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An Automated Sample Extraction and Processing Method Combining Immuno-Affinity and Mass Spectrometry Analysis for Biotherapeutics Quantitation

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ABSTRACT

A generic method for the quantitation of humanized monoclonal antibodies (mAb) was developed using the SCIEX BioBA solution. The method, which combines immunocapture using high capacity magnetic beads followed by protein digestion and signature peptide LC-MS/MS analysis was then automated by transferring to a Beckman Coulter Biomek FX^P automated liquid handling station. Testing and characterization of the performance of the automated method was conducted using a generic humanized monoclonal antibody in rat plasma. Finally, a rat dosing study was performed to generate real samples for comparative analysis of the automated method and an ELISA method. Samples from the study were split and analyzed by the two different techniques in two different labs and the results were compared.

INTRODUCTION

Protein based biotherapeutics are a growing component of pharmaceutical companies' drug pipeline. In order to support this growing class of new drug molecules robust and reliable bioanalytical methods are required. The signature peptide approach is the most commonly used LC-MS based strategy for protein quantitation due to its high sensitivity and specificity. When this strategy is combined with immuno-affinity sample preparation to concentrate the target analyte and reduce the matrix background the sensitivity and selectivity of the technique is greatly expanded. There is however several steps in immuno-affinity sample preparation plus several incubation wait times that consume an analyst's valuable time. This creates a new bottleneck in sample preparation compared to traditional small molecule workflows. Added to this bottleneck is the fact that reproducible sample preparation is critical for delivering high quality pre-clinical and clinical study results.

Magnetic beads offer several advantages for immuno-affinity workflows including: ease of handling, scalability, improved sample recovery, parallel processing of samples and use in high-throughput formats with robotics. Using automation reduces the variability of multi-step sample preparations within a batch and between day to day preparations. It also reduces the labour required to process sample batches and liberates scientists to do other work in order to deliver study results. In this poster we demonstrate successful method transfer of the BioBA sample preparation protocol to the Beckman Coulter Biomek FX^P automated workstation and show its ability to deliver robust results from real study samples.

MATERIALS AND METHODS

Dosing Study:
Four male Sprague-Dawley rats were given a sub-cutaneous dose of rituximab at 10 mg/kg and blood samples were collected at: predose, 0.5, 2, 6, 24 h, 2, 3, 6, 8, 10, 14, 17, 21, 24 and 28 days and kept frozen. The samples were analyzed by QPS using a previously validated ELISA assay in the range of 100 to 10 000 ng/mL and samples were pre-diluted 5 or 10 fold prior to analysis. The remainder of the samples was shipped to SCIEX in Concord for analysis by immuno-affinity LCMS.
Calibration standards were prepared in the range of 100 to 100 000 ng/mL and QC samples at 300, 3250 and 75000 ng/mL. Samples from rats 1,2: Day 2, rat 3: 0.5 hr, Day 2,14, rat 4: 0.5 hr, Day 2,14 were diluted 5-fold with blank rat plasma prior to analysis due to low sample volume. Twenty-five microliters of each standard, QC and study sample was then processed following the procedure outlined below using SILuMab (Sigma-Aldrich) internal standard, 1.0 µg/mL.
Chromatography:
Separation of the signature peptides of the digested samples was performed on a Shimadzu LC-20 system consisting of the following components: CBM-20A system controller, LC-20AD isocratic pumps (2), SIL-20AC autosampler, CTO-20AC column oven (50 ° C) using a Phenomenex 2.6 µm, Kinetex C18 Column, (50 x 2.1 mm). A short gradient was used and 5 µL of sample was injected onto the column and run at 400 µL/min. The gradient conditions were: 0.00, 10%B, 4.00 min, 40%B, 4.25 min, 95%B, 5.50 min, 95%B, 5.60 min, 10%B, 6.30 min, 10%B.
Mass Spectrometry:
The MRM analysis was performed on a SCIEX QTRAP 6500® system equipped with an IonDrive™ Turbo V source. The following source/gas parameters were used, IS 5500, CUR 25 psi, TEM 500 ° C, GS1 85 psi, GS2 80 psi and CAD High. The analyte MRM parameters used for signature peptide quant using a conserved signature peptide from the Fc region of rituximab and SIGMAMAB are shown in Table 1.

Table 1. MRM parameters.

Q1	Q3	Dwell	DP	CE	CXP	Retention Time (min)	Peptide
560.1	708.8	25	60	22	28	2.1	Signature Peptide 1_1
560.1	615.7	25	60	23	15	2.1	Signature Peptide 1_2
562.9	713.3	25	50	23	28	2.1	Heavy Signature Peptide 1_1

RESULTS

Automated Sample Preparation:
The SCIEX BioBA sample preparation protocol was automated for 96 samples on the Biomek FX^P Workstation. The Biomek FX^P Workstation is setup with a Peltier heater with deep well plate adapter, a MagnaBot® 96 Magnetic Separation Device and an orbital shaker. Pipetting techniques were optimized for all transfer steps to ensure accurate delivery of all reagents. The workflow is divided into two milestones: capture and digestion. In the capture workflow the deck of the Biomek FX^P Workstation is loaded with streptavidin magnetic beads conjugated with capture antibody, isotopically-labeled internal standard, bind/wash buffer, elution buffer and neutralization buffer. A sample plate containing 25 µL calibration standards, QC samples, blank and double blank controls and subject samples was prepared and placed on the deck of the Biomek FX^P Workstation. First, the Biomek FX^P Workstation transferred 2 x sample volume of internal standard (1.0 µg/mL SILuMAB) to the sample plate. 25uL of the beads were added to the capture plate and the sample plus internal standard was transferred to the beads. After incubation for 1 hour the sample supernatant was removed from the beads and transferred to a storage plate. The beads were then rinsed three times with buffer. After washing was complete the beads were then incubated with 50 µL of elution buffer (0.1% TFA) for 10 minutes. After elution was complete the acidic supernatant was transferred to a clean elution plate and neutralization buffer (500 mM ammonium bicarbonate) was added. The analyte in the elution plate was now ready for digestion.
At the end of the capture milestone the deck of the Biomek FX^P Workstation was then cleared of the capture reagents and digestion labware were placed on the deck. Digestion reagents (TCEP, IAM, anionic surfactant and trypsin/lys-C) were placed in 2 mL sample tubes and formic acid and water were placed in divided reservoirs. The digest reagents were stamped out into 96 v-bottom well plates using the Span-8 head and the 96-channel head was used to deliver the required volume to the elution plate. The digestion reagents were not stamped out until required in 'just-in-time' delivery fashion to minimize evaporation loss of the small volume reagents. Performing reagent delivery in this way and taking advantage of both the 96-channel and Span-8 heads also minimizes the amount of dead time in the method and synchronizes reagent addition across the 96-samples. The automated digestion workflow begins with addition of the reducing reagent (100 mM TCEP) and the elution plate is heated at 50 ° C for 1 hour. Next the alkylation reagent (100 mM iodoacetamide) was added to the elution plate and mixed for 30 minutes at room temperature. Next the anionic mass spec compatible surfactant was added followed by trypsin-lysC and the elution plate was incubated for 3 hours at 37° C. At the end of the digestion 3 µL of formic acid was added to the elution plate to stop digestion. Lastly, 50 µL of water was added to the digested samples and 75µL of the diluted samples were transferred to a clean 96-well LC injection plate.

To test the reproducibility of the digestion protocol a plate was prepared containing samples of SILuLite (Sigma-Aldrich) antibody standard plus SILuMAB internal standard (3.6 µg/mL) in 50 mM ammonium bicarbonate buffer. Columns 1, 4, 6, 9 and 11 contained SILuLite at 540 ng/mL, columns 2, 5, 7, 10 and 12 contained SILuLite at 180 µg/mL and columns 3 and 8 contained blank buffer. Three universal signature peptide peak area ratios were monitored for reproducibility. The data in figure 1 represent the DTLMISR universal signature peptide. The %CV across 40 wells of the 540 ng/mL sample was 5.9% and 4.4% for the 180 µg/mL sample. Two other universal peptides MRM area ratios were monitored (data not shown) and the %CVs were 8.2% and 5.4% for the low concentration sample and 5.2% and 4.1% for the high concentration sample. There was no signature peptide MRM response from the blank samples in columns 3 and 8 in the middle of the plate indicating there was no cross contamination of sample during the liquid handling steps.

With the reproducibility of the digestion protocol established we moved to test the reproducibility of the entire workflow. A large QC sample of rituximab 3.25 µg/mL was prepared in rat plasma and 50 µL was aliquoted in all wells of a 96- well plate. The data in figure 2 represent the peak area ratio of a universal peptide and its heavy labelled internal standard. The %CV across all wells of the extracted plasma sample was 8.7%.

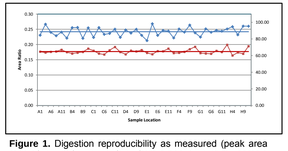

Figure 1. Digestion reproducibility as measured (peak area ratio of DTLMISR/DTLMISR*) from a neat 180 µg/mL sample (red) and neat 540 ng/mL sample (blue).

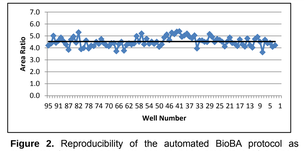

Figure 2. Reproducibility of the automated BioBA protocol as measured from the signature peptide peak area ratio of a single 3.25 µg/mL rituximab plasma sample aliquoted to 94 wells of a 96-deep well plate.

Software Workspace:
The software contains a dashboard workspace where one of the two workflows is selected. When a method is launched the Guided Labware Setup (Figure 3) takes the user through the setting up of the deck step by step to ensure no labware is misplaced. The Guided Labware Setup also informs the user of the reagent volumes required for the batch. Once a method is launched the Milestone View informs the user of the current status of the labware as it moves through the workflow as well as the overall progress and time remaining for the entire method (Figure 3). When the Biomek Workstation is networked, this information can be viewed in a standard web browser, thereby allowing remote monitoring of the system.

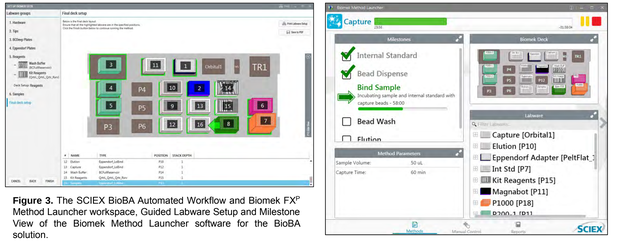

Figure 3. The SCIEX BioBA Automated Workflow and Biomek FX^P Method Launcher workspace, Guided Labware Setup and Milestone View of the Biomek Method Launcher software for the BioBA solution.

Study Data and Comparison with ELISA:

Finally, to demonstrate the power and utility of the automated protocol the validity of the method was tested on real study samples, not just QC samples. A study was commissioned with QPS to dose animals with rituximab. Samples were first analyzed by QPS using an ELISA method with a range of 100 to 10 000 ng/mL and study samples were pre-diluted prior to analysis. After analysis by ELISA, samples were shipped to SCIEX for analysis using a universal signature peptide MRM. Figure 4 shows the peaks from the LLOQ standard and double blank sample. The signal to noise ratio was ~95, indicating excellent sensitivity. Figure 4 also shows the calibration curve from the sample batch. Curve values ranged in accuracy from 92.4-108% of expected values and %CVs ranging from 0.8-12.6%. The calibration curve showed excellent linearity as evidenced by an r value of 0.9985. Figure 5 shows the average sample concentration (4 rats) at each time point from measured using both techniques. The data from the rat dosing study show excellent agreement (<15%) between the two analytical techniques and shows that the immuno-affinity LCMS assay provides equivalent results to the ELISA in this case while the IA-LCMS assay had the advantage of a wider linear dynamic range and required no sample pre-dilution. Although not explored in this study the LCMS method can be used to do simultaneous quantitation of antibody catabolism from the same sample set.

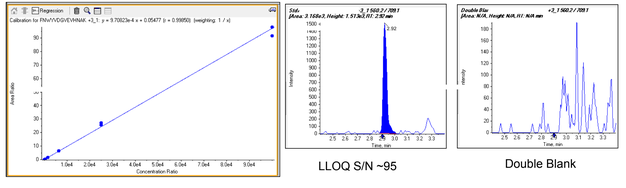

Figure 4. Calibration curve and chromatograms of the LLOQ standard and double blank sample processed using the automated BioBA protocol on the Beckman Coulter FX^P.

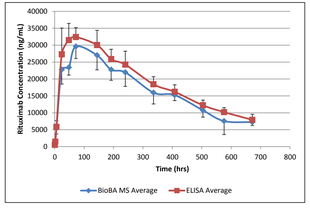

Figure 5. The average Rituximab concentration (4 rats) at each time point on the PK curve. The blue points were measured by BioBA automated immuno-affinity LCMS. The red points were measured by ELISA by QPS

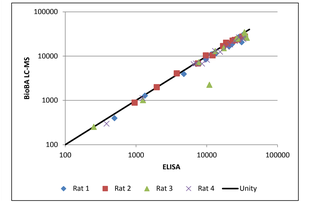

Figure 6. Correlation of each individual rat time point between BioBA automated immuno-affinity LCMS and ELISA.

CONCLUSIONS

Fully automated sample preparation is critical to reducing bottlenecks in immuno-affinity sample preparation workflows for signature peptide quantitation. Using automation reduces the variability of multi-step sample preparations within a batch and between day-to-day preparations and liberates scientists to do other work required to deliver study results.

Here, a fully automated solution has been developed for immuno-affinity sample preparation and signature peptide quantification and been successfully demonstrated for a monoclonal antibody therapeutic in real dose samples. Excellent sensitivity, accuracy and precision were achieved by the assay. The results of real dose samples analyzed by two different techniques, ELISA and immuno-affinity LCMS analysis of the samples agreed to within 15% which demonstrates the automated BioBA solution to be a robust and accurate solution for mAb quantitation. The BioBA solution including the Biomek FX^P automated protocol and ready to use consumables from BioBA kits will increase productivity and accelerate biologics bioanalysis.

TRADEMARKS/LICENSING

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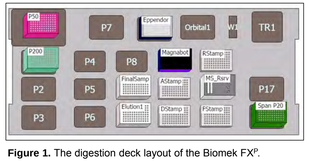


Figure 1. The digestion deck layout of the Biomek FX^P.

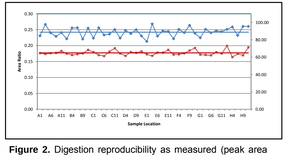
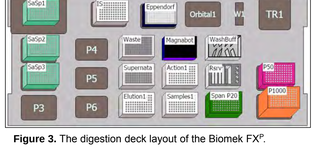


Figure 2. Digestion reproducibility as measured (peak area ratio of DTLMISR/DTLMISR*) from a neat 180 µg/mL sample (red) and neat 540 ng/mL sample (blue).

Next the capture workflow was transferred to the Biomek FX^P. The immuno-capture workflow begins with the plasma samples aliquoted in a 96-deep well plate and streptavidin coated magnetic beads pre-conjugated to an anti-human Fc antibody. Figure 3 shows the deck layout of the capture workspace. The layout consists of the sample plate, the final elution plate, the immuno-capture plate, 96-deep well plates for waste and sample supernatant and the boxes of tips required. The reagents required: beads, internal standard, wash buffer, elution and neutralization buffers are placed in divided reservoirs.



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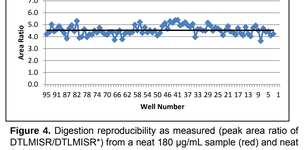

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