Application Note

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Ultra-Sensitive Intact Monoclonal Antibody Quantification Using AssayMAP Bravo Liquid Handling Platform and 6545XT AdvanceBio LC/Q-TOF Mass Spectrometer

Authors

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Abstract

Traditionally, LC/MS for large molecule drug analysis required an enzymatic digestion to cleave larger proteins into smaller peptides more amenable to mass analysis. The disadvantages to this approach are many and include low throughput, incomplete enzymatic reaction, and surrogate peptide bias due to modifications in the molecules. Modern mass spectrometers have allowed researchers to analyze large molecules at their intact level. We demonstrate an automated LC/MS workflow to quantify intact mAb drugs in mouse plasma. The workflow uses an Agilent AssayMAP Bravo automated liquid handling platform and an Agilent 6545XT AdvanceBio LC/Q-TOF system. Our results show that this automated LC/MS assay can be used for quantitative analysis of biologic therapeutics with excellent sensitivity and reproducibility.

Introduction

In recent years, pharmaceutical research and development companies have shifted their focus from small molecule drugs to biologic therapeutics. These therapeutics include antibody-drug conjugates (ADCs), recombinant fusion proteins, monoclonal antibodies (mAbs), oligonucleotide drugs, etc. The traditional analytical method for quantification of these large molecules is the ligand binding assay (LBA) because LBA is sensitive, high-throughput, low-cost, and easily automated. In the past two decades, liquid chromatography mass spectrometry (LC/MS) has become an alternative method to analyze these large molecules because of its high specificity, sensitivity, wide dynamic range and fast method development.[1] At the same time, LC/MS can avoid cross-reactivity, improves productivity, and can reduce costs and delays related to reagent/antigen availability.

Traditionally, LC/MS for large molecule drug analysis required an enzymatic digestion to cleave larger proteins into smaller peptides more amenable to mass analysis. These peptides were used as surrogates to quantitate these large molecule drugs. This approach was very sensitive, highly specific, and had no special reagent requirement. However, there were disadvantages to this approach: low throughput due to enzymatic reaction, potential incomplete enzymatic reaction, and surrogate peptides bias due to modifications in other parts of the large molecules.

In the past ten years, with the advancement of modern mass spectrometers, more researchers are exploring the detection of large molecules at their intact level to address the disadvantages of the surrogate peptides approach.[2,3] We demonstrate an automated LC/MS workflow (Figure 1) to quantify intact mAb drugs in mouse plasma using an AssayMAP Bravo automated liquid handling platform and a 6545XT AdvanceBio LC/Q-TOF system. Our results show that this automated LC/MS assay can be used for quantitative analysis of biologic therapeutics with excellent sensitivity and reproducibility.

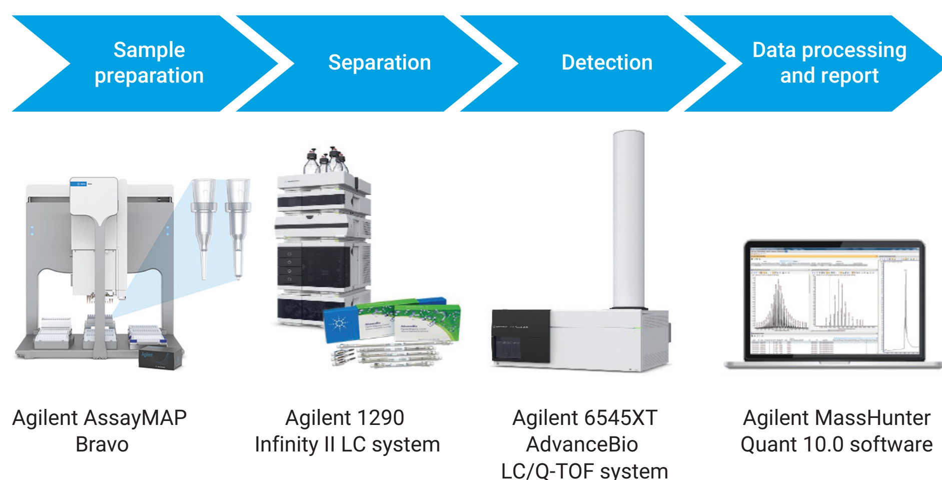

Figure 1. Agilent intact protein quantitation workflow configuration.

Experimental

Materials and methods

Formulated Herceptin (trastuzumab) and Rituxan (rituximab) were from Genentech (South San Francisco, CA). Formic acid (FA), bovine serum albumin (BSA), PBS buffer, and biotinylated anti-human Fc antibody were purchased from Sigma-Aldrich (St. Louis, MO). 96-well LoBind plates were purchased from Eppendorf USA (Hauppauge, NY) and Streptavidin cartridges were from Agilent Technologies (Santa Clara, CA).

Instrumentation

- Agilent AssayMAP Bravo system (G5571AA)
- Agilent 1290 Infinity II LC system including:
  - Agilent 1290 Infinity II high speed pump (G7120A)
  - Agilent 1290 Infinity II multisampler (G7167B)
  - Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 6545XT AdvanceBio LC/Q-TOF system

Software

- Agilent MassHunter Acquisition 9.0 software
- Agilent MassHunter Quant 10.0 software

Sample preparation

All sample preparation steps were performed using the AssayMAP Bravo automation platform. First, 2.5 µg of biotinylated anti-human Fc antibody was immobilized onto Streptavidin cartridges at 3 µL/min. The cartridges were then washed twice with 100 µL PBS buffer at 20 µL/min. Then, 50 µL of plasma aliquots fortified with different concentrations of mAb were loaded onto each cartridge at 3 µL/min. The cartridges were washed twice again with 150 µL PBS buffer at 20 µL/min. The final elution step was carried out by loading 25 µL of 0.1% trifluoroacetic acid to the cartridge at 2 µL/min. Then, 5 µL of 50 mM ammonium bicarbonate buffer was added to the final elution and 15 µL was injected into the LC/MS for intact protein analysis.

LC/MS analysis

Data acquisition was performed using an Agilent 1290 Infinity II LC coupled to a 6545XT AdvanceBio LC/Q-TOF system with dual Agilent Jet Stream source. Sample chromatographic separation was obtained with an Agilent PLRP-S column (2.1 × 50 mm, 1000 Å, 5 µm). The large molecule SWARM autotune feature was used before MS data acquisition. Tables 1 and 2 list the LC and MS parameters used for this workflow.

Data processing

All MS data of the intact mAbs were processed using MassHunter Quantitative Analysis 10.0 software.

Table 1. Liquid chromatography parameters.

LC Conditions	
Column	Agilent PLRP-S 1000Å, 2.1 × 50 mm, 5 µm (p/n PL1912-1502)
Column Temperature	80 °C
Injection Volume	15 µL
Autosampler Temperature	4 °C
Needle Wash	3 seconds in wash port (50:50/water:methanol)
Mobile Phase	A) Water + 0.1% formic acid B) Acetonitrile + 0.1% formic acid
Flow Rate	0.4 mL/min
Gradient Program	Time (min) %B 0 5 1 20 6 50 7 70 7.1 90 8 90 8.1 5
Stop Time	10 min

Table 2. MS acquisition parameters.

MS Conditions	
Gas Temperature	360 °C
Drying Gas Flow	13 L/min
Nebulizer Gas	45 psi
Sheath Gas Temperature	380 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	5,500 V
Nozzle Voltage	2,000 V
Fragmentor	380 V
Skimmer	140 V

Results and discussion

Method optimization for intact protein quantitative analysis

To improve the sensitivity and reproducibility for intact protein quantitative analysis, the sample preparation, LC, and MS conditions were optimized for intact protein quantitation.

The AssayMAP Bravo automated liquid-handling platform has prewritten programs for many applications, including immobilization (Figure 2) and affinity purification (Figure 3), demonstrated in this workflow.[4] In each step, the volume, flow rate, and wash cycles were optimized to achieve the best immobilization and affinity purification efficiency when using a Streptavidin affinity cartridge (SA-W) for the biological samples.

The HPLC method was optimized using a PLRP-S column with high temperature (80 °C) and flow rate (0.4 mL/min) to achieve the best reproducibility for intact protein analysis. The higher column temperature significantly improved the MS sensitivity under this condition.

The 6545XT AdvanceBio LC/Q-TOF system was also optimized including source parameters and large molecule SWARM autotune to achieve best MS sensitivity for intact mAb analysis. The optimized source parameters are listed in Table 2. Large molecule SWARM autotune is a special feature designed for intact protein analysis, and it increased the MS signal fourfold compared to standard SWARM autotune.

Immobilization: Using AssayMAP v1.0

Application Settings

Number of Full Columns of Cartridges: 4

Step	Conduct Step?	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Initial Syringe Wash	☑			3
Prime	☑	100	300	1
Equilibrate	☑	100	5	1
Load Sample	☐	100	5	3
Collect Flow Through	☐			
Cup Wash 1	☑	25		1
Internal Cartridge Wash 1	☑	50	10	3
Collect Flow Through	☐			
Load Blocking Reagent	☑	100	5	3
Collect Flow Through	☐			
Cup Wash 2	☑	25		1
Internal Cartridge Wash 2	☑	50	10	3
Collect Flow Through	☐			
Stringent Syringe Wash	☐	50		1
Re-Equilibrate	☐	50	10	1
Final Syringe Wash	☐			3

Deck Layout

1. Wash Station	2. Cartridges	3. Priming & Equilibration Buffer
4. Sample	5. Cartridge Wash Buffer 1	6. Cartridge Wash Buffer 2
7. Flow Through Collection	8. Stringent Syringe Wash Buffer	9. Blocking Reagent

Labware Table

Deck Location	Labware Type
1	96AM Wash Station
2	96AM Cartridge & Tip Seating Station
3	12 Column, Low Profile Reservoir, Natural PP
4	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro
5	12 Column, Low Profile Reservoir, Natural PP
6	12 Column, Low Profile Reservoir, Natural PP
7	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro
8	12 Column, Low Profile Reservoir, Natural PP
9	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro

Figure 2. AssayMAP Immobilization application setup screen.

Affinity Purification: Using AssayMAP v1.0

Application Settings

Number of Full Columns of Cartridges: 4

Step	Conduct Step?	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Initial Syringe Wash	☑			3
Prime	☐	100	300	1
Equilibrate	☐	50	10	1
Load Sample	☑	100	3	3
Collect Flow Through	☑			
Cup Wash 1	☑	25		1
Internal Cartridge Wash 1	☑	150	10	3
Collect Flow Through	☐			
Cup Wash 2	☐	25		1
Internal Cartridge Wash 2	☐	50	10	3
Collect Flow Through	☐			
Stringent Syringe Wash	☐	50		1
Elute	☐	25	5	1
Eluate Discard	☐	0		
Add to Flow Through	☐			
Existing Collection Volume		0		
Final Syringe Wash	☑			3

Deck Layout

1. Wash Station	2. Cartridges	3. Prime & Equilibrate Buffer
4. Samples	5. Cartridge Wash Buffer 1	6. Cartridge Wash Buffer 2
7. Flow Through Collection	8. Elution & Syringe Wash Buffer	9. Eluate Collection

Labware Table

Deck Location	Labware Type
1	96AM Wash Station
2	96AM Cartridge & Tip Seating Station
3	12 Column, Low Profile Reservoir, Natural PP
4	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro
5	12 Column, Low Profile Reservoir, Natural PP
6	12 Column, Low Profile Reservoir, Natural PP
7	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro
8	12 Column, Low Profile Reservoir, Natural PP
9	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro

Figure 3. AssayMAP affinity purification application setup screen.

Quantitative analysis of Intact mAbs from mouse plasma:

Using the AssayMAP Bravo automation platform, a concentrated final elution volume (25 µL versus 100 µL in bead-based) with higher recovery can be achieved with increased sensitivity and reproducibility compared to the magnetic bead-based workflow. Figures 4A and 4D show the total ion chromatograms (TICs) of trastuzumab and rituximab from mouse plasma samples prepared by the AssayMAP Bravo system. The baseline separation of target peak from interference peaks demonstrated good selectivity from the AssayMAP Streptavidin cartridge and LC conditions. Representative spectra (top four charge states) of trastuzumab and rituximab in mouse plasma after immunoaffinity purification are shown in Figures 4C and 4F. Note the excellent spectral quality using the AssayMAP Bravo automation platform for sample preparation and the 6545XT AdvanceBio LC/Q-TOF for data acquisition. The extracted ion chromatogram (EIC) of the targeted mAb was generated (Figures 4B and 4E) by summing a total of the eight most intense peaks over four charge states, with a mass extraction window of 1.0 *m/z* unit.

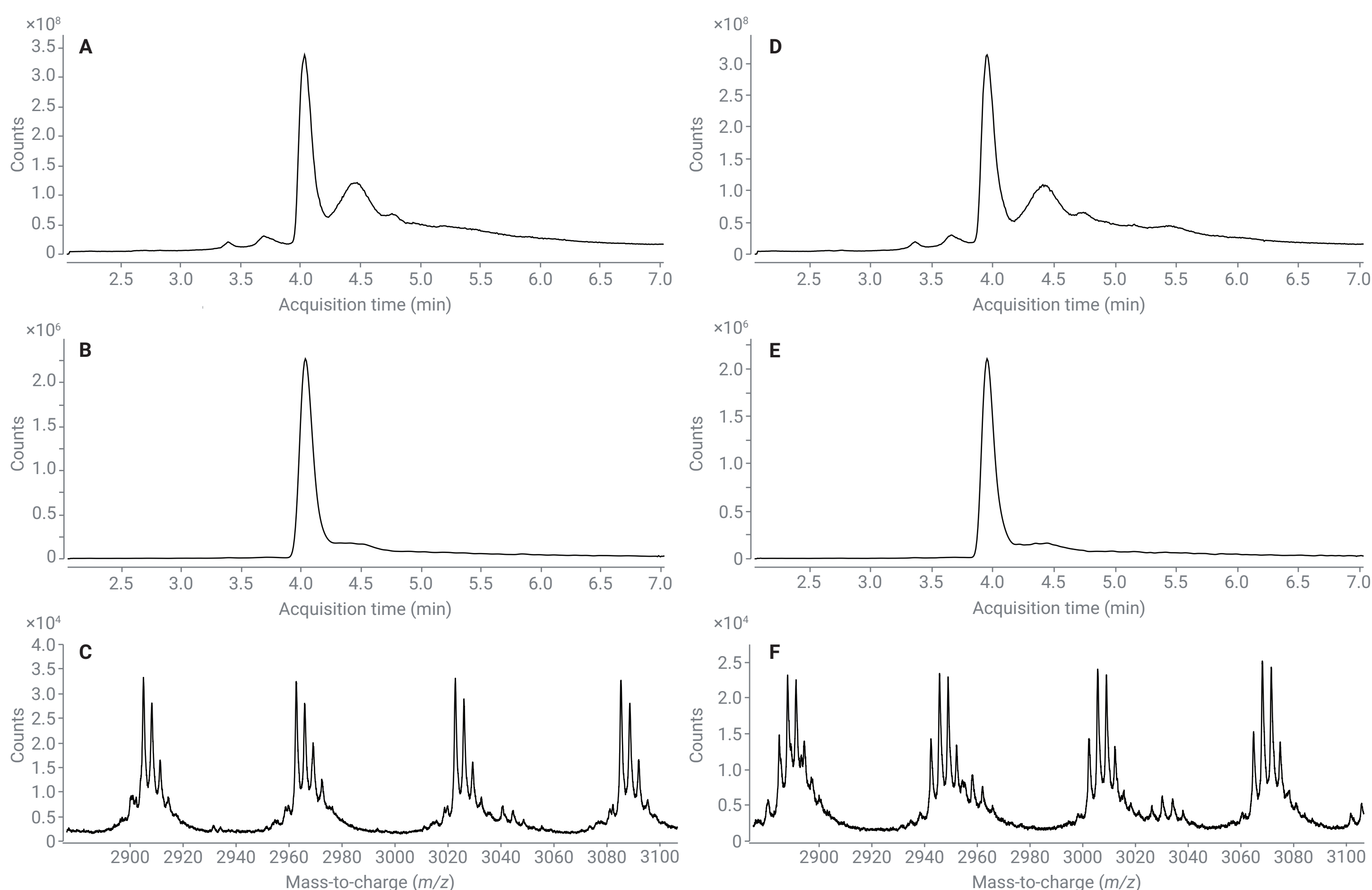

Figure 4. Representative chromatograms and mass spectra of trastuzumab and rituximab in mouse plasma. (A) TIC of trastuzumab in mouse plasma; (B) EIC of trastuzumab in mouse plasma; (C) Raw spectrum of trastuzumab top four charge states in mouse plasma; (D) TIC of rituximab in mouse plasma; (E) EIC of rituximab in mouse plasma; (F) Raw spectrum of rituximab top four charge states in mouse plasma.

MassHunter Quant 10.0 software was used to perform quantitative analysis. The EICs of two glycoform peaks from the top four charge states were summed with an extraction window of 1.0 *m/z* unit for each peak. In Figures 5A and 5C, the low limit of quantification for both trastuzumab and rituximab in mouse plasma was 0.02 µg/mL. By using 50 µL of plasma as starting material, the calibration curve was linear up to 10 µg/mL with quadratic fit and $1/x^2$ weight (Figures 5B and 5D). The bending at the top of the curve was probably caused by immunoaffinity cartridge saturation.

The intraday and interday analytical precision and accuracy were determined from three independent preparations performed over three days. The precision and accuracy results for both mAb drugs in mouse plasma are shown in Table 3. All levels of quality control samples (n = 6) met the acceptance criteria of 20% (25% for LLOQ) recommended by regulatory agencies. The results demonstrate excellent assay performance using automated sample preparation and a highly sensitive and accurate mass spectrometer for intact protein quantification.

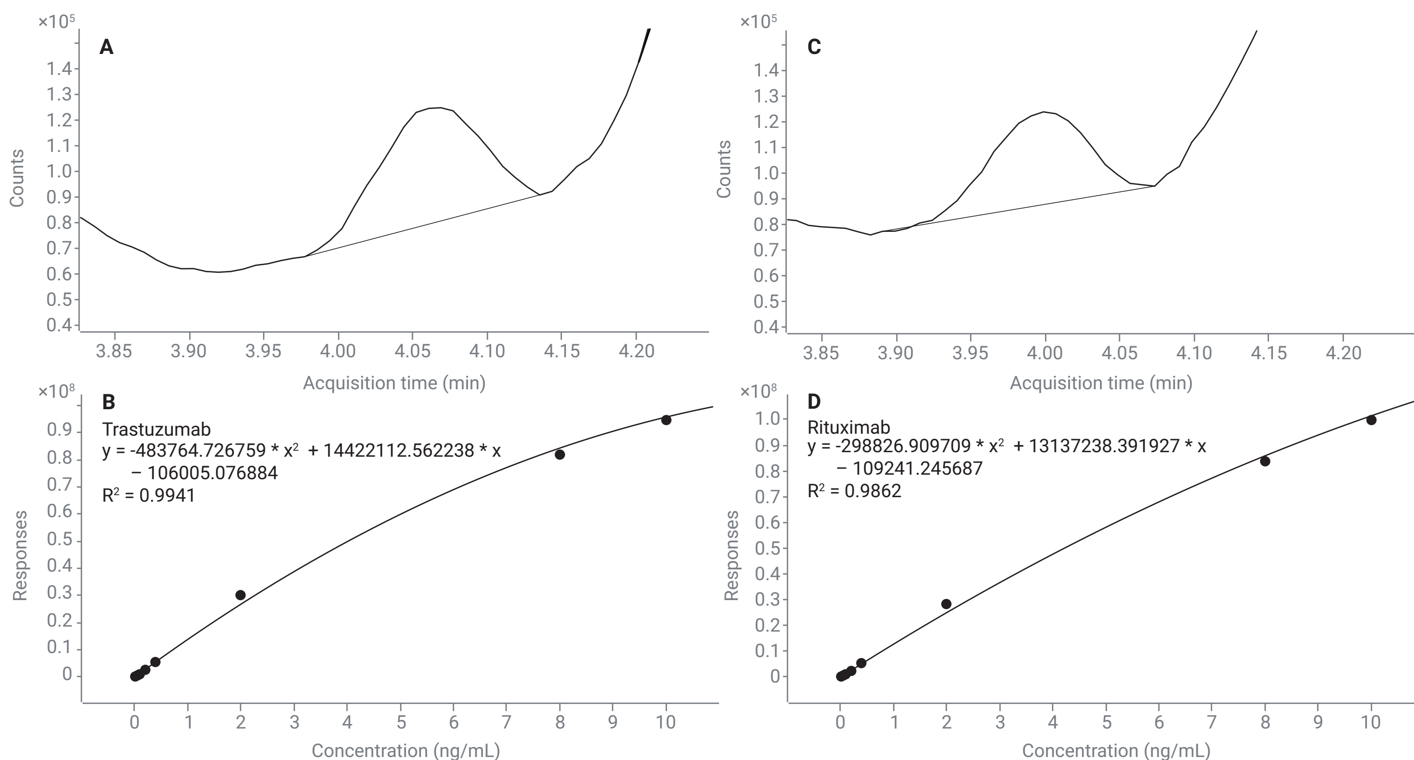

Figure 5. Representative extract ion chromatograms of lowest calibration point and full calibration curve. (A) EIC of lowest calibration point of trastuzumab in mouse plasma; (B) Calibration curve of trastuzumab from 0.02 to 10 µg/mL in mouse plasma; (C) EIC of lowest calibration point of rituximab in mouse plasma; (D) Calibration curve of rituximab from 0.02 to 10 µg/mL in mouse plasma.

Table 3. Precision and accuracy of quality control samples in mouse plasma (n = 6).

Day	Statistic	LLOQ QC (0.02 µg/mL)		Low QC (0.06 µg/mL)		Mid QC (1.0 µg/mL)		High QC (7.5 µg/mL)	
		Trastuzumab	Rituximab	Trastuzumab	Rituximab	Trastuzumab	Rituximab	Trastuzumab	Rituximab
Day 1	Mean	0.0234	0.0240	0.0519	0.0508	1.08	1.11	8.08	7.70
	% Bias	17.0	20.0	-13.5	-15.3	8.0	11.0	7.8	2.7
	% CV	11.3	3.1	9.7	4.9	5.0	5.4	5.9	5.1
Day 2	Mean	0.0180	0.0199	0.0540	0.0568	1.19	1.19	7.85	6.97
	% Bias	-10.0	-0.5	-10.0	-5.3	18.5	19.0	4.6	-7.1
	% CV	6.7	9.3	11.7	13.3	3.3	5.0	8.0	2.8
Day 3	Mean	0.0176	0.0207	0.0494	0.0540	1.03	1.18	7.42	7.78
	% Bias	-12.0	3.5	-17.7	-10.0	3.4	18.0	-1.1	3.7
	% CV	10.2	6.5	6.2	5.3	2.4	4.3	6.4	5.2
Interday	Mean	0.0197	0.0215	0.0518	0.0539	1.10	1.16	7.78	7.48
	% Bias	-1.7	7.7	-13.7	-10.2	10.0	16.0	3.8	-0.2
	% CV	16.5	10.1	4.4	5.6	7.0	3.8	4.3	6.0

Analyte extraction efficiency

A recovery test to evaluate the extraction efficiency was performed to ensure optimal analyte recovery during sample preparation. Three concentrations, 0.06, 1.0, and 7.5 µg/mL, were evaluated. As shown in Table 4, the average recovery of trastuzumab in mouse plasma was 64.9% for mid and high QC samples. The average recovery of rituximab in mouse plasma was 53.7% for mid and high QC samples. Due to nonspecific binding of mAb to the tubes at low concentrations, the low QC sample showed more than 100% recovery (data not shown). Based on the recovery percentage of trastuzumab and rituximab in mouse plasma, the actual sample on-column quantification limits were only 0.32 and 0.27 ng for trastuzumab and rituximab, respectively.

Table 4. Recovery of quality control samples in mouse plasma (n = 4).

	Mid QC	High QC	Average
Trastuzumab	60.1	69.6	64.9
Rituximab	51.4	56.0	53.7

Conclusion

The AssayMAP Bravo automated sample preparation platform and an 6545XT AdvanceBio LC/Q-TOF system are ideal for intact protein LC/MS analysis. The workflow is ultrasensitive and highly automated as well as providing excellent assay reproducibility. We demonstrated a sensitive LC/MS workflow for the quantitative analysis of intact mAbs from biological matrix. The workflow achieved a lower limit of quantification of 0.02 µg/mL using 50 µL of sample for two mAb drugs in biological matrix. The final on-column quantification limit was 0.32 ng for trastuzumab and 0.27 ng for rituximab, which is as good as or better than published literature. In three qualification runs, the intraday and interday QC sample's precision and accuracy all met regulatory acceptance criteria, demonstrating excellent assay performance and reproductivity.

References

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