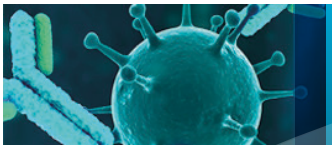

Sensitive and Accurate Quantitation of the Antibody-Drug Conjugate Ado-Trastuzumab Emtansine in Rat Plasma

High-Sensitivity Bioanalysis of ADC using BioBA with M3 MicroLC and QTRAP® 6500+ LC-MS/MS system

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Overview

Who Should Read This: Senior Scientists, Lab Directors

Focus: Improved quantitation of total antibody-drug conjugates (ADCs) using BioBA solution and microflow liquid chromatography-mass spectrometry (LC-MS/MS).

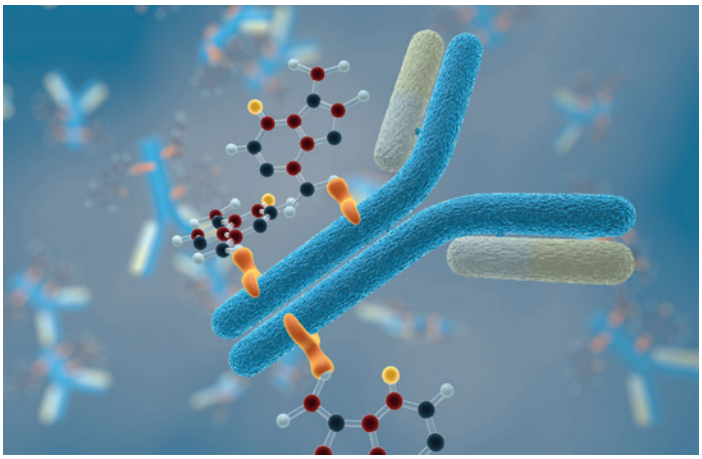
Goal: Develop an accurate and sensitive trap-and-elute microflow LC-MS/MS method to improve sensitivity and linear dynamic range of the BioBA High Capacity Sample Enrichment method for quantitation of antibody-drug conjugates in complex matrices.

Problem: Traditional LC-MS/MS methods for the quantitation of antibody-drug conjugates and similar biotherapeutics often deliver insufficient sensitivity. While we have seen improvement in sensitivity for the antibody-drug conjugate ado-trastuzumab emtansine quantitation in rat plasma using direct injection microflow LC-MS/MS,[1] this approach results in long sample loading times, and MS contamination as a divert valve could not be used.

Results: The SCIEX M3 MicroLC with a QTRAP 6500+ mass spectrometer provides up to 5X lower limit of quantitation for the antibody-drug conjugate ado-trastuzumab emtansine in rat plasma. Quantitation over 5 orders of magnitude linear dynamic range was achieved with r value of 0.995.

Key Challenges:

- Ligand binding assays (LBAs) like ELISA have been utilized in the past for the quantitation of biotherapeutics such as monocolonal antibodies (mAbs) and ADCs. These methods have limited linear dynamic range, cross reactivity and poor reproducibility
- Quantitation of mAbs over a wide dynamic range at low nanogram levels often requires greater sensitivity than can be achieved with LC-MS/MS with traditional LC flow rates
- Scientists who are familiar with quantitation of traditional small molecule therapeutics and new to analysis of these large and heterogeneous proteins conjugates require a robust and sensitive work flow which is easy to implement and reproducible in different laboratories



Key Features:

- The SCIEX BioBA Kit includes a generic method and all the reagents necessary for immunocapture and digestion to quantitate ADCs in plasma
- Microflow LC-MS/MS allows quantitation over a wide linear dynamic range of 5 orders of magnitude (1ng/ml-100ug/ml)
- Up to 4X improvement in s/n ratio enables quantitation at 5x lower concentration than can be achieved with traditional flow LC-MS/MS
- Robust on-line trap-and-elute sample loading and desalting increases column lifetime and reduces solvent consumption and costs

Fast-Growing Field: Protein biotherapeutics like immunoglobulin G (IgG)-derived monoclonal antibodies and antibody-drug conjugates occupy a rapidly increasing share of the pharmaceutical industry due to their lower toxicity, higher potency and target specificity. Ado-trastuzumab emtansine (Kadcyla) is the first HER2-targeted treatment for metastatic breast cancer. It is made of two cancer-fighting drugs, a monoclonal antibody trastuzumab (Herceptin) which targets HER2 and a lysine conjugated chemotherapy drug. It is made to bring chemotherapy inside HER2-positive cancer cells and kill them with less harm to normal cells.

Due to the heterogeneous nature of lysine conjugated adotrastuzumab emtansine, several bioanalytical assays,

including ligand binding assays (LBAs), are required during the drug development process. Ligand binding assays have limited dynamic range, which makes hybrid LBA LC-MS/MS assays a more attractive choice for quantitation of the total antibody. These hybrid assays are based on quantitation of signature peptides which provide a wide linear dynamic range.

The BioBA High Capacity Sample Enrichment Kit provides a hybrid LBA LC-MS/MS workflow with a sample preparation protocol, and a generic method for pharmacokinetic studies of biotherapeutics during pre-clinical or phase I-IV studies. The BioBA workflow (Figure 1) includes the enrichment of mAbs from plasma using magnetic streptavidin beads conjugated with an anti-Human IgG for enrichment and quantitation of the total antibody with a generic or specific signature peptide. A target specific immunocapture strategy can also be employed with a recombinant target protein or an anti-idiotype antibody.

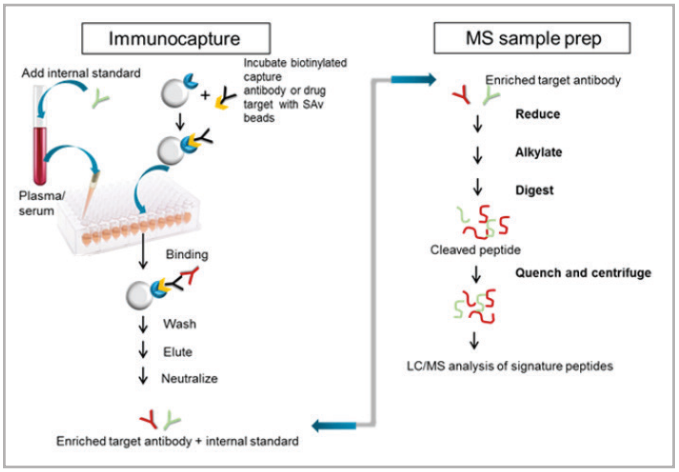


Figure 1. BioBA Immuno-Enrichment and Sample Processing Workflow. BioBA workflow includes immuno-enrichment of mAbs and ADCs, reduction, alkylation, digestion and a generic traditional flow LC-MS/MS method for the quantitation of the mAbs and ADCs in plasma.

BioBA magnetic beads offer several advantages including: ease of handling, scalability, improved sample recovery, parallel processing of samples using a variety of magnetic stands and use in high-throughput formats with robotics.[3] Following enrichment, the proteins are eluted, reduced, alkylated, and digested before detection using MRM based LC-MS/MS analysis of generic or signature peptides of mAbs of interest. The trypsin/lys-C enzyme mixture allows for the most efficient digestion. Digestion can be completed within three and half hours, enabling the workflow to be completed in a single day.[1]

In this application note we describe a robust and sensitive workflow for total antibody quantitation of ado-trastuzumab emtansine in rat plasma using a hybrid LBA LC-MS/MS approach. The BioBA sample preparation kit and BioBA generic immunocapture strategy is employed, and then followed by analysis of signature peptides on a M3 MicroLC coupled to a QTRAP 6500 mass spectrometer. This method takes advantage of a trap-and-elute strategy for fast sample injection and desalting to increase throughput of lower flow rate microflow LC analysis.

Experimental Design

Sample Preparation: 10x spiking solutions of ado-trastuzumab emtansine were first prepared in 1X BioBA bind/wash buffer containing 0.01% BSA (bovine serum albumin), then spiked into Sprague-Dawley rat plasma, K2EDTA (BioreclamationIVT) at the final concentrations of 0.5-100000 ng/ml. SILuMab (Sigma-Aldrich), was used as internal standard (IS) and was added to the plasma samples prior to BioBA immunocapture processing.

A blank and double blank sample was also prepared. The double blank only had 1X BioBA bind/wash buffer containing 0.01% BSA and the blank sample had rat plasma with additional internal standard. Spiked plasma samples (50 µL) were mixed with internal standard and processed based on the BioBA generic method[2] with some modified steps to reduce background signal and improve signal to noise (s/n) ratio in order to utilize the advantage of higher sensitivity provided by microflow LC. The modified steps include; an extra hour of incubation of conjugated beads with sample (2 hrs vs 1 hr in generic method) followed with two wash steps with 500 µL of BioBA bind/wash buffer and a 500 µL of 50 mM ammonium bicarbonate instead of the two wash steps with 200 µL of BioBA bind/wash buffer in generic method. Also a 1:10 dilution of stock BioBA digestion buffer (500 mM ammonium Bicarbonate) was used for preparing iodoacetamide, Trypsin/Lys-C and a mass spec compatible surfactant was used, with 1ug total Trypsin/Lys-C for each sample digestion.

Traditional Flow LC		Microflow LC	
Time (min)	%B	Time (min)	%B
0	5	0	3
0.7	5	0.7	5
0.8	10	0.8	10
3.5	25	3.5	25
5	40	5	40
5.1	95	5.1	95
5.9	95	10.0	95
6	3	10.1	3
7	3	15	3

Table1. Gradients used for Traditional and Microflow LC-MS/MS.

Traditional Flow Liquid Chromatography: A Shimadzu Prominence HPLC system with two LC-20AD pumps, CTO-20A column oven and a SIL-20AC autosampler was used. The column was a 100 x 2.1 mm Kinetex C18 2.6 µm 100 Å column (Phenomenex). Mobile phase A, water with 0.1% formic acid, and mobile phase B, acetonitrile with 0.1% formic acid was used at a flow rate of 0.5 ml/min. Wash solvent for the autosampler was 20/20/60 methanol/acetonitrile/IPA. Injection volume was 25 µL, and the column was kept at 40° C.The gradient method used is listed in Table 1.

Microflow Liquid Chromatography: A SCIEX M3 MicroLC-TE system, with two microLC gradient pumps and an integrated autosampler was used in combination with a source mounted column oven (SCIEX). A 10 x 0.3 mm trap column packed with 5 µm 120 Å ChromXP C18 CL and an analytical column 50 x 0.3 mm HALO Peptide ES-C18 2.7 µm 160 Å column was used (SCIEX). Mobile phase A in the analytical gradient was water with 0.1% formic acid, mobile phase B was acetonitrile with 0.1% formic acid (Table 1) with flowrate of 10 µL/min. The column temperature was set to 40° C. Injection volume was 25 µL, and the autosampler needle and valve wash consisted of two cycles using mobile phase B, followed by one cycle using mobile phase A. For trapping conditions, mobile phase A in the loading gradient was water with 0.1% formic acid, mobile phase B was acetonitrile with 0.1% formic acid. Sample was loaded from the injection loop onto the trap column using 100% A for two and half minute at 50 µL/min flow rate. The trap was then washed with 95% B followed by 100% A each at 50 µL/min for 5 minutes after every injection (Table 2).

Mass Spectrometry and Data Processing: A SCIEX 6500 QTRAP with IonDrive™ Turbo V source was used. For the microflow LC experiments, the standard electrode was replaced with a 25 µm ID electrode (SCIEX). The transitions and MS parameters were optimized using DiscoveryQuant software (SCIEX) and kept constant for both the traditional flow and microflow LC experiments. The source and gas parameters are listed in table 3. The MS parameters are listed in table 4, and MultiQuant™ 3.0.2 software (SCIEX) was used for data analysis. Sample for both microflow and traditional flow LC-MS/MS analysis was prepared on the same day to exclude variations in response due to sample preparation. Three replicate LC-MS/MS injections were acquired for both the traditional flow and trap-and-elute microflow LC analysis.

Time (min)	%B
0	0
1	0
2.5	0
2.6	95
8	95
8.1	0
15	0

Table2. Gradient used for the Trap Wash Workflow.

MS Parameters	Traditional Flow LC	Microflow LC
Electrode ID	100 µm	25 µm
Curtain Gas	30	20
Collision GAS	High	High
IonSpray Voltage	5500	5000
Temperature	600	250
Ion Source Gas 1	50	20
Ion Source Gas 2	65	20

Table3. Source Parameters

Results and Discussion

Improved Sensitivity: The calibration curve of Ado- trastuzumab Emtansine standards in rat plasma matrix (0.5-100,000 ng/ml) was generated using MultiQuant Software (Table 5, 6) for data acquired by traditional and microflow LC using signature tryptic peptides IYPTNGYTR and FTISADTSK. Figure 2 shows the calibration curve for signature peptide IYPTNGYTR using Microflow LC. Figure 3 shows the extracted ion chromatograms (XIC's) of the signature peptide (FTISADTSK) used for quantitation of trastuzumab in both methods at the 5 ng/ml and 10 µg/ml level. S/N ratio was improved by 4 fold for both peptides using microflow LC. The LLOQ's for both methods were determined using the requirements of precision < 20% and accuracy between 80 and 120% at LLOQ, and at any higher concentration a precision <15% and accuracy between 85% and 115%. LLOQ improved by a factor of 5 using the microflow LC trap-and-elute method using both signature peptides and the generic peptide DTLMISR. For the traditional LC method the limit of quantification (LOQ) was 5 ng/ml where the LOQ of 1 ng/ml was achieved by microflow LC. Both the traditional flow and microflow LC methods showed good linearity with r >0.99.

Transitions	Q1 Mass (Da)	Q3 Mass (Da)	Time (msec)	Signature Peptide Sequences	DP (volts)	CE (volts)	CXP (volts)
1	**423.2**	629.4	20	**S-DTLMIS[R].heavy 1**	40	24	18
2	423.2	516.3	20	S-DTLMIS[R].heavy 2	60	22	17
3	**418.2**	619.3	20	**G-DTLMISR.1**	60	22	15
4	418.2	506.2	20	G-DTLMISR.2	40	20	18
5	542.8	405.8	20	S-IYPTNGYTR.1	120	23	10
6	**542.8**	808.2	20	**S-IYPTNGYTR.2**	60	16	11
7	485.2	608.2	20	S-FTISADTSK.1	50	25	25
8	**485.2**	721.3	20	**S-FTISADTSK.2**	90	20	15

Table 4. Transitions and MS Parameters for Signature Peptides of Ado-Trastuzumab Emtansine and Internal Standard. Peptide transitions in bold were used for quantification and the 2nd peptide transitions were used for confirmation with S-DTLMIS[R].heavy 1 transition used for internal calibration.

	Microflow LC			Traditional Flow LC		
Actual Concentration	Mean Measured Concentration (ng/ml)	Accuracy (%)	CV (%)	Mean Measured Concentration	Accuracy (%)	CV (%)
1	1.00	100.42	3.24	-	-	-
5	4.87	96.92	7.61	4.63	92.63	7.48
10	10.43	104.29	9.37	11.50	115.00	7.30
50	46.94	93.89	6.35	49.65	99.29	8.05
100	89.38	89.38	2.41	99.52	99.52	1.62
1000	921.6	92.16	4.38	921.6	92.16	0.71
10000	10930	109.27	0.56	10120	101.24	4.00
25000	27720	110.86	3.04	26270	105.06	2.04
50000	54780	109.57	3.04	52720	105.44	1.41
100000	93250	93.25	4.35	89650	89.65	2.93

Table 5. Quantitation Curve for Standard Ado-Trastuzumab Emtansine using Traditional Flow and Microflow LC-MS/MS. MultiQuant analysis based on peptide IYPTNGYTR resulted in an accurate quantitation with single digit CV% and r value of 0.995. Micro LC-MS/MS provides 5X increased in sensitivity with wider linear dynamic range as compared to Traditional LC-MS/MS.

	Microflow LC			Traditional Flow LC		
Actual Concentration (ng/ml)	Mean Measured Concentration (ng/ml)	Accuracy (%)	CV (%)	Mean Measured Concentration (ng/ml)	Accuracy (%)	CV (%)
1	0.9694	96.94	8.04	-	-	-
2	2.109	105.43	6.80	-	-	-
2.5	2.552	102.10	4.65	-	-	-
5	4.73	94.71	8.04	4.15	83.12	19.83
10	10.44	104.35	3.27	10.44	104.42	15.65
50	49.91	99.82	2.14	52.25	104.49	2.23
100	112.6	112.59	6.81	122.4	122.41	4.11
1000	1052	105.18	1.18	1017	101.67	0.91
10000	10150	101.49	3.68	10430	104.25	4.00
25000	23040	92.15	3.59	26230	104.93	3.63
50000	42620	85.24	3.94	48300	96.60	2.68

Table 6. Quantitation Curve for Standard Ado-Trastuzumab Emtansine using Traditional Flow and Microflow LC-MS/MS. MultiQuant analysis based on peptide FTISADTSK resulted in an accurate quantitation with single digit CV% for micro LC data and CV of 20% at LLOQ for Traditional LC data with r value of 0.995 and 0.998 for Microflow and traditional flow LC. Micro LC-MS/MS provides 5X increased in sensitivity with wider linear dynamic range as compared to Traditional LC-MS/MS.

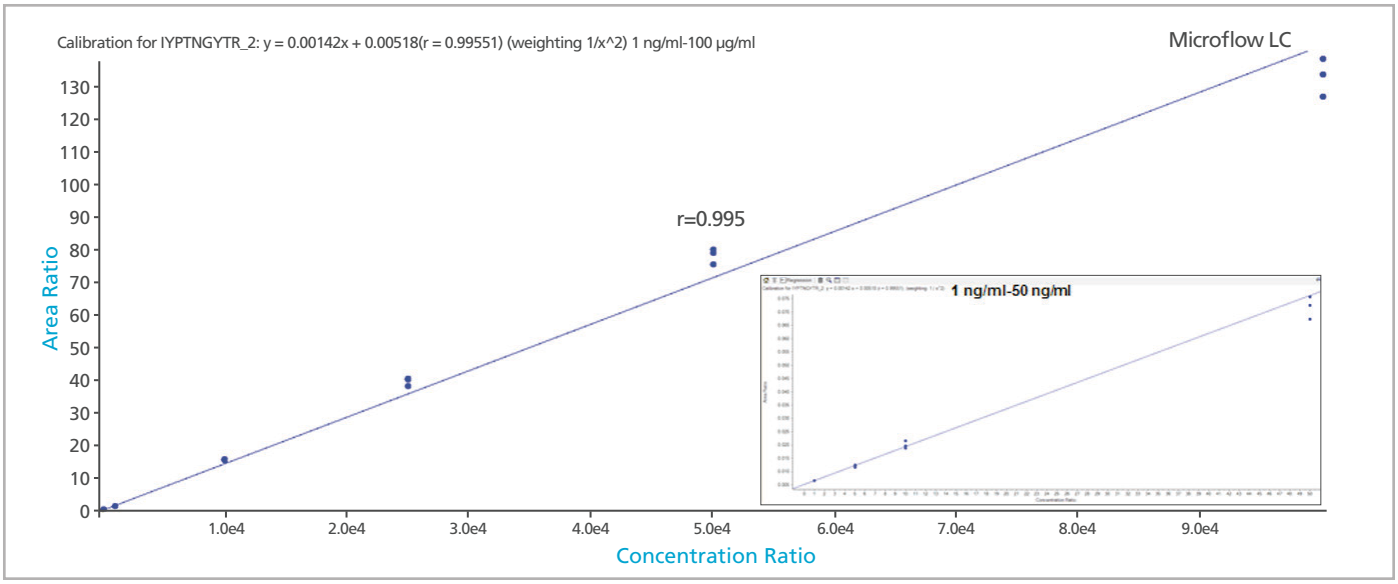


Figure 2. Quantitation Curve for Standard Ado-Trastuzumab Emtansine using Signature Peptide IYPTNGYTR. MultiQuant quantitation curve using peptide IYPTNGYTR resulted in an accurate quantitation with single digit CV% and r value of 0.995 using peak area and 1/x^2 weighting.

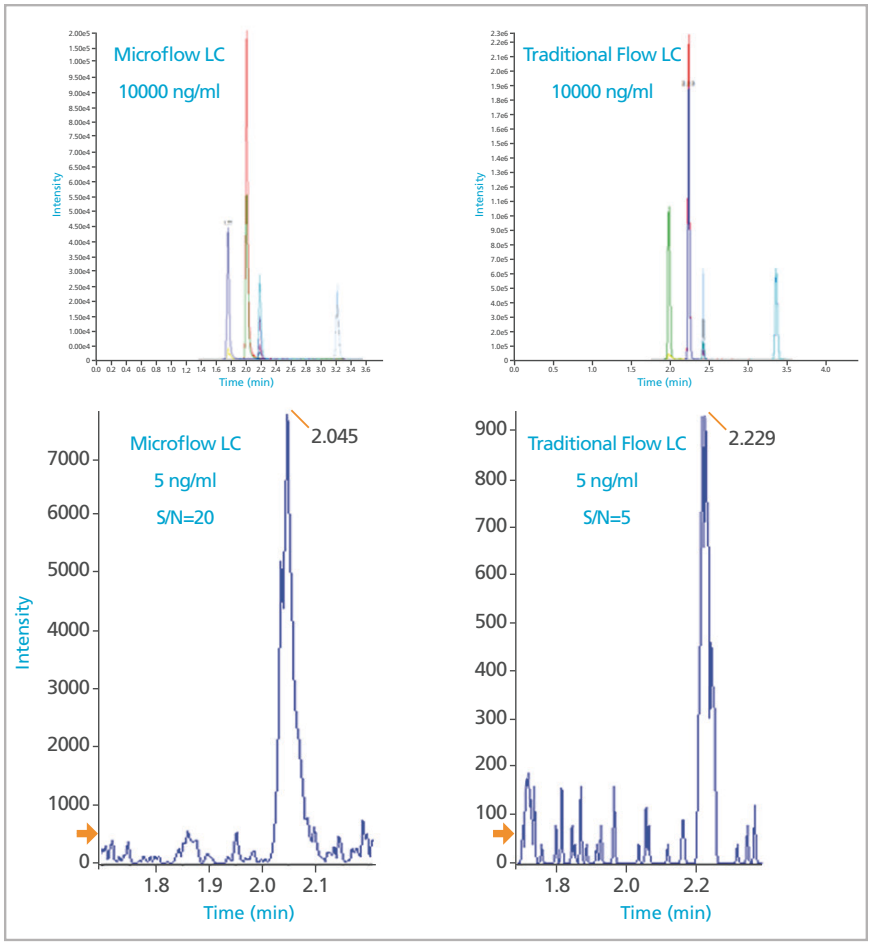


Figure 3. Signal Intensity Improvement using M3 MicroLC. A 4 fold improvement in s/n ratio was achieved using micro flow LC. XIC data for micro flow and traditional flow LC for 10 µg/ml (Top) and 5ng/ml (Bottom) is shown here with improved s/n.

Column Carryover: Carryover was determined by injecting the digest of the immunocaptured extract from a blank+IS plasma sample after an injection of the ULOQ of 100,000 ng/ml using traditional flow LC-MS/MS, and The trap-and-elute microflow LC method. Observed carryover was 0.0075 and 0.007 respectively. The carryover was 1/3 of the response at the LLOQ (1 ng/ml) which is slightly higher than required 20%. Additional washing of trap and column is required to reduce the carryover if wider dynamic range is required.

Conclusion

Up to 5x lower LLOQ was achieved using a trap-and-elute microflow LC-MS/MS method at 10 µL/min, compared to using a direct inject traditional flow LC-MS/MS method at 500 µL/min for the quantitation of ado-trastuzumab emtansine using the signature tryptic peptides IYPTNGYTR and FTISADTSK in samples prepared using BioBA magnetic bead based immunocapture.

The trap-and-elute method ensures similar throughput while injecting the same 25 µL of extracted sample, while protecting the column and MS from contamination. Similar results were observed for infliximab using M3 MicroLC with SCIEX 6500 QTRAP.[4,5]

Optimized BioBA sample preparation protocol in combination with microflow LC provides wider linear dynamic range of 4.5 for traditional flow and linear dynamic range of 5 for microflow as compared to the data obtained from generic method optimized for traditional LC using IYPTNGYTR peptide transitions.[1]

This workflow offers a solution for applications where mAb's need to be quantified in low concentrations and/or when sample volumes are limited.

References

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