Antibody Drug Conjugate Bioanalysis using the BioBA Solution

A Robust and Accurate Quantification of Ado-Trastuzumab Emtansine Using the BioBA Solution and the SCIEX QTRAP® 6500+ System

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Key Challenges of Antibody-Drug Conjugate Bioanalysis

- **Structurally complex analytes** – The complex and heterogeneous nature of ADCs require multiple bioanalytical assays during drug development studies
- **Numerous assay possibilities –** Many workflow options can be complex making life difficult for the bioanalytical scientist tasked with ADC bioanalysis.
- **Limitations of LBAs** – Limited linear dynamic range, cross reactivity and poor reagent reproducibility can lead to poor assay performance and inaccurate results.

Key Benefits and Features of BioBA Solution for ADC Bioanalysis

- **Ready-to-use kit –**BioBA kits provide all the reagents necessary from high capacity streptavidin beads to digestion enzyme compiled into one kit that provides a generalized approach for the immuno-capture and signature-peptide quantitation of any ADC.
- **Improved selectivity and expanded linear dynamic range –** Signature-peptide-based LC/MS/MS analysis provides wider dynamic range, enhances selectivity and specificity and provides the ability to multiplex a second analyte (payload) or catabolite for ADC quantification.
- **Comprehensive and universal solution**: Six key instrument and software components for easy transition from small molecule bioanalysis to ADC bioanalysis: 1) BioBA High-Capacity Enrichment Sample Preparation Kit, 2) Exion LC system, 3) QTRAP® 6500+ System, 4) MultiQuant™ Software, 5) Biopharma vMethods and 6) for automation the Biomek Fx automated liquid handling workstation.

Figure 1. BioBA reagents kit and the SCIEX QTRAP® 6500+ System used in this study for the bioanalysis of the antibody drug conjugate ado-trastuzumab emtansine.

Introduction

Protein based biotherapeutics including monoclonal antibodies (mAbs) and antibody-drug conjugates (ADCs) are a growing component of pharmaceutical companies' drug pipelines. The growth of ADCs in particular is due to their ability to selectivity target and deliver a potent molecule to a cancer cell based on a specific tumor marker. In order to support this growing class of new drug molecules, robust and reliable bioanalytical methods are required. While ligand binding assays (LBAs) like ELISA have been the most popular platform for biotherapeutic

quantitation, bioanalytical scientists have been increasingly adopting hybrid LBA-LCMS methods in this area. The strengths of hybrid LCMS assays for this application include: selectivity, broad LDR, the ability to multiplex a second analyte or catabolite and the quick method development time afforded by a generic method.

For a bioanalytical scientist inexperienced in hybrid LBA LCMS signature peptide quantitation the various workflows can appear complex and difficult. The BioBA sample preparation kits are designed to make this complex process simple by enabling a magnetic bead based approach to immunoaffinity sample preparation and providing all the reagents necessary (buffers, reagents, enzyme and bead) to complete the workflow.

Most ADCs are heterogeneous mixtures of species and an example is ado-trastuzumab emtansine, a lysine linked ADC that is an approved treatment for patients with $Her2^{+}$ breast cancer. Due to the nature of the chemistry involved in lysine conjugation the drug product is a heterogeneous mixture of species with a drug to antibody (DAR) of 0 to 8. Due to the heterogeneous nature of ADCs like ado-trastuzumab emtansine several bioanalytical assays are required during the drug development process. Some of these include: unconjugated payload, conjugated antibody (DAR 1 to 8) and total antibody (DAR 0 to 8). Hybrid LBA LCMS assays can be used to address conjugated and total antibody assays by choosing an appropriate immunocapture reagent. An anti-payload antibody can be used to immunopurify and assay conjugated ADC species. To assay the total antibody a generic anti human Fc antibody can be employed for immunocapture or a target specific immunocapture strategy can be employed with recombinant target protein or an anti-idiotype antibody.

In this application note we demonstrate a total antibody assay of ado-trastuzumab emtansine using hybrid LBA LCMS approach employing the BioBA sample preparation kit and a generic immunocapture strategy.

Experimental

Sample Description: To prepare calibration standards and QC samples 10x spiking solutions of ado-trastuzumab emtansine were first prepared in BioBA bind/wash buffer containing 0.01% BSA, then spiked into rat plasma (Sprague-Dawley, K_2EDTA) at the concentrations listed in Table 1.

Table 1. Concentration of ado-trastuzumab emtansine calibration standard and QC samples prepared in rat plasma.

Name	Concentration (ng/mL)	Name	Concentration (ng/mL)
Std A	10	QC-LL	10
Std B	50	QC-L	30
Std C	100	QC-M	500
Std D	1000	QC-H	75 000
Std E	10 000		
Std F	50 000		
Std G	100 000		

Hybrid LBA LC/MS Sample Preparation: Plasma samples (50 µL) were processed for LCMS analysis using the BioBA sample preparation kit and following the included protocol. An intact heavy labeled human antibody internal standard was used (SILuMab, Sigma-Aldrich) and added to the plasma samples prior to processing. Briefly the steps involved in sample preparation are shown in Table 2.

Table 2. Sample Preparation.

Step 1	BioBA high-capacity bead preparation with anti-human IgG.
Step 2	Samples diluted in buffer containing internal standard (1.0 µg/mL).
Step 3	Samples with internal standard added to prepared and washed beads. Binding time was 1 hr.
Step 4.	Beads washed 3x with BioBA bind/wash buffer.
Step 5.	Elution of analyte and internal standard for 10 minutes at 37 °C.
Step 6.	Neutralization and addition of BioBA digest surfactant.
Step 7.	Reduction for 1 hr at 50 °C.
Step 8.	Alkylation for 30 minutes at room temperature.
Step 9.	Digestion for 3.5 hours at 37 °C.
Step 10.	Acidification with formic acid.
Step 11.	Dilution of acidified sample 2 fold with water.

The signature peptides IYPTNGYTR and DTLMISR were used for quantitation and the heavy labeled signature peptide DTLMIS[R] from SILuMAB was used as the internal standard.

Chromatography: Separation of the signature peptides of the digested samples was performed on a Shimadzu Prominence system. The total runtime for the method was 7 minutes and an injection volume of 5 µL was used for all samples.

Table 3. Gradient profile for signature peptide quantitation.

Column	Phenomenex 2.6 µm, Kinetex C18 Column, (50 x 2.1 mm)	
Mobile Phase A	0.1% formic acid in water (v/v)	
Mobile Phase B	0.1% formic acid in acetonitrile (v/v)	
Flow rate	350 µL/min	
Column temperature	40°C	
Injection volume	5 µL	
Gradient profile	**Time (min)**	**% B**
	0.70	5
	0.80	10
	3.50	25
	5.00	40
	5.10	95
	5.90	95
	6.00	5
	7.00	End

Mass Spectrometry: The signature peptide MRM analysis was performed on a SCIEX QTRAP 6500+® system equipped with an IonDrive™ Turbo V source in positive Electrospray Ionization (ESI) mode. The optimized MRM conditions for the analytes are summarized in Table 4.

Table 4. MRM transitions for signature peptide analysis.

Name	Q1	Q3	DP	CE	CXP
DTLMIS[R] Heavy	423.2	516.3	40	22	17
DTLMISR	418.5	506.2	40	20	18
IYPTNGYTR	542.8	808.4	60	16	11

Data Processing: After acquisition data was imported into MultiQuant™ software for peak integration, calibration and calculation of unknown sample and QC calculations.

Results and Discussion

The signature peptides IYPTNGYTR from the CDR region of trastuzumab and the conserved Fc peptide DTLMISR were chosen for quantitation due to the absence of a lysine residue. Lysine containing signature peptides should be avoided for signature peptide quantitation of lysine linked ADCs because the presence of a payload molecule will result in a miscleavage. If a lysine containing peptide was chosen the heterogeneous nature of lysine conjugation may result in lower calculated concentrations than a signature peptide without lysine.

Peptide IYPTNGYTR contains an asparagine residue adjacent to glycine and is prone to deamidation and rearrangement to aspartic acid and iso-aspartic acid. The deamidation and rearrangement is pH dependent and neat digests of ado-trastuzumab emtansine were used to determine the level of deamidation. Neat digests were performed in ammonium bicarbonate buffer adjusted to pH 8.2 and pH 7.5 and the LC-MS chromatograms of the IYPTNGYTR MRM (542.8/249.2) are shown in Figure 2.

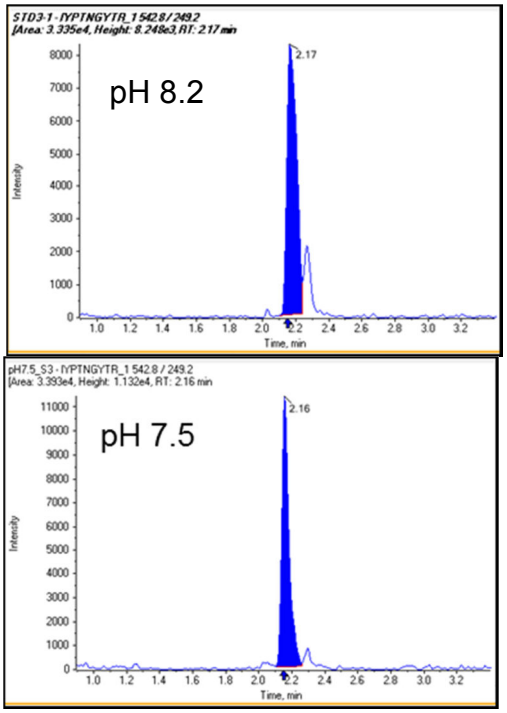

Figure 2. Deamidation and rearrangement of the signature peptide IYPTNGYTR (2.17 mins). The deamidated product iso-aspartic acid (2.3 mins) is reduced by performing the digestion at a lower pH.

Deamidation and rearrangement to iso-aspartic acid was found to be reduced at lower pH. The pH of samples from a scaled up elution from the BioBA beads and neutralization was measured and found to be ~ pH 7.6.

The BioBA sample preparation kit includes an anionic mass spec compatible surfactant to improve digest efficiency and signature peptide yield. The surfactant is acid and heat labile and decomposes during the course of the digestion reaction and sample work-up that follows. Using neat digests of ado-trastuzumab emtansine the signature peptide yield from digests using the BioBA surfactant was compared to other available mass spec compatible surfactants. A neutral mass spec surfactant N-octyl-β-D-glucoside (OGS) was chosen along with another commercially available anionic surfactant that contains a ketal structure and is also acid labile. All surfactants were added prior to reduction, alkylation and digestion and the final concentration of each surfactant in the enzymatic digestion was: 0.025% (w/v) BioBA, 0.63% OGS and 0.063% for the anionic ketal surfactant. The yield of the signature peptides IYPTNGYTR, DTLMISR and the CDR peptide FTISADTSK (485.2-721.3) were followed. A graph of the peak area counts from each reaction along with a control containing no surfactant is shown in Figure 3.

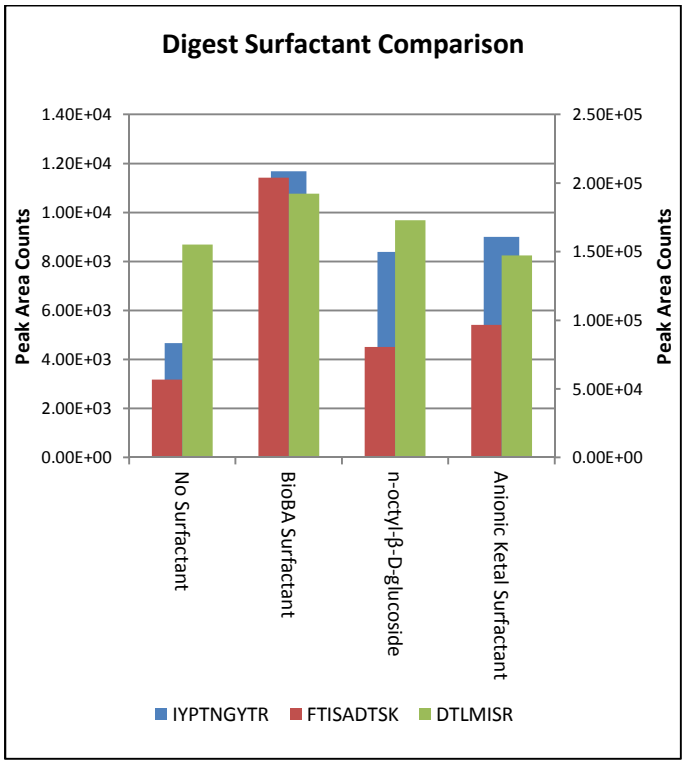

Figure 3. Signature peptide yield from three different mass spec compatible surfactants.

It can be seen from the graph in Figure 3 that the effect of a surfactant is dependent on the peptide. The Fc peptide DTLMISR is not sensitive to the presence or identity of surfactant while the CDR peptides IYPTNGYTR and FTISADTSK gave better yields with all three surfactants compared to the control without surfactant. When comparing the signature peptide yields between the three surfactants the highest yield for each peptide was observed using the BioBA surfactant.

Immunoenrichment from plasma was performed with goat anti-human IgG antibody coated BioBA magnetic beads. This antibody will capture all ADC species including those without payload attached and gives a total antibody measurement. After immunoenrichment from plasma the samples were processed following the BioBA protocol which included: washing, elution of the ADC from the immunocapture beads, neutralization to pH 7.5, addition of BioBA surfactant, reduction, alkylation, and digestion with trypsin/LysC. Samples were prepared for LC injection by acidifying the reaction with formic acid and diluting 2-fold with water.

Adding an immuno-affinity capture step in the sample preparation workflow yields a sensitive and functional assay. Sensitivity, linearity and accuracy and precision data met typical method validation requirements. Standard curves were linear over 4 orders of magnitude from 10 to 100 000 ng/mL. The average accuracy of the IYPTNGYTR QC samples was 96% and the average %CV for the QC samples was 8.5%. For the signature peptide DTLMISR the average accuracy of the QC samples was 104.5% and the average %CV for the QC samples was 8.6%.Table 5 and 6 summarizes the statistics from both the standard curves and QC samples for both the IYPTNGYTR and DTLMISR signature peptides. The response of the DTLMISR heavy labelled internal standard signature peptide released from SILuMAB was consistent across all standards and QCs, the %CV of the peak area was and gave an average peak area was 13.8%. Examples chromatograms of the for the IYPTNGYTR signature peptide from the QC samples are shown in Figure 4.

Conclusions

The BioBA sample preparation kit and the SCIEX QTRAP® 6500+ System were successfully employed to perform a hybrid immunoaffinity LC/MS assay of the ADC ado-trastuzumab emtansine. Using a generic anti-human F_C immunocapture and digestion accurate and precise limits of quantitation of 10 to 100 000 ng/mL were achieved from 50 µL of plasma sample.

A hybrid immunoaffinity LC/MS assay allows users to achieve higher sensitivity than direct plasma digestion methods. The kit-based approach with easy to follow protocol allows bioanalytical scientists to achieve rapid and reproducible results in drug development studies.

Table 5. The accuracy and precision statistics for the signature peptide IYPTNGYTR from the total antibody assay of ado-trastuzumab emtansine.

Row	Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Accuracy
1	IYPTNGYTR_2	10.00	3 of 3	10.180	0.254	2.50	101.80
2	IYPTNGYTR_2	50.00	3 of 3	46.470	4.549	9.79	92.94
3	IYPTNGYTR_2	100.00	3 of 3	95.914	3.640	3.80	95.91
4	IYPTNGYTR_2	1000.00	3 of 3	1008.177	31.410	3.12	100.82
5	IYPTNGYTR_2	10000.00	3 of 3	11294.023	226.072	2.00	112.94
6	IYPTNGYTR_2	100000.00	3 of 3	95588.543	729.261	0.76	95.59

Row	Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Accuracy
1	IYPTNGYTR_2	10.00	6 of 6	8.286	1.202	14.51	82.86
2	IYPTNGYTR_2	30.00	6 of 6	27.762	3.609	13.00	92.54
3	IYPTNGYTR_2	500.00	6 of 6	516.598	13.794	2.67	103.32
4	IYPTNGYTR_2	75000.00	6 of 6	79364.180	3133.539	3.95	105.82

Table 6. The accuracy and precision statistics for the signature peptide DTLMISR from the total antibody assay of ado-trastuzumab emtansine.

Row	Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Accuracy
1	DTLMISR +2_1	10.00	3 of 3	10.169	2.164	21.29	101.69
2	DTLMISR +2_1	50.00	3 of 3	44.806	5.181	11.56	89.61
3	DTLMISR +2_1	100.00	3 of 3	103.073	8.517	8.26	103.07
4	DTLMISR +2_1	1000.00	3 of 3	1074.473	24.078	2.24	107.45
5	DTLMISR +2_1	10000.00	3 of 3	11210.686	176.501	1.57	112.11
6	DTLMISR +2_1	100000.00	3 of 3	86076.102	1801.306	2.09	86.08

Row	Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Accuracy
1	DTLMISR +2_1	10.00	6 of 6	10.388	1.703	16.39	103.88
2	DTLMISR +2_1	30.00	6 of 6	29.169	3.005	10.30	97.23
3	DTLMISR +2_1	500.00	6 of 6	586.341	11.333	1.93	117.27
4	DTLMISR +2_1	75000.00	6 of 6	75892.469	4491.733	5.92	101.19

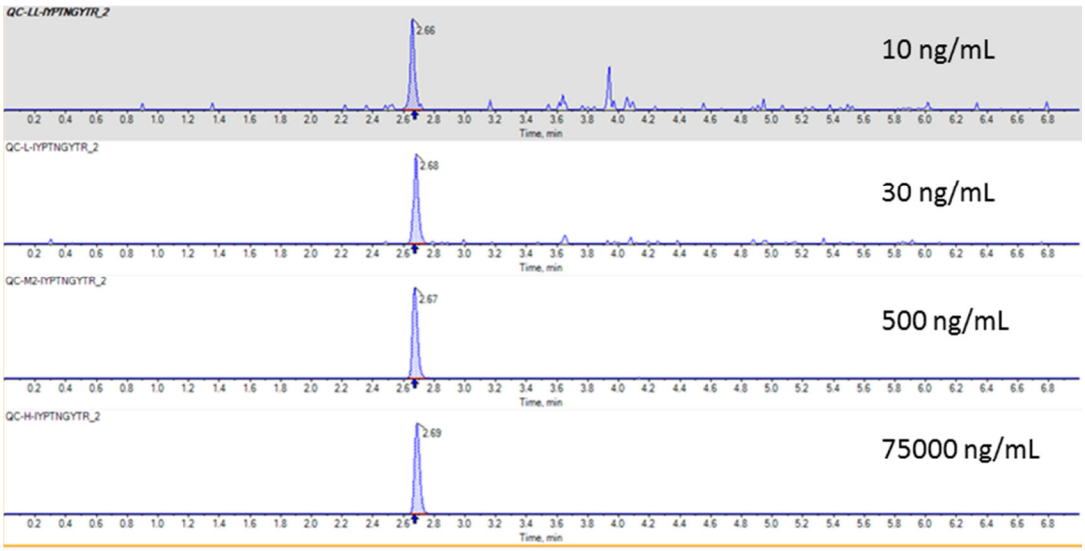

Figure 4. Example QC chromatograms of ado-trastuzumab emtansine for the IYPTNGYTR signature peptide (542.8/808.4).

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