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Total Antibody Quantification of the Antibody Drug Conjugate, Ado-Trastuzumab Emtansine in Rat Plasma Using the BioBA Solution

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INTRODUCTION

Due to their heterogeneous nature, ADCs require multiple bioanalytical assays to quantify the various forms. One important assay is the total antibody measurement. The total antibody measurement is the sum of all the conjugated antibody species plus the unconjugated antibody. Different protein sample preparation techniques can be combined with LC-MS/MS analysis to make a total antibody measurement. By combining immunoenrichment sample preparation with LC-MS/MS detection more selective assays with lower LLOQs are possible over a direct plasma or pellet digest. The purpose of this study was to develop an immunoaffinity LC-MS/MS method for the selective quantitation of the ADC, ado-trastuzumab emtansine using the BioBA sample prep kit.

MATERIALS AND METHODS

Sample Preparation:

Ado-trastuzumab emtansine spiking solutions (10x) were serially diluted from a neat stock solution (20 mg/mL) and used to spike rat plasma (K_2EDTA). Immunocapture beads were prepared with goat anti-human IgG (Southern Biotech) following the BioBA kit procedure. Each calibration standard and QC sample was processed with a 25 µL aliquot of prepared beads. A 50 µL aliquot of plasma was diluted 2-fold with internal standard solution (SILuMAB, Sigma-Aldrich) and added to the beads. Samples were incubated with beads for 1 hr with mixing. Samples were eluted from the beads for 10 minutes at 35 ˚ C, then neutralized, reduced, alkylated and digested using the components of the BioBA kit following the example protocol. The reaction times and temperature were as follows: reduction for 1 hour at 50 ˚ C, alkylation for 30 minutes at room temperature and digestion at 37 ˚ C for 3.5 hours.

HPLC Conditions:

System	Shimadzu LC-20
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
Run Time	7 minutes
Rinsing Solution	60:20:20 IPA:Methanol:Acetonitrile

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

System	SCIEX M3 MicroLC System
Column	Eksigent, HALO Peptide-ES, C18, 0.3 x 50 mm, 2.7 µm
Mobile Phase A	0.1% formic acid in water (v/v)
Mobile Phase B	0.1% formic acid in acetonitrile (v/v)
Flow rate	8 µL/min
Column temperature	40 °C
Injection volume	5 µL
Run Time	7 minutes
Rinsing Solution	60:20:20 IPA:Methanol:Acetonitrile

Time (min)	% B
3.5	25
5.0	40
5.1	95
5.9	95
6.0	5
7.0	End

MS/MS Conditions:

The MRM analysis was performed on a SCIEX QTRAP 6500+® system equipped with an IonDrive™ Turbo V source. The source parameters plus probe and electrode positions were optimized prior to the analytical run.

Analyte	Q1	Q3	DP (V)	CE (V)	CXP (V)
DTLMIS[R]_Heavy	423.2	516.3	40	22	17
DTLMISR	418.5	506.2	40	20	18
FTISADTSK	485.2	721.3	90	20	15
IYPTNGYTR	542.8	808.4	60	16	11

RESULTS

Signature Peptide Selection and Digest Optimization

The signature peptides IYPTNGYTR from the CDR region of trastuzumab and the conserved peptide DTLMISR were chosen for quantitation due to the absence of a lysine residue. Due to the heterogeneous nature of lysine conjugation signature peptides containing a lysine residue could be occupied with the payload and will not be cleaved by trypsin.

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 trastuzumab emtansine were tested at pH 7.5 and pH 8.2 for deamidation. Deamidation was reduced at the lower pH.

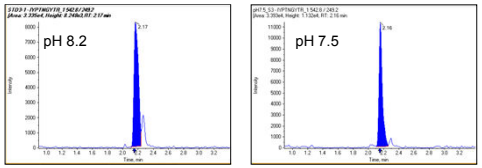


Figure 1. Deamidation and rearrangement of the signature peptide IYPTNGYTR. Performing the digestion at a lower pH reduced the amount of deamidation.

Digest Surfactant Comparison

The BioBA sample preparation kit includes an anionic mass spec compatible surfactant to improve digest efficiency and signature peptide yield. The signature peptide yield from digests using the BioBA surfactant (0.02%) was compared to a neutral mass spec compatible surfactant OGS (0.6%) and a second commercially available anionic surfactant (0.06%).

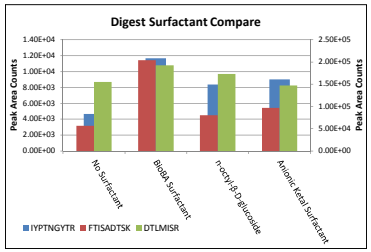


Figure 2. Signature peptide yield from three different mass spec compatible surfactants.

Immunocapture

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.

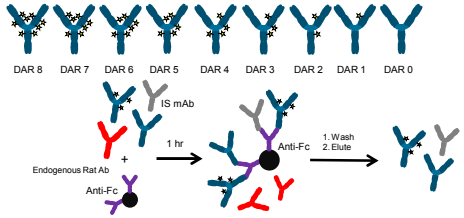
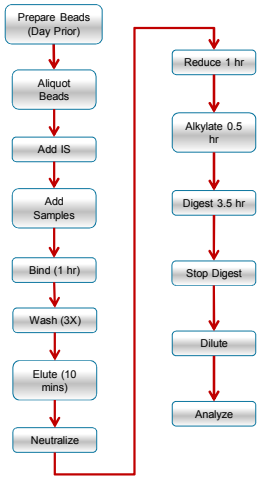


Figure 3. Total immunocapture strategy to capture all ADC species including those without payload.

After immunoenrichment from plasma the samples were processed following the BioBA protocol which included: washing, elution (pH ~2.2), neutralization, reduction (TCEP), alkylation (IAM), and digestion with trypsin/LysC and surfactant. Samples were prepared for LC injection by stopping the digestion with formic acid and diluting 2-fold with water. Figure 4 shows the steps in the BioBA sample processing workflow and the calibration standard and QC sample concentrations analyzed.

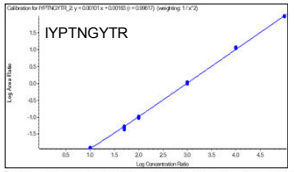


Name	Concentration (ng/mL)
Std A	10
Std B	50
Std C	100
Std D	1000
Std E	10 000
Std F	50 000
Std G	100 000

Name	Concentration (ng/mL)
QC-LL	10
QC-L	30
QC-M1	500
QC-H	75 000

Figure 4. The steps of the BioBA sample processing workflow and the calibration standard and QC sample concentrations analyzed in this study.

After dilution with water the samples were analyzed on the SCIEX QTRAP 6500+® system . The results of the accuracy and precision batch are shown below in figures 5A,B for signature peptides IYPTNGYTR and DTLMISR. The heavy labeled internal standard peptide DTLMIS[R]_Heavy was used as an internal standard for both.

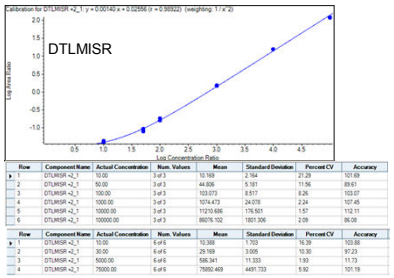


SCIEX QTRAP 6500+® system

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

Figure 5A. The calibration curves and 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	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	5.101	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	11210.606	176.921	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	10.366	1.703	16.39	103.66
2	DTLMISR_2_1	30.00	6 of 6	29.169	3.005	10.30	97.23
3	DTLMISR_2_1	5000.00	6 of 6	586.341	11.333	1.93	11.73
4	DTLMISR_2_1	75000.00	6 of 6	76892.469	4491.733	5.92	101.19

Figure 5B. The calibration curves and accuracy and precision statistics for the signature peptide DTLMISR from the total antibody assay of ado-trastuzumab emtansine.

Analysis Using Microflow

The samples were tested using a microflow method and the SCIEX M3 MicroLC System. Analysis using microflow showed a gain in sensitivity over standard LC flow (~4.5). Samples from high flow analysis were diluted 3-fold further and analyzed.

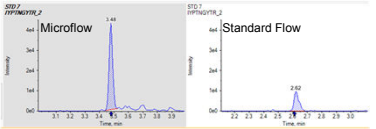
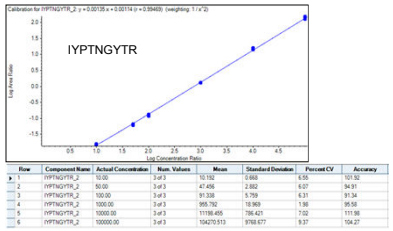


Figure 6. The comparison in peak height between a 50 ng/mL samples analyzed by standard flow and microflow.



Row	Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Accuracy
1	IYPTNGYTR_2	10.00	3 of 3	10.162	0.660	6.50	101.62
2	IYPTNGYTR_2	50.00	3 of 3	47.456	2.882	6.07	94.91
3	IYPTNGYTR_2	100.00	3 of 3	91.338	5.759	6.31	91.34
4	IYPTNGYTR_2	1000.00	3 of 3	955.792	18.969	1.98	95.58
5	IYPTNGYTR_2	10000.00	3 of 3	11198.455	786.421	7.02	111.98
6	IYPTNGYTR_2	100000.00	3 of 3	104270.513	9758.677	9.37	104.27

Figure 7. The calibration curves and accuracy and precision statistics for the signature peptide IYPTNGYTR from the total antibody assay of ado-trastuzumab emtansine analyzed using microflow LC conditions.

CONCLUSIONS

1. **Robust bioanalysis of ADCs**
   Achieved accurate quantitation of ado-trastuzumab emtansine.
   BioBA sample prep kits provide the necessary reagents and protocols for enrichment and detection of a wide range of large molecules.

2. **Gold Standard & Proven LC-MS performance**
   Integrated LC/MS capabilities for both standard flow and microflow.
   M3 MicroLC System offers sensitivity increase.
   QTRAP® 6500+ System offers optimal sensitivity and dynamic range and for complex peptide quantitation utilize the MRM^3 or SelexION+ Ion Mobility options

TRADEMARKS/LICENSING

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