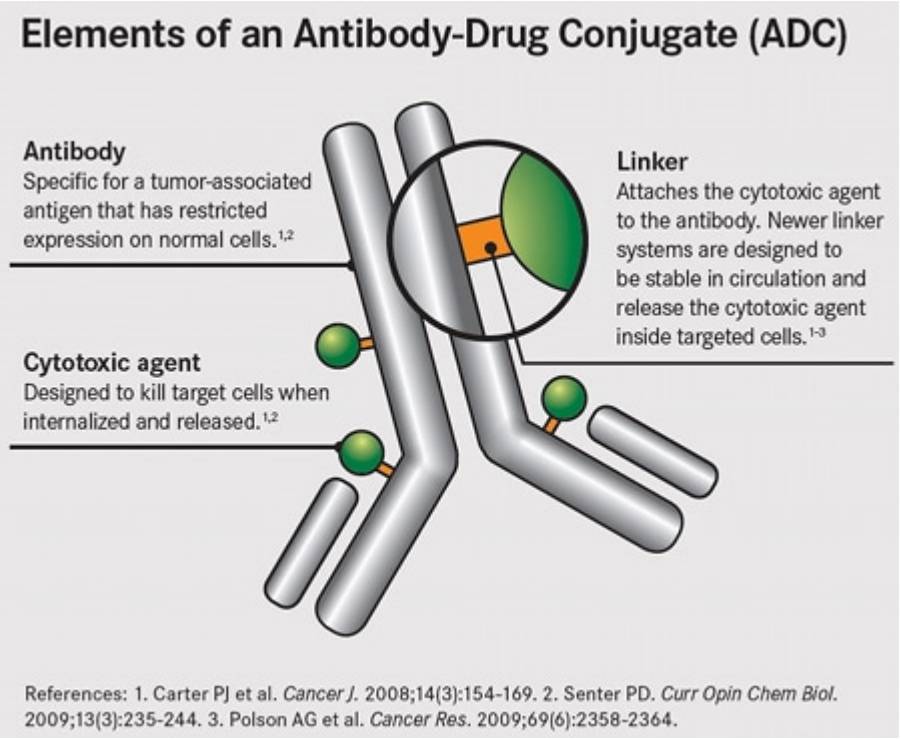
An opportunity for LBA and LC-MS Synergy

WHAT ARE ANTIBODY DRUG CONJUGATES?

Antibody-drug Conjugates (ADCs) are monoclonal antibodies (mAbs) linked to cytotoxic small molecules

The mAbs provide selectivity to reduce the systemic toxicity of the cytotoxic small molecule.

There are several ADCs already on the market with the most commonly known being the cancer treatments Brentixumab Vedotin (Adcetris) and Trastuzumab Entansine (Kadcyla).



ADC BIOANALYSIS REQUIREMENTS

Why should we be interested in ADC Bioanalysis?

The ADC market is growing:

- Over 250 ADCs currently under development
- Industry expected to be worth $15 billion by 2030

charles river

ADC BIOANALYSIS REQUIREMENTS

What do we actually need to quantify?

Since ADCs are generally complex heterogeneous mixtures of multiple species, no single assay can provide a full picture of the ADCs behaviour in vivo.

Table 1. Analytes commonly assessed for antibody–drug conjugate bioanalysis.

Analyte type	Analyte(s) details	Typical analytical method(s)
Conjugated antibody[†]	Antibody with minimum of DAR ≥1	LBA
Total antibody[‡]	Conjugated, partially unconjugated and fully unconjugated (DAR ≥0)	LBA
Antibody-conjugated drug[§]	Total small-molecule drug conjugated to antibody	Affinity LC–MS/MS, LBA
Unconjugated drug[¶]	Small-molecule drug not conjugated to antibody	LC–MS/MS
Total drug[#]	Total unconjugated and conjugated drug	LC–MS/MS
Antitherapeutic antibody	Antibodies directed against antibody component of ADC, linker or drug (binding/neutralizing)	LBA

[†]Conjugated antibody analyte may be used as an assessment of the conjugate exposure.
[‡]The total antibody analyte provides an assessment of the protein component of the ADC.
[§]Antibody-conjugated drug analyte is an alternative assessment of the conjugate exposure.
[¶]Unconjugated drug analyte may be used as an assessment of safety characteristic.
[#]Total drug analyte has been reported previously [33]. The analyte is not broadly used at this time.
ADC: Antibody–drug conjugate; Affinity LC–MS/MS: Affinity capture followed by linker cleavage and LC–MS/MS; DAR: Drug-to-antibody ratio; LBA: Ligand-binding assay.

As ADCs are a developing class of molecules there are no specific guidelines to support their bioanalysis.

ADC BIOANALYSIS REQUIREMENTS

What do we actually need to quantify?

An understanding of the ADC structure and the purpose of the study is critical to determining which assays are most appropriate.

For example in addition to these established assay types, for cysteine linked ADCs it is known that the payload can migrate from the ADC to other plasma proteins and therefore it may be useful to measure this.

BIOANALYSIS STRATEGY USED

Assays used on recent ADC study carried out at Charles River.

Recently we were asked to develop assays for 5 cysteine linked MMAE ADC for use on early stage biosimilar pre-clinical studies. The assays developed were:

Assay Name	Assay Description
Unconjugated Drug	Small-molecule drug not conjugated
Total Drug	Total small-molecule drug, both conjugated and unconjugated
Conjugated Antibody	Antibody with minimum of DAR ≥1
Total Antibody	Conjugated, partially unconjugated and fully unconjugated (DAR ≥0)
Total Non-ADC Conjugated Drug	Total small-molecule drug not conjugated to the antibody of the ADC

UNCONJUGATED DRUG ASSAY

Challenge 1: Very low LLOQ required but limited sample volume

ADCs concept is to reduce systemic exposure to the unconjugated drug

Size difference between and an ADC and the unconjugated drug is significant (~150kDa vs ~750Da)

Five separate assays from each sample and the test system being rat severely limited possible spike volume

Solution: 25 pg/mL LLOQ achieved using 10 µL plasma

- Most sensitive mass spectrometer (API6500+)
- Sum multiple transitions
- Maximise recovery by using a simple protein precipitation extraction

UNCONJUGATED DRUG ASSAY

Challenge 2: Ensure extraction does not cause the release of the drug

All though theoretically only enzyme action should release the drug, how do we confirm this?

Solution: Assess % Unconjugated drug present in ADC.

Very high ADC concentration added to blank plasma, extracted and assessed against unconjugated calibration line.

All ADCs tested contained <0.0005% unconjugated drug, therefore only QCs containing >5 µg/mL ADC would contain a quantifiable level of unconjugated drug.

	Concentration of Free Payload pg/mL				
	ADC 2	**ADC 3**	**ADC 4**	**ADC 5**	**ADC 6**
	1000000	**1380000**	**1200000**	**1200000**	**1100000**
	4.77	2.53	1.98	6.16	1.58
	4.83	2.74	2.11	5.85	1.50
	5.19	3.32	2.27	6.13	1.35
Mean	**4.93**	**2.86**	**2.12**	**6.05**	**1.48**
CV %	**4.6**	**14.3**	**6.9**	**2.8**	**7.9**
% Free	**0.000493**	**0.000207**	**0.000177**	**0.000504**	**0.000135**

UNCONJUGATED DRUG ASSAY

Method Summary

Extraction:

1. 10 µl Serum Sample +10 µl IStd
2. PPT in 500 µL Acetonitrile
3. Transfer 450 µL and evaporate
4. Reconstitute in 100 µL H_2O/ACN/HCOOH (80/20/0.2,v/v/v)

LC-MS/MS:

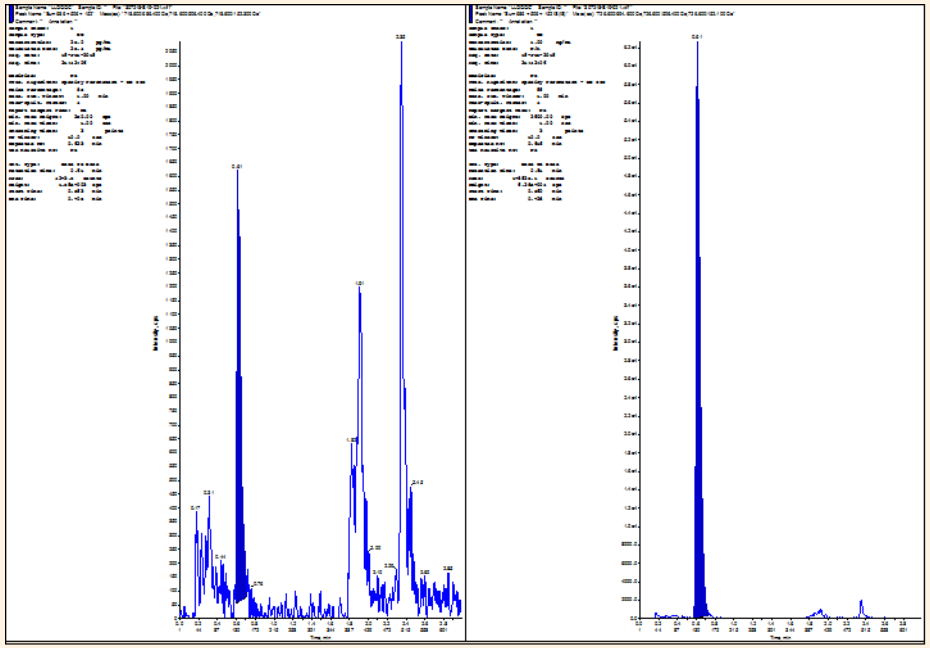
Column: Acquity BEH C18 50 x 2.1 mm, 1.7 µm

Mobile Phases: ACN and H_2O with 0.2% HCOOH

Run Time: 3 minutes

Performance:

- **Range:** 0.0250-250 ng/mL
- **Accuracy & Precision:** ±15% (±20% at LLOQ)
- **Example Chromatogram:**



TOTAL DRUG ASSAY

Purpose and Design

Assay designed to measure all drug, whether that be free, conjugated to an ADC or any other type of protein.

Plasma Sample | Enzymatic digestion | Protein Precipitation | LC-MS/MS

Samples quantified against unconjugated drug calibration standard line.

TOTAL DRUG ASSAY

Challenge 1: How do we ensure our assay achieves total release of conjugated drug.

Solution: ADC QCs extracted and their actual drug concentration compared with theoretical

Drug concentration of an ADC calculated based upon DAR and ADC concentration.

ADC QCs extracted using total drug assay and quantified against an unconjugated drug calibration standard line. Calculated concentration then compared with theoretical.

TOTAL DRUG ASSAY

Challenge 2: What can be done if theoretical 100% release cannot be achieved?

Example Summary of Release Achieved:

	Concentration of MMAE ng/mL									
	ADC 2		ADC 3		ADC 4		ADC 5		ADC 6	
	Low	High	Low	High	Low	High	Low	High	Low	High
	1.50	400	1.50	400	1.50	400	1.50	400	1.50	400
	1.28	405	0.752	246	1.42	371	1.50	377	1.46	403
	1.25	404	0.838	253	1.37	369	1.58	366	1.57	412
	1.21	428	0.881	252	1.32	371	1.39	371	1.52	413
	1.25	421	0.872	251	1.33	369	1.50	360	1.50	410
	1.36	423	0.878	245	1.37	363	1.49	364	1.53	416
	1.31	423	0.878	244	1.30	378	1.44	372	1.56	394
Mean	**1.28**	**417**	**0.850**	**249**	**1.35**	**370**	**1.48**	**368**	**1.52**	**408**
RE %	**-14.7**	**4.3**	**-43.3**	**-37.8**	**-10.0**	**-7.5**	**-1.3**	**-8.0**	**1.3**	**2.0**
CV %	**4.1**	**2.4**	**5.9**	**1.6**	**3.2**	**1.3**	**4.3**	**1.7**	**2.7**	**2.0**

Solution: Prepare calibration standards and QCs using the ADC instead of the unconjugated drug.

As standards and QCs experience the same conditions as samples, providing percentage release is consistent across range, they will account for the incomplete release.

TOTAL DRUG ASSAY

Final Method Summary

Extraction:

1. 10 µl Sample +10 µl Istd in 0.2 mL PCR tube
2. Add 50 µl papain enzyme solution to eluent and incubate overnight at 40°C
3. PPT in 500 µL Acetonitrile
4. Transfer 450 µL and evaporate
5. Reconstitute in 100 µL H_2O/ACN/HCOOH (80/20/0.2,v/v/v)

(LC-MS/MS Conditions as per unconjugated drug assay)

Performance:

- **Range:** 0.500-500 ng/mL
- **Accuracy & Precision:** ±15% (±20% at LLOQ)

TOTAL ANTIBODY ASSAY

Final Method Summary

Extraction:

- **Sample:** 5 µL
- **Capture:** Recombinant Human CD30/TNFRSF8 Protein
- **Wash:** PBST
- **Block:** 2% BSA in PBST
- **Detection:** Anti-Human IgG (γ-chain specific) Perioxidase antibody
- **Detector:** VarioskanFlash Plate Reader

Performance:

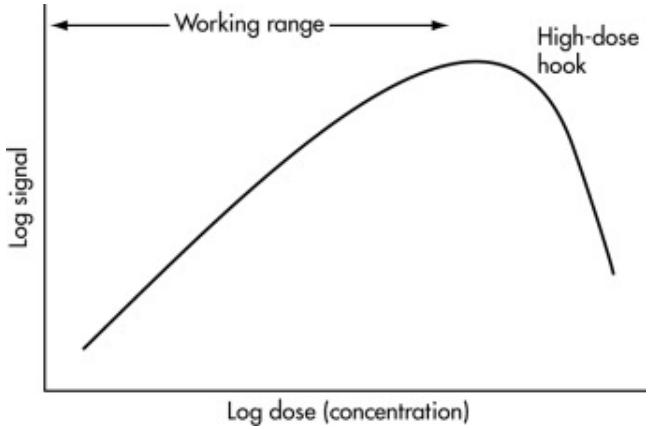
- **Range:** 31.3-2000 ng/mL
- **Accuracy:** < ±35% RE
- **Precision:** ≤ 25% CV
- **Prozone:** No effect observed

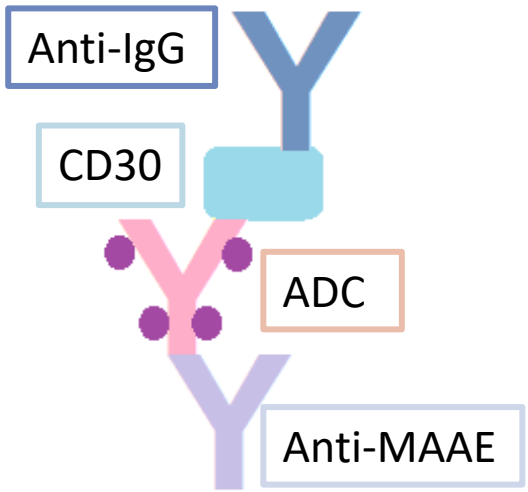
CONJUGATED ANTIBODY ASSAY

Challenge: Prozone effect encountered

The Prozone or hook effect is name given to the phenomena that causes very high analyte concentration samples to read as falsely low.

In ELISA this effect is caused by the reagent antibodies being saturated and therefore being unable to form the required sandwich complex





Solution: Invert capture and detection antibodies

Using the more specific Anti-MMAE antibody as the capture means the assay is not overwhelmed by non-conjugated antibodies.

CONJUGATED ANTIBODY ASSAY

Final Method Summary

Extraction:

- **Sample:** 5 µL
- **Capture:** Rabbit Anti-vc-PAB-MMAE pAb
- **Wash:** PBST
- **Block:** 2% BSA in PBST
- **Detection:** Recombinant Human CD30/TNFRSF8 Protein and Goat Anti-Human IgG (H+L)
- **Detector:** VarioskanFlash Plate Reader

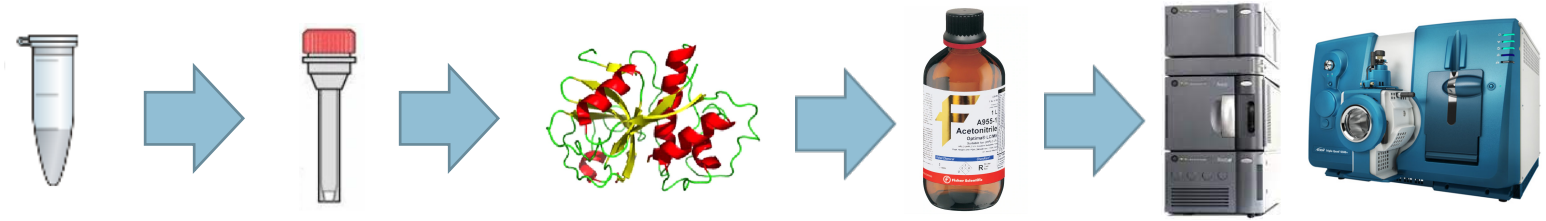
Performance:

- **Range:** 31.3-2000 ng/mL
- **Accuracy:** < ±35% RE
- **Precision:** ≤ 25% CV
- **Prozone:** No prozone effect observed

ADC DEPLETED TOTAL DRUG ASSAY

Purpose and Design

Assay designed to measure non-ADC protein drug conjugates.



Plasma Sample → ADC Removal → Enzymatic digestion → Protein Precipitation → LC-MS/MS

Samples quantified against unconjugated drug calibration standard line.

ADC DEPLETED TOTAL DRUG ASSAY

Challenge 1: How to measure depletion?

Solution: Prepare unconjugated drug and ADC QCs. Extract them both with and without the depletion procedure and assess the difference with the following equation:

$$\text{Percentage Depletion} = 100 - \left(\left(\frac{\text{Depleted}}{\text{Non Depleted}}\right) * 100\right)$$

ADC DEPLETED TOTAL DRUG ASSAY

Challenge 2: How much is sufficient depletion?

Ideal scenario would be 100% ADC removal however is this really feasible?

Solution: Optimise depletion as far as possible and evaluate the impact of the level of non-depleted in collaboration with client.

Due to the relative size of drug to ADC, even a very small percentage non-depleted ADC could have a significant detrimental impact on drug quantitation.

i.e. 1000 ng/mL ADC that is depleted 95% will still contain 50 ng/mL ADC.

50 ng/mL ADC with a DAR of 4 (1 mole ADC: 4 mole drug) will contain 0.935 ng/mL drug

ADC DEPLETED TOTAL DRUG ASSAY

Challenge 3: How to account for batch to batch variability

Solution: Include depletion QCs in every batch.

- 3 x Non-Depleted ADC QC and 3 x Depleted ADC QC extracted on each analytical occasion to enable calculation of depletion.
- This depletion result is then assessed by applying a common sense approach rather than firm acceptance criteria.
- The percentage depletion is then reported alongside in-vivo sample results as additional information to aid interpretation.

ADC DEPLETED TOTAL DRUG ASSAY

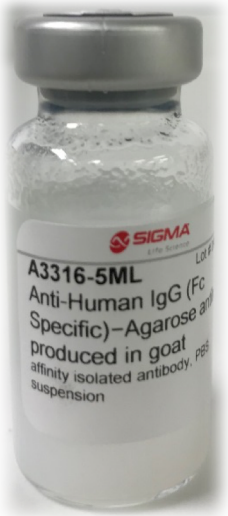
Challenge 4: How do we deplete the ADC?

Initially utilised the capture reagent used for the total antibody assay.

However we found that the quantity required to remove the relatively vast quantities of ADC was not practical.

Solution: Use a more generic antibody

As the ADCs are based on human IgG and this was a rat assay the only human IgG in the samples will be ADC. Therefore we can use an antibody that targets human IgG.



ADC DEPLETED TOTAL DRUG ASSAY

Challenge 5: How do we check that the depletion procedure doesn't simply remove all conjugated forms of the drug not just the ADC form?

No non-ADC protein-drug conjugate reference standard available to quantitatively assess if it is depleted along with ADCs. Therefore alternative method required to provide an indication.

Solution: Incubated QCs

Analyse both incubated and non-incubated ADC QCs using both unconjugated drug assay and ADC depleted total drug assay.

Expected drug concentration if no non-ADC conjugates present is calculated as:

$$\left((\textbf{Total Drug} * \%\ \textbf{Not Depleted}) + \textbf{Unconjugated Drug}\right)$$

Concentration > Calculated Expected Concentration = Presence of non-ADC conjugates

ADC DEPLETED TOTAL DRUG ASSAY

Final Method Summary

Extraction:

1. 10 µl Sample +10 µl Istd in 0.2 mL PCR tube
2. 50 µl µl Anti-Human IgG (Fc specific) antibody coated agarose bead slurry and incubate at RT whilst mixing
3. Filter sample through microcentrifuge column to remove beads
4. Add 50 µl papain enzyme solution to eluent and incubate overnight at 40°C
5. PPT in Acetonitrile
6. Evaporate and reconstitute in 100 µL H_2O/ACN/HCOOH (80/20/0.2,v/v/v)

Example Summary of Depletion Achieved:

	Drug Concentration (ng/mL)									
	ADC 2		ADC 3		ADC 4		ADC 5		ADC 6	
	Non-Depleted	Depleted	Non-Depleted	Depleted	Non-Depleted	Depleted	Non-Depleted	Depleted	Non-Depleted	Depleted
	126	1.86	105	1.56	109	2.70	50	0.89	65	0.62
	121	1.65	105	1.48	115	3.10	49	1.02	63	0.77
	124	1.84	103	1.56	114	2.78	49	0.90	63	0.69
Mean	**124**	**1.78**	**104**	**1.53**	**113**	**2.86**	**50**	**0.94**	**63**	**0.69**
CV %	**2.0**	**6.5**	**1.1**	**3.0**	**2.8**	**7.4**	**0.6**	**7.5**	**1.8**	**11.3**
% Depleted	**N/A**	**-98.6**	**N/A**	**-98.5**	**N/A**	**-97.5**	**N/A**	**-98.1**	**N/A**	**-98.9**

CONCLUSION

ADC bioanalysis is an ever expanding field that comes with additional challenges for the bioanalyst.

Main Challenges:

- Determining what measurements are required
- Designing experiments to appropriately characterise assay performance

Neither LBA or LC-MS alone can effectively provide a complete picture of an ADCs behaviour in-vivo.

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