

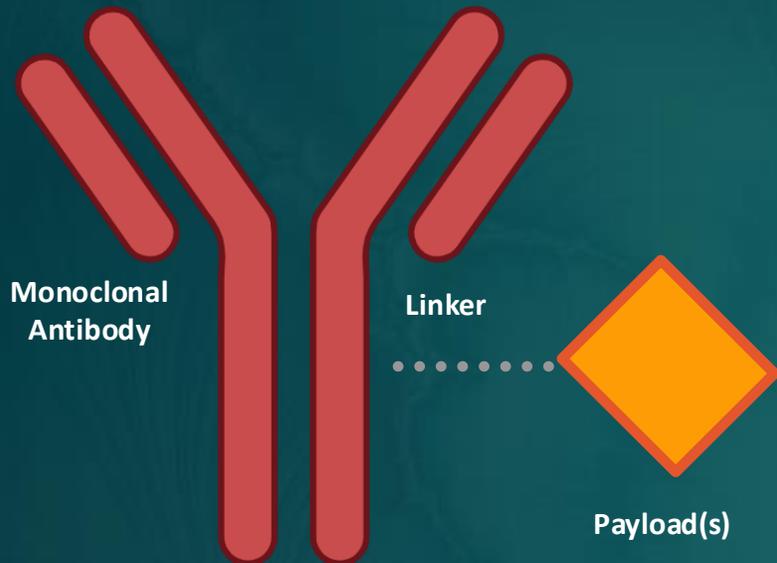
Exploring Insights into the Development & Validation of PK Assays for ADCs Using LC/MS Platform Technology

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EBF Open Symposium 2025



Bioanalytical Considerations for ADCs



What to measure?

Pharmacokinetics

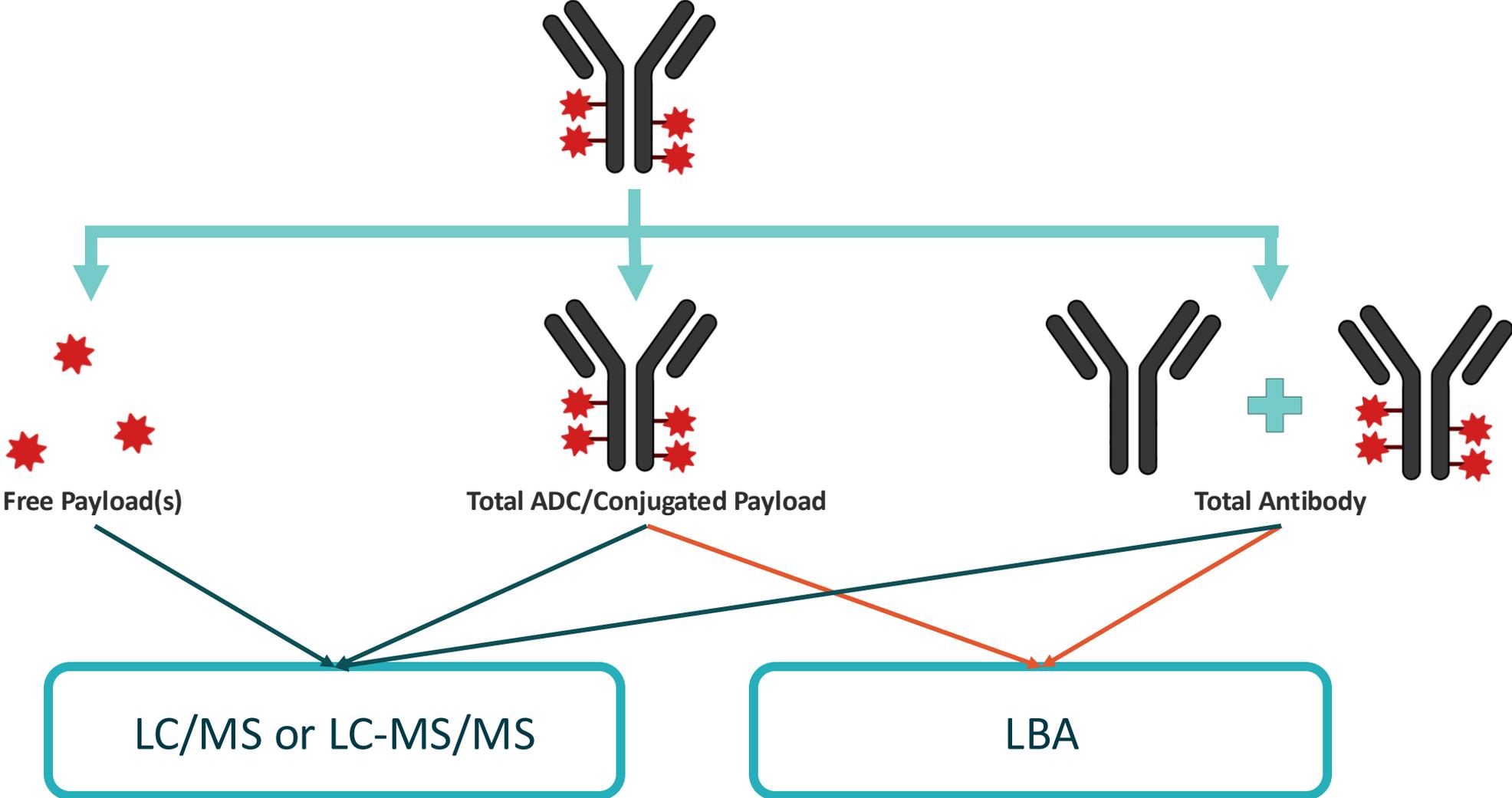
1. Free payload(s)
2. Total antibody
3. Conjugated antibody (Total ADC or conjugated payload)
4. *In vivo* DAR and metabolite ID*

Immunogenicity

1. Anti-drug antibodies
2. Neutralizing antibodies

Pharmacokinetics for ADCs

Three Components for ADC PK Measurement



ADC Linker Types

| Linker Type | Cleavage Mechanism |
|----------------------------|--|
| Acid Cleavable Linker | Cleavage by low pH of tumor acidic microenvironment |
| Cathepsin Cleavable Linker | Cleavage by Cathepsin in lysosomes |
| Enzymatic Cleavable Linker | Cleavage by phosphatase, phosphodiesterase, Sulfatase, Glycosidase in lysosomes |
| GSH Cleavable Linker | Cleavage by reducing disulfide bridge with high level of GSH in cytoplasm (~10 mM v.s. ~5 μ M in plasma) |
| Photo Responsive linker | Cleavage by irradiation with NIR ($\lambda = 650-900$ nm) or with UV ($\lambda = 365$ nm) |
| Non-cleavable Linker | |

- **Understand the linker chemistry to select optimal conditions that maximize linker stability and minimize ADC interference in the free payload assay.**
- **Understand the linker chemistry to select optimal strategy that cleavage and release conjugated payload for total ADC quantitative assay by LC/MS.**

Free Payload(s)

PK Measurement – Free Payload by LC/MS

- Targeted delivery of ADC allows much higher doses with lower systemic exposure of the payload.
 - Low levels of payload detected in plasma
 - Payload can be metabolized, and/or payload can be released with partial linker attached
 - Net result is that sensitive LC/MS methods (100 pg/mL or lower) are typically needed
- Even with a sensitive method, payload levels can be below the quantifiable limit (BQL) when conjugated with a stable linker or when extensive metabolism occurs

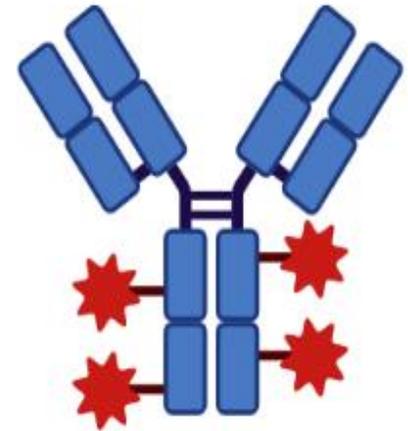


Toxic Payload with linker

Assessing Interference and Stability for an ADC Free Payload Assay

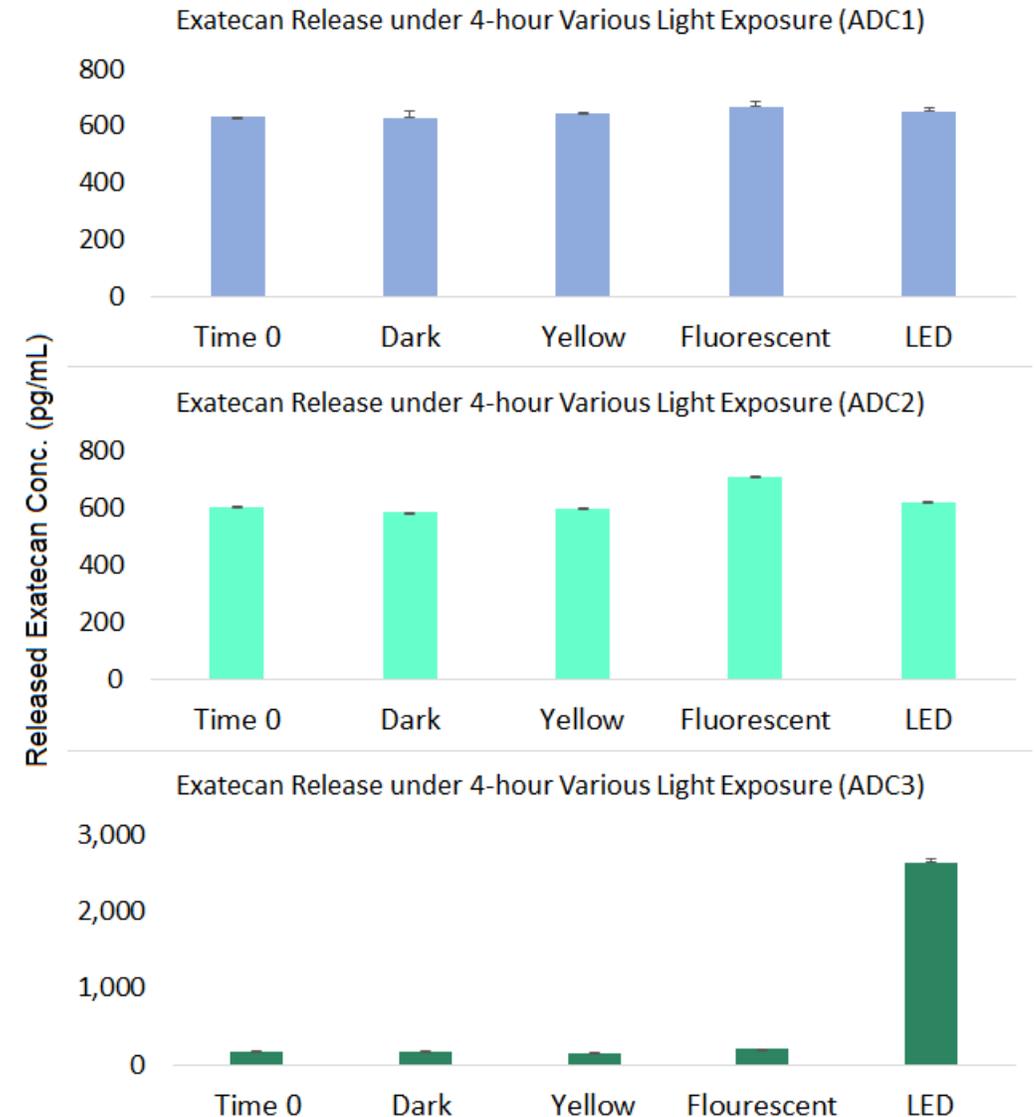
Per ICH M10 the impact of the administered ADC at C_{max} must be established for assay interference and stability

- Non-interference testing
 - Evaluate ADC at C_{max} for free payload
 - Payload at low, high and OTC QCs both with and without ADC
- Test ADC stability under various conditions for assay impact and release of free payload
 - Light sensitivity - LED and yellow light and limited light (dark hood) comparison
 - Temperature sensitivity - ambient vs Ice Bath, time courses for whole blood and plasma at various temperatures
 - F/T conditions (ambient vs Ice Bath) and cycles
 - Post-extraction stability in the autosampler from any remaining ADC or linker payload
 - Fresh vs. aged plasma samples may impact stability and PK results



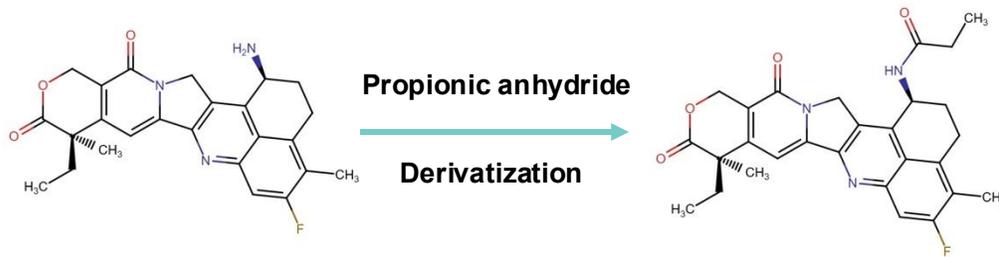
Linker Stability and ADC Interference (Pre-extraction)

- Test for interference and stability impact when expected C_{max} concentration of ADC is present
 - Spike ADC at 400 $\mu\text{g}/\text{mL}$ into blank matrix
- Non-interference: Spike ADC at C_{max} (400 $\mu\text{g}/\text{mL}$) in free Exatecan QC samples at 30, 90, 600 and 1,600 pg/mL . Curve range 30 – 2,000 pg/mL .
- Stability testing (ADC + Exatecan)
 - Freeze/Thaw Sample Stability (ambient vs Ice Bath)
 - **Short Term Light Stability (4 hrs exposure)**
 - Sample Storage Stability (-20 °C and -70 °C)
 - Whole Blood Stability (ADC only)
- Re-injection Integrity (post-extraction)



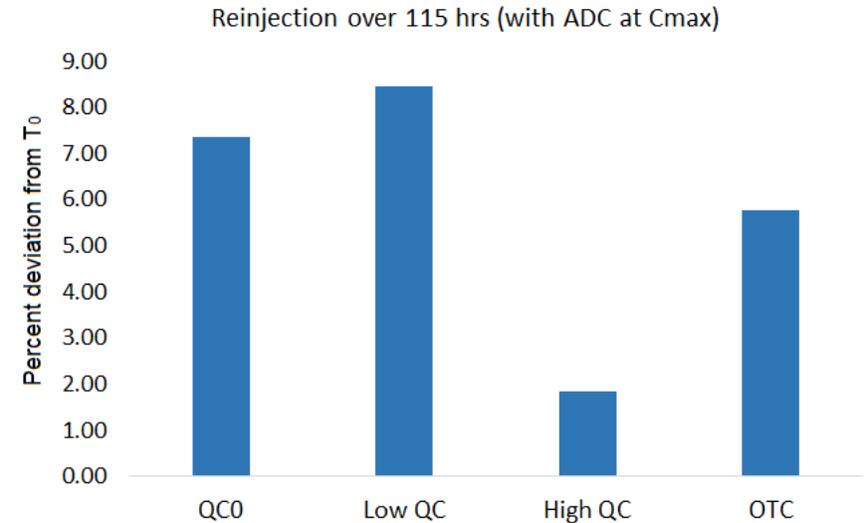
ADC/Linker Payload Interference (Post-extraction)

- Proteins and antibodies are efficiently removed during extraction; however, trace ADC carryover can significantly interfere with free payload assays due to its high concentration.
- Residual linker-payload is a main source of post-extraction interference.
- The primary amine of exatecan is the linker attachment site; its derivatization effectively reduces post-extraction interference.

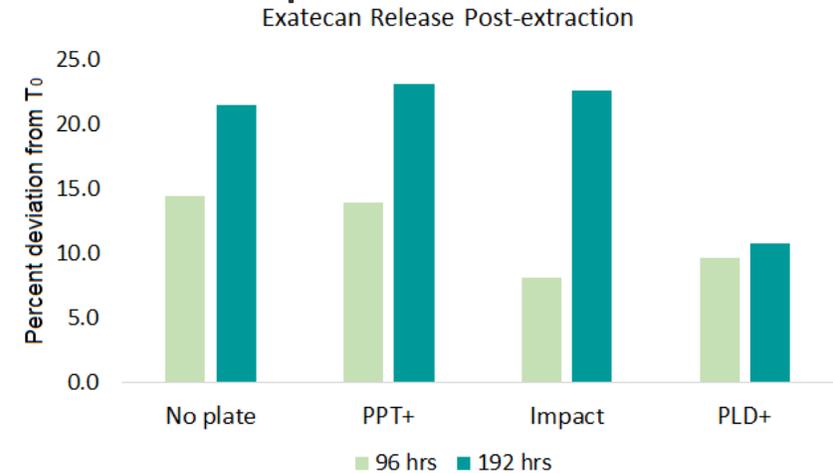


- Cytotoxin such as Dxd has no primary amine to be derivatized. Various protein precipitation plates were evaluated to simplify the free payload assay; some yielded acceptable performance.

1. Exatecan Derivatization



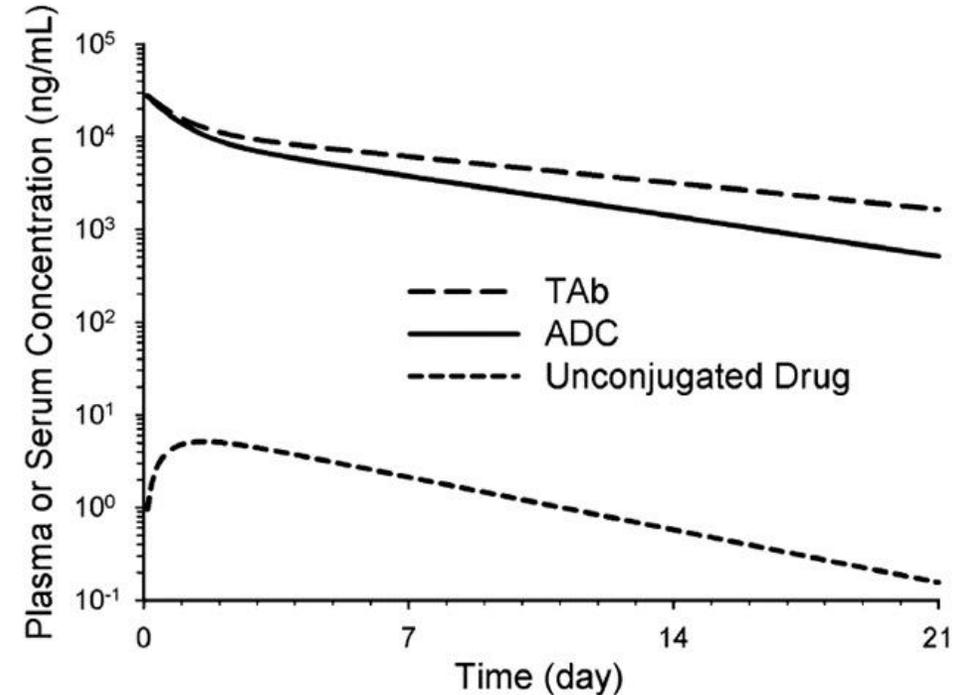
2. Protein Precipitation Plates



Total ADC and Total Antibody

PK Measurement – Total ADC, Total Ab by LC/MS

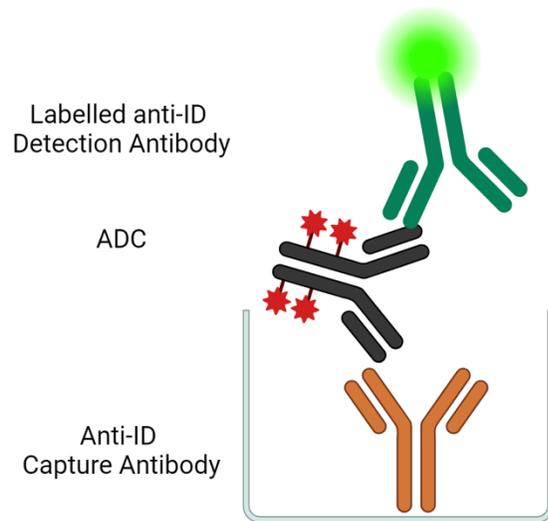
- The structural complexity of ADCs requires distinct analytical strategies from those used for small molecules or naked mAbs
 - Complex ADCs require LC/MS for all PK assays to assess *in vivo* DAR and stability of the ADC
- Even stable linker designs can deconjugate *in vivo*, leading to a complex distribution of DAR species and increased free payload exposure
 - ADCs are inherently heterogeneous, consisting of DAR0 and multiple DAR species, which evolve over time *in vivo*.
 - ADCs with multiple payloads can be assessed for total antibody and conjugated payloads from a single aliquot, offering more data than traditional LBA methods
 - LC/MS quantifies total antibody using signature peptides as surrogates, and total ADC using cleaved payloads as surrogates.
 - Estimate *in vivo* average DAR by comparing total antibody vs. total ADC data over time.



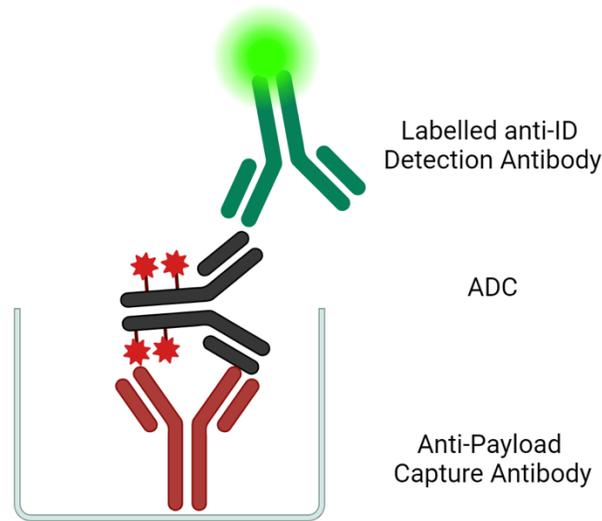
- Cleavage strategy is determined by linker type
- Linker payloads may be used as surrogates, though suboptimal

Ligand Binding Assays for ADC

Total Antibody LBA Format

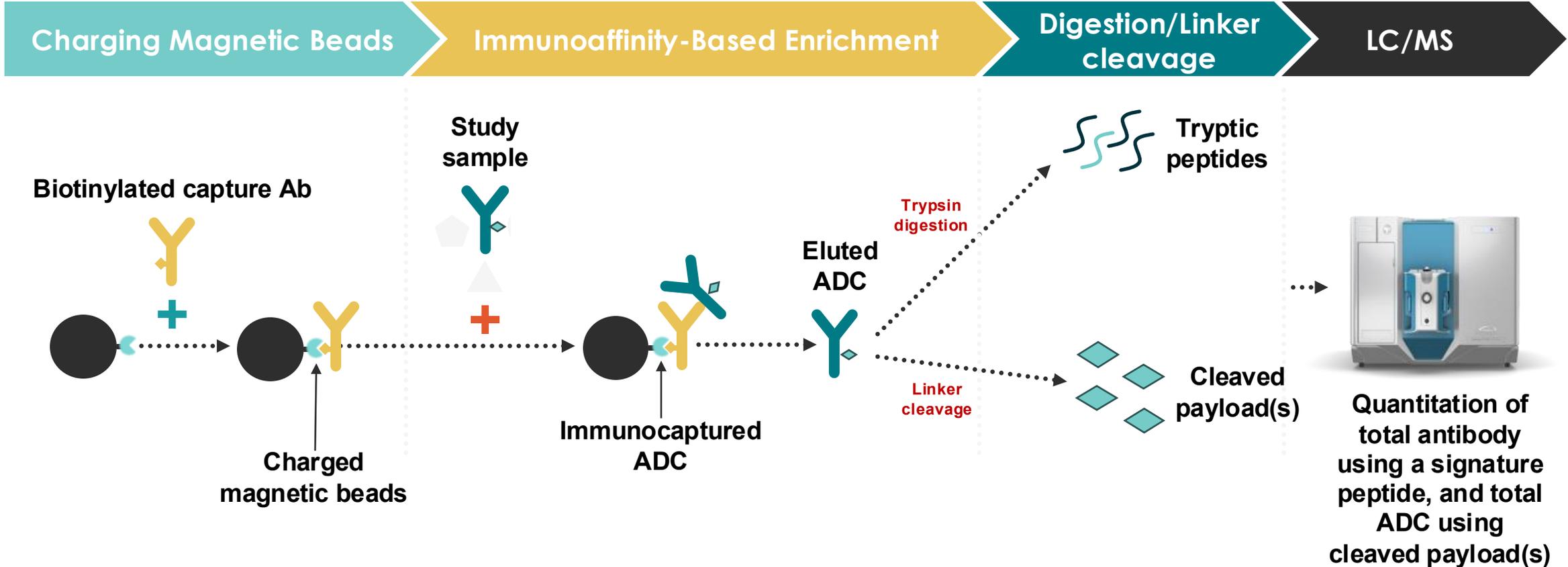


Antibody Conjugated Drug LBA Format

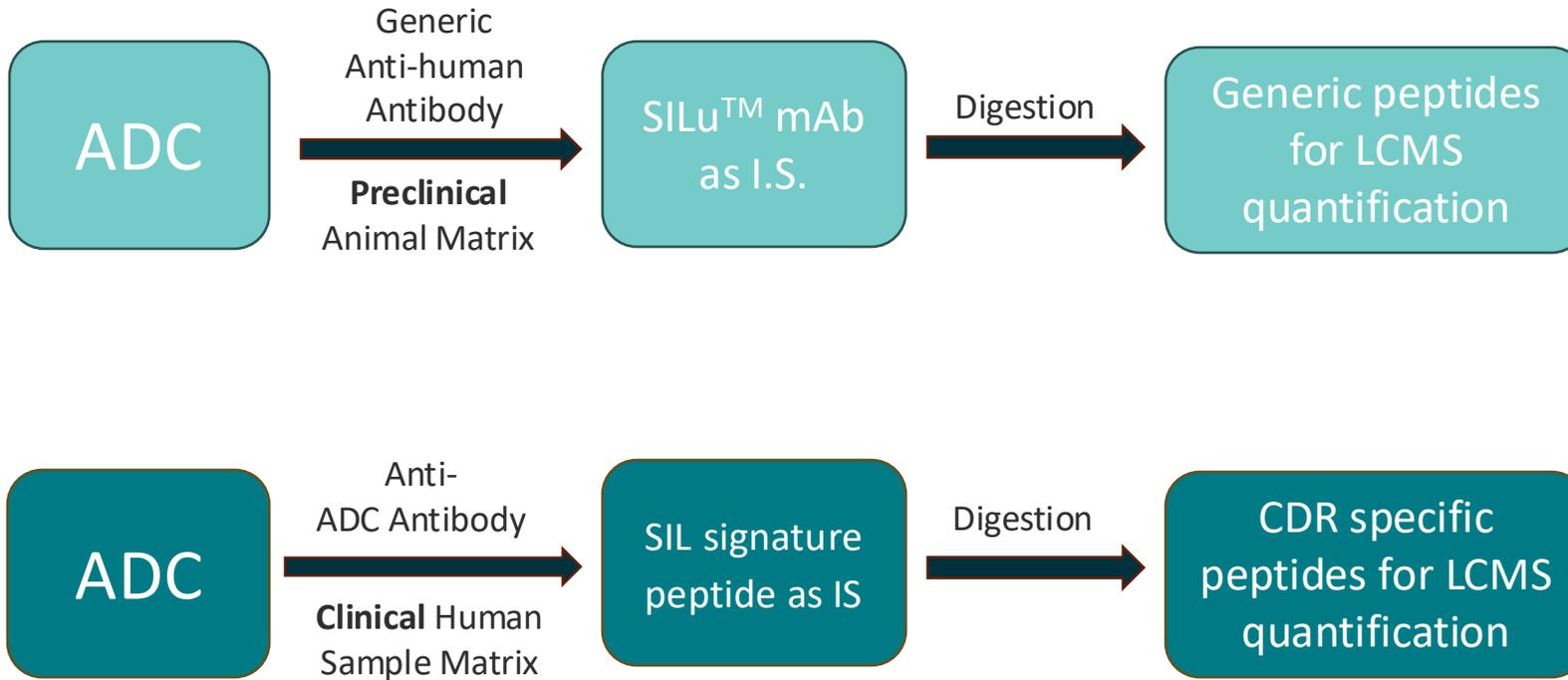


- Highly dependent on critical reagents.
- Requires a minimum of three antibodies.
- Always two separate assays
- Assay quality is driven by reagent quality.
 - High-quality antibodies ensure strong sensitivity and selectivity.
 - Poor-quality antibodies compromise sensitivity (low affinity) and selectivity (off-target cross-reactivity).

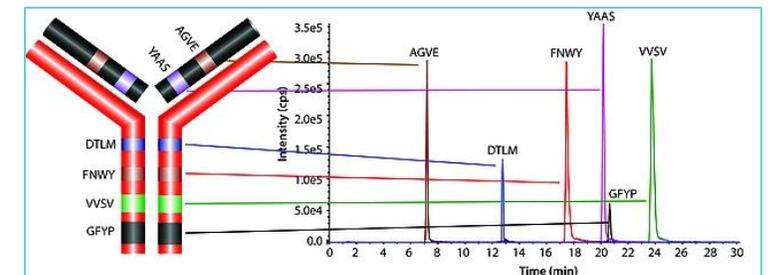
Hybrid IA-LC/MS Workflow for ADCs



Workflow Difference Between Preclinical and Clinical PK



- ADC usually contains a humanized antibody.
- In preclinical species, generic anti-human antibody can be used for capturing (either Fc or Fab specific), whereas anti-ID antibody is needed for clinical studies.
- Generic peptides in human antibody constant regions can be used for LCMS quantification in preclinical studies. Commercially available SILumAb can be used as I.S. where multiple generic peptides are stable isotope labeled.
- Variable CDR specific signature peptides need to be identified for LCMS quantification in clinical studies. Made-to-order SIL signature peptides are needed as I.S.



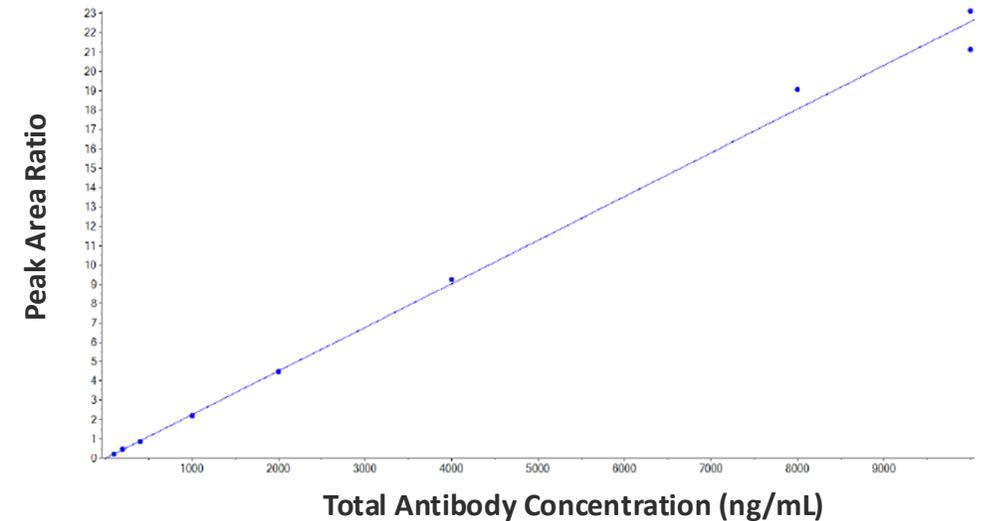
Generic peptides in human IgG constant regions

Case Study: Total Antibody and Total ADC Clinical Assay

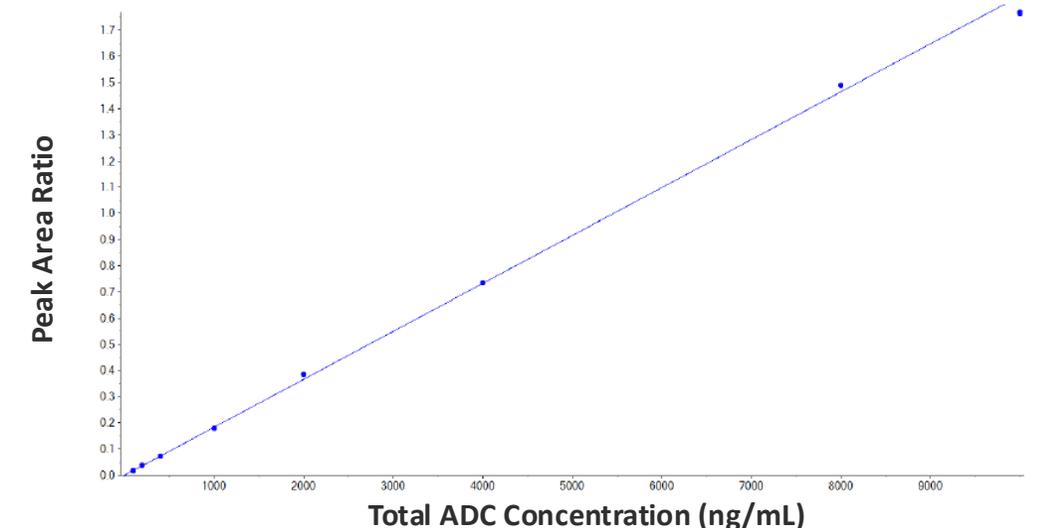
Analytical Method (Single aliquot for two assays)

- ADC DS were used as reference standard.
- Human plasma samples were incubated with a biotinylated anti-ADC antibody (anti-ID).
- Magnetic beads were added for capture.
- Beads were washed using a KingFisher system. ADCs were eluted with acidic buffer and neutralized with Tris-HCl.
- Stable isotope-labeled signature peptide and exatecan were spiked into the eluate as internal standards.
- The eluate was split:
 - Trypsin digestion for total antibody
 - Papain digestion for total ADC
- Digested samples were analyzed by LC-MS/MS.

Calibration Curve for Total Antibody (100 to 10,000 ng/mL)



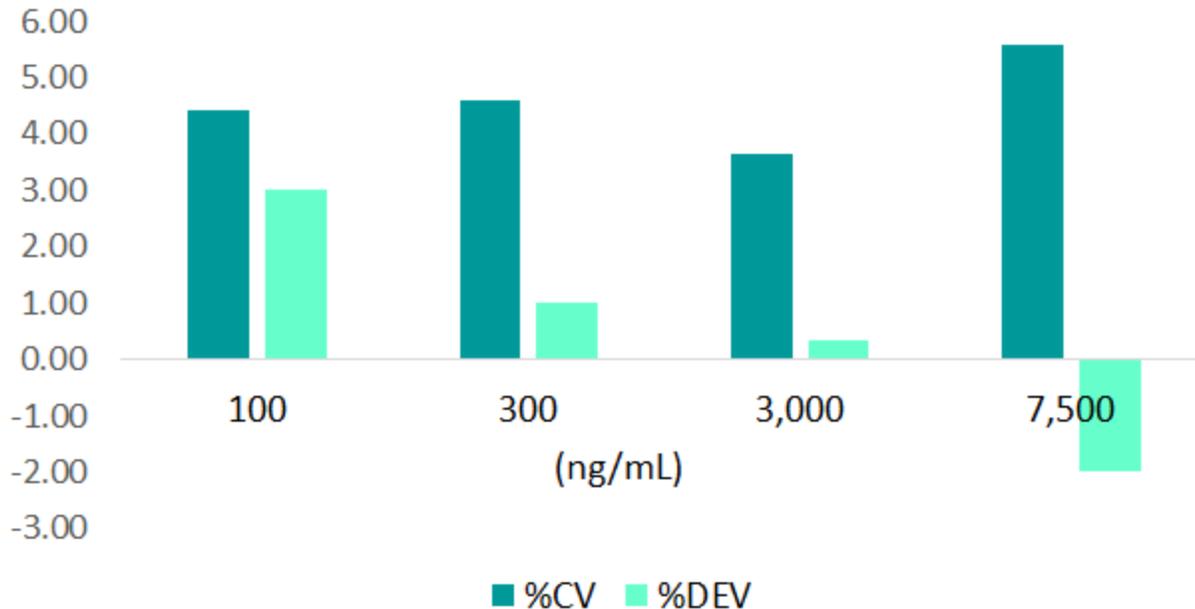
Calibration Curve for Total ADC (100 to 10,000 ng/mL)



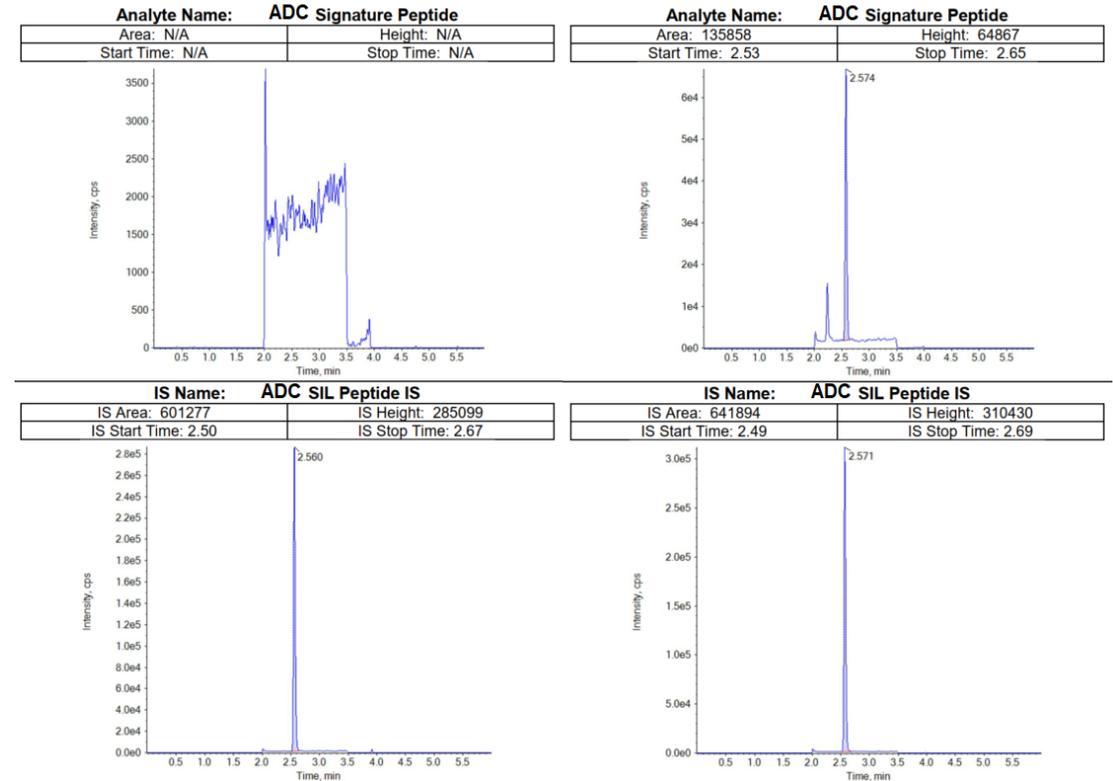
Total Antibody Assay Quantification Performance

- Sample aliquot: 10 μ L, the same aliquot for both total antibody and conjugated exatecan quantification.
- The signature peptide was identified in CDR region based on its selectivity and sensitivity.

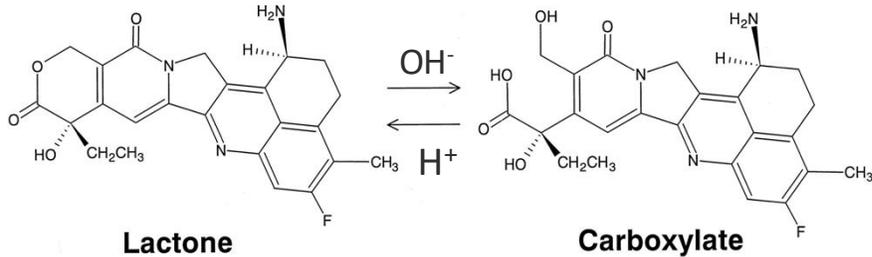
Total Antibody Inter Day Statistics of 3 Validation A&P Runs



Example Chromatograms (Control Zero v.s. LLOQ) – Total Antibody Signature Peptide

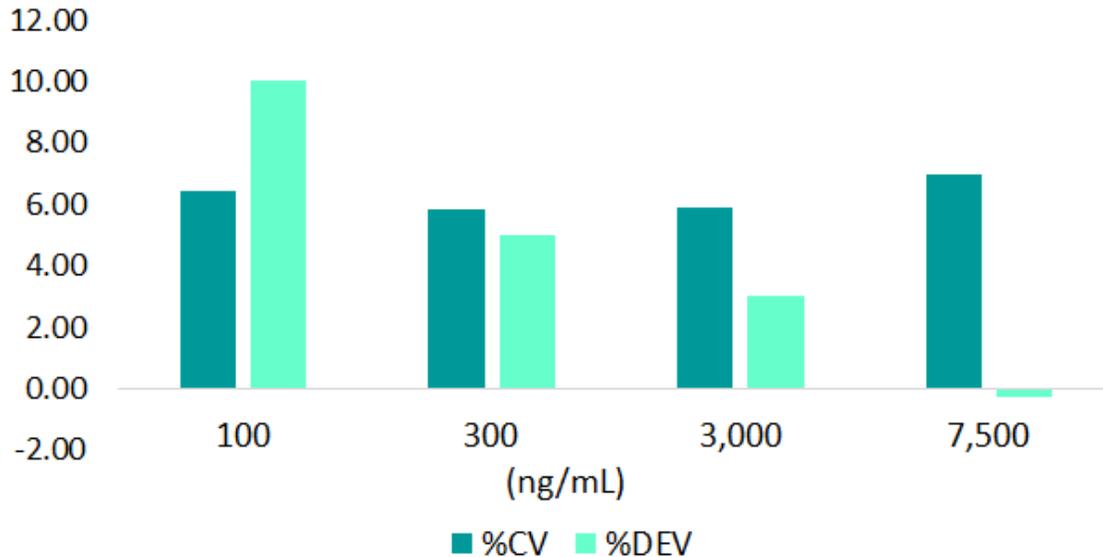


Total ADC Assay Quantification Performance

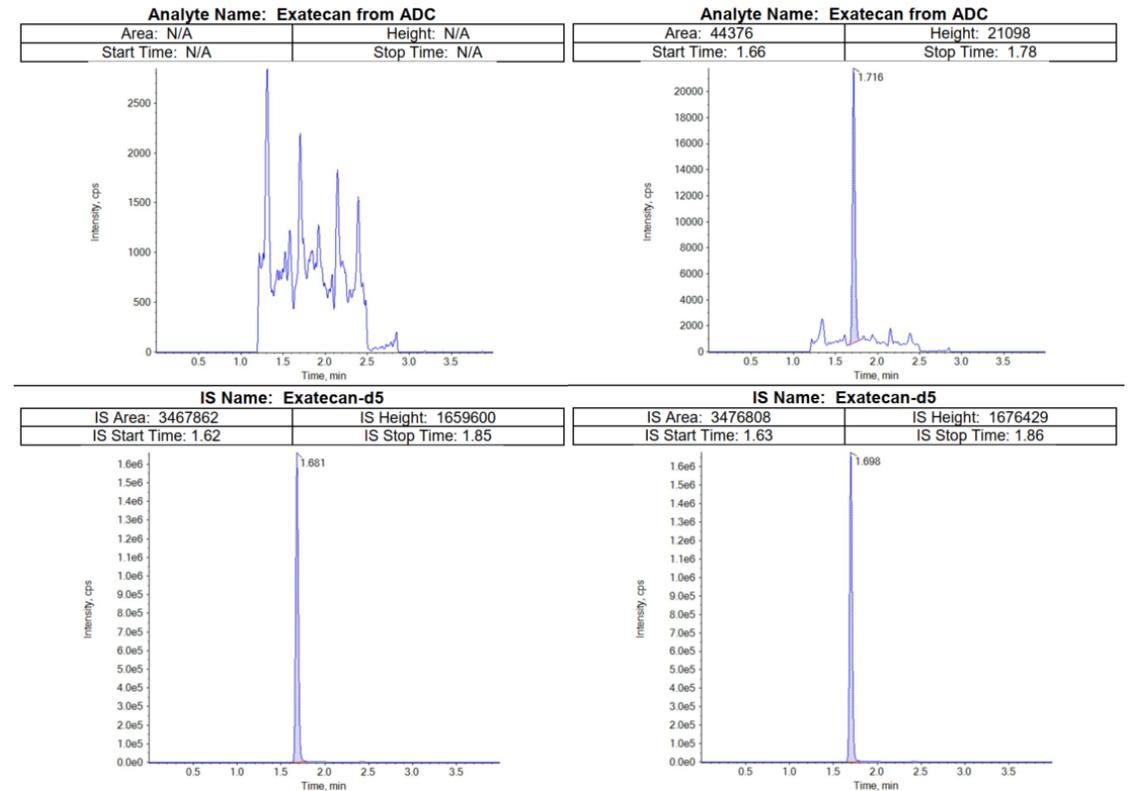


Samples were acidified after papain digestion to maintain lactone form

Total ADC Inter Day Statistics of 3 Validation A&P Runs



Example Chromatograms (Control Zero vs. LLOQ) - Conjugated Exatecan



Summary

Three Key Assays:

- **Free payload(s)**
- **Total ADC**
- **Total antibody**

All analyzable on **LC/MS platform**

Linker Chemistry:

- Critical determinant of assay strategy
- Directly impacts **free payload** and **total ADC** quantification
- Defines **linker cleavage strategy**

Free Payload Assay:

- Requires **high sensitivity**
- Must address interference from the presence of high concentrations of ADC
- Strongly influenced by **linker stability**

Sample Efficiency:

- **Single sample aliquot** can support both total antibody and total ADC assays

Preclinical vs. Clinical:

- **Preclinical:** Generic method feasible
- **Clinical:** Requires **anti-ID antibodies** and **CDR-specific signature peptides**

Any Questions?